Molecular Mechanisms of the Activation of the Pattern Recognition Receptor Dectin-1 and its Induction of Immune Responses

by

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Abstract

In the past two decades, a rising number of fungi have emerged as serious human pathogens and the incidence of invasive fungal infections has increased enormously. Millions of people contract fungal diseases that kill at least as many people as tuberculosis or malaria, yet the global burden of fungal disease is largely unrecognized. This rise in fungal disease burden has become a public health concern and major threat to human health, especially in immunocompromised patients, whose numbers are increasing due to the advent of HIV, organ transplantations, and the use of immuno-suppressive therapies for auto-immune diseases and cancer. Despite the high mortality rates of invasive fungal infections, they remain understudied compared to other infections, and to date the molecular mechanisms of antifungal immune responses are still poorly understood. It is therefore a healthcare priority and an exciting challenge *to understand at the molecular level the mechanisms that our immune system utilizes to fight fungal infections.* With this goal in mind, I have been particularly interested in studying the first and crucial step of this process: the recognition of specific molecules of the fungal pathogen by receptors expressed on the surface of innate immune cells.

Work presented in this thesis focuses on Dectin-1, an innate immune receptor that plays a key role in antifungal immunity. Dectin-1 is the major receptor for β-glucans, which are polysaccharides that are largely present in the fungal cell wall. The *exact molecular mechanisms of how Dectin-1 is activated by β-glucans and how it induces intracellular signaling are still not clear* and need to be elucidated. Previous studies have shown that depending on the size of the ligand, Dectin-1 can activate, inhibit or modify receptor signaling. Based on these studies, we hypothesized that binding of β-glucans to Dectin-1 would cause it to cluster into multimeric complexes, which would act as signaling nodes to initiate intracellular signaling and subsequent

immune responses. To perform this analysis, we employed conventional biochemical approaches and cell biological approaches, combined with quantitative superresolution microscopy. We were able to characterize the relationship between receptor clustering and Dectin-1 signal-transduction. Herein, results from this thesis provide a **"Receptor Clustering Model"** for Dectin-1 activation and induction of antifungal immune responses.

With this better understanding of the mechanisms of antifungal immunity activation by Dectin-1, we sought to design, in collaboration with the group of Dr. Bundle (Dept. of Chemistry, U of A), a new anti-*Candida* vaccine. We were able to show that a multimeric β-glucan conjugate, developed by this group, is capable of producing enhanced antifungal immunostimulatory effects.

Overall, findings from my PhD thesis provide valuable mechanistic insights into the early signaling and molecular events that lead to Dectin-1 activation and its subsequent capacity to induce antifungal immune responses. Finally, work presented here sets a framework for sophisticated development of novel and more effective antifungal therapeutics, and provides considerations for better design of antifungal vaccines, a significant area to be pursued in the near future. Therefore, findings from this thesis will ultimately establish a foundation for hopefully effective treatment and prevention of fungal infections.

Preface

This thesis is entirely an original work by myself, Amira Fitieh, unless otherwise stated. I started the work presented in this thesis in May 2008, as a PhD student in Dr. Nicolas Touret's laboratory at the Biochemistry Dept. (U of A). Dr. Touret's lab is interested in studying membrane receptors, specifically immune receptors, using innovative cell imaging techniques. The research-training environment that I encountered during my Doctoral studies for preparation of this thesis was very interactive and fruitful. Dr. Touret's lab is part of the Membrane Protein Disease Research Group (MPDRG), where we would have weekly meetings with researchers and members of the group. This interaction greatly enhanced my knowledge about membrane proteins and related experimental procedures, which were were relevant to research covered in this thesis. More specific and relevant to research presented in this thesis were our collaborations that were established with the groups of Dr. Hanne Ostergaard and Dr. David Bundle, in the Departments of Medical Microbiology and Immunology (U of A), and Chemistry (U of A), respectively. In the lab of Dr. Ostergaard, I developed and learnt immunological approaches, including animal dissection and the preparation of primary immune cells, which are techniques of great significance for my results presented in *Chapter 4* of this thesis. Animal work shown in *Chapter 4* was done in collaboration with Dr. Hanne Ostergaard and Dr. Bundle. The animals that I personally used for dissection and preparation of primary immune cells (BMDCs) were provided by Dr. Ostergaard, who received ethical approval by the U of A for the use of these animals for research. All animal experimental procedures were approved by the Health Sciences Animal Care and Use Committee at the University of Alberta (Protocol Number 055) and conform to guidelines put forward by the Canadian Council on Animal Care. Dr. David Bundle's group, mainly Dr. Tomasz Lipinski (formerly a research associate in Dr. David Bundle's lab, Dept. of Chemistry, U of A), assisted us in the design and synthesis of β-glucan derivatives and fluorescent probes for Dectin-1 stimulation, that were extremely pivotal and indispensable to research presented in this thesis. Through direct interaction with the Bundle group, I learnt new experimental and analytical methods that were critical to the research project of this thesis. Interaction with these two groups allowed me to acquire a unique set of multidisciplinary competencies that not only provided me with a larger set of research skills, but also expanded my catalogue of scientific knowledge. *Chapter 4* of this thesis has been published in the Journal of Immunology as "*Enhanced Immunogenicity of a Tricomponent Mannan Tetanus Toxoid*

Conjugate Vaccine Targeted to Dendritic Cells via Dectin-1 by Incorporating β-Glucan" (Lipinski et al., 2013). I am the co-lead author (co-first author) together with Dr. Tomasz Lipinski of this research article, and accordingly it has been reproduced with permission from the *Journal of Immunology* for *Chapter 4* of my thesis. I have mainly (by more than 90%) conceived, designed, performed, optimized, interpreted, and analyzed the immunological, biochemical, cell biology and molecular biology experiments for research conducted in this paper. I have also written 70% of the manuscript. Overall, my experiments have produced 4 figures (Figures 2 to 5 in the paper) out of the 6 figures presented in the paper. Specifically, I performed the biochemical, cell biological, and immunological characterization of anti-*Candida* vaccine conjugates prepared by Dr. Tomasz Lipinski (Bundle lab). These vaccine conjugates included a multivalent β-glucan vaccine conjugate against *Candida*. I executed immunological, biochemical and cell biology experiments on primary immune cells that I extracted from mice provided by Dr. Ostergaard. Dr. Lipinski's main contribution to the paper that is covered in *Chapter 4* was synthesizing anti-*Candida* vaccine conjugates and testing the *in vivo* effect of these vaccine conjugates in animals. Specifically, he vaccinated mice with these vaccine conjugates and analyzed the antibody titer that was produced by the animals in response to vaccination. The contribution of Joelle St. Pierre, one of the co-authors of this paper and a former member of the Ostergaard lab, was merely training me in animal dissection and preparation of primary immune cells. Overall, my results from the paper reproduced for *Chapter 4* provided important mechanistic insight into the molecular and biochemical basis of the enhanced immune response provided by a multimeric β-glucan vaccine conjugate. Lastly, our collaboration with Dr. Khuloud Jaqaman (UT Southwestern Medical Center, Dallas) with the help of John Maringa, a member of the Touret lab, combined with the unique expertise of Dr. Touret in the field of microscopy, enabled us to perform high-end image analysis of our super-resolution data and provide modeling tools to extract the single molecule behavior of individual Dectin-1 receptors at the surface of innate immune cells. In this regard, Dr.Touret and John Maringa, in collaboration with Dr. Khuloud Jaqaman, performed the "Dectin-1 clustering analysis" shown in *Chapter 3*. My Ph.D. research has taken me into a fascinating journey of discovery, while offering moments of genuine analysis and thorough interpretation along the way. The first level collaborations listed above, in addition to the unique research-training environment that I encountered during my PhD, had direct influence and beneficial impact on the research presented in this thesis.

Dedication

In memory of my late loving father, Dr. Mohammed Fitieh, my role Model

"The real voyage of discovery consists not in seeking new landscapes, but in having new eyes."

Marcel Prous

"There is nothing, Sir too little for so little a creature as man. It is by studying little things that we attain the great art of having as little misery and as much happiness as possible"

Samuel Johnson 1763

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This doctoral thesis would not have been possible without the support of many people around me, whom I would like to acknowledge. First and foremost, I would like to sincerely thank my supervisor, Dr. Nicolas Touret for his support, motivation, and encouragement throughout this process and for being a very instilling scientific mentor and source of inspiration during my long PhD journey. Thanks for accepting me in your lab as a graduate student and introducing me to the exciting world of Microscopy and Cell Biology. Your guidance and supervision throughout my doctoral studies has been invaluable to my scientific development and academic career. It has also been a great privilege having Drs. Charles Holmes and Marek Michalak in my supervisory committee, and I would like to express my deepest thanks to them for their support, valuable advice, encouragement, and incredible mentorship throughout my doctoral program and for providing constructive criticism and feedback at each meeting. Thanks for your insightful Biochemistry courses, which greatly enhanced my scientific background for work covered in this thesis. Likewise, I extend my thanks to my PhD examining committee Drs. Christopher Mody, Charles Holmes, Shairaz Baksh, and Todd Alexander for their fruitful discussions during my PhD defense. Special thanks to Dr. Mody for accepting my invitation to examine my thesis, despite his busy schedule, and great thanks for his valuable scientific feedback and insight for my PhD thesis work. Thank you all for reading my very long thesis! I am grateful to our collaborators Drs. David Bundle and Tomasz Lipinski who were a huge inspiration to me during my PhD. The β-glucans provided by them were extremely indispensible to the work done in this thesis. Thanks for introducing me to the interesting field of carbohydrate chemistry. Without you this thesis wouldn't have seen the light! Also, I would like to express my thanks to Dr. Hanne Ostergaard for allowing me to train in immunological techniques and mice dissection in her lab under the supervision of Dr. Joelle St.Pierre. Thanks all for the great work that we did together! I am also very grateful for all the Touret lab members, Andrew Locke, John Maringa, David Kramer, Gurpaul, and Sandra Ungarian, for providing a wonderful atmosphere full of support and innovation, and for contributing to research presented in this thesis. Thanks for very great scientific discussions! Thanks John for being our cool superresolution guy! Thanks Andrew for your great sense of humour, the cheer and laughter that you gave me were the driving force that kept me going during the bad days of my experiments. I will never forget the funny scientist that I used to meet everyday at work and have chats with about the mysterious world of β-glucans and

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List of Abbreviations

Ab	Antibody
Ab x-linking	Antibody cross-linking
AF	Alexa Fluor (Invitrogen)
AMPs	Antimicrobial peptides
AP-1	Activator protein 1
AIDS	Acquired Immunodeficiency Syndrome
B cells	B lymphocytes
BCR	B cell receptor
β-glucans	Beta-glucans
Bcl10	B cell lymphoma 10
BIR	Baculovirus inhibitor of apoptosis protein repeat
BMDCs	Bone marrow-derived dendritic cells
BMDMs	Bone marrow-derived macrophages
BSA	Bovine Serum Albumin
CARD	Caspase-associated recruitment domain
Cav1	Caveolin-1
CCL	Chemokine (C-C motif) ligand or CC-chemokine ligand
CLRs	C-type lectin receptors
CM	Carboxymethylated
CR	Complement receptor
CTL	Cytotoxic T lymphocytes
CXCL	Chemokine (C-X-C motif) ligand
DAP	DNAX activation protein
DAPI	4´,6-diamidino-2-phenylindole
DC	Dendritic Cell
Dectin-1	Dendritic Cell-associated C-type Lectin
DED motif	Aspartate-glutamate-aspartate motif
DMSO	Dimethyl sulfoxide (vehicle for dissolving pharmacological inhibitors)
DNA	Deoxyribonucleic acid
DS	Donkey Serum
DTAF	5-(4,6-dichlorotriazinyl)aminofluorescein, a fluorescent dye
DTSSP	3,3´-dithiobis(sulfosuccinimidylpropionate) – chemical cross-linker
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug adminstration
GFP	Enhanced green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTPase	Guanosine nucleotide triphosphatases
HA	Human influenza hemagglutinin epitope tag

HCV	Hepatitis C virus
hDectin1	Human Dectin-1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
HRP	Horseradish peroxidase
HRPMI	HEPES-buffered RPMI media
IFN	Interferon
Ig	Immmunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
LB	Luria-Bertani medium
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation gene 1
MAPK	Mitogen-Activated Protein Kinase
MHC	Major histocompatibility complex
Mincle	Macrophage-inducible C-type lectin
MIP-2	Macrophage inflammatory protein-2
MOPS	3-(N-morpholino)propanesulfonic acid
MW	Molecular Weight
MyD88	Myeloid differentiation factor 28
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T cells
NK	Natural Killer Cell
NLR	Nucleotide-binding oligomerization domain (NOD)-like receptors
NLRP3	Nucleotide-bindingoligomerization domain-like receptor containing pyrin domains 3
NOD	Nucleotide-binding oligomerization domain
PALM	Photo-Activated Localization Microscopy
PAMPs	Pathogen Asscoiated Molecular Patterns
PBS	Phosphate buffered saline solution (137 mM sodium chloride, 2.7 mM Potassium chloride, 10 mM sodium hydrogen phosphate, 2 mM potassium dihydrogen phosphate, pH 7.4)
PBST	Phosphate buffered saline + 0.1% Tween-20
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PG	Prostagalandin
PIP2	Phosphatidylinositol 4,5-bisphosphate)
PKCδ	Protein Kinase C, δ isozyme

PLCγ	Phospholipase C, γ isozyme
PRR	Pattern recognition receptor
RAW – Dectin-1	RAW 264.7 cells stably expressing full length human Dectin-1
RAW WT	The original RAW 264.7 cells, or “RAW Wild-Type”
RIG-1	Retinoic acid-inducible gene-I
RLR	Retinoic acid-inducible gene-I (RIG-1)-like receptors
ROS	Reactive oxygen species
S.D.	Standard deviation
S.E.	Standard Error
S.E.M	Standard Error of the Mean
SDS-PAGE	Sodium dodecyl sulfate–Polyacrylamide Gel Electrophoresis
siRNA	Short interfering ribonucleic acids
SR	Scavenger Receptor
Src	Cellular sarcoma tyrosine protein kinase
SYK	Spleen Tyrosine Kinase
T cells	T lymphocytes
Tc	T cytotoxic cell
TBST	Tris Buffered Saline with Tween 20
TCEP	Tris(2-carboxyethyl)phosphine
TCR	T cell receptor
TDM	Trehalose-6,6’-dimycolate
Th	T helper cell
TIR domain	Toll/IL-1R homology domain
TLR	Toll-like receptor
TNFα	Tumour Necrosis Factor alpha
Treg cells	regulatory T cells
Tris	Tris (hydroxymethyl)aminomethane
Tris	Tris (hydroxymethyl)aminomethane
WGP	Wellmune whole glucan particles (Biothera)
WGPsol	WGP soluble (Biothera)

Chapter 1. INTRODUCTION

1.1. Overview of the Immune System

1.1.1. Role of the Immune System

Multicellular organisms are constantly threatened by the invasion of an enormous number of pathogenic microorganisms during their lifetime, and have developed systems of immune defense to combat such infectious agents. In most cases, we are able to resist these infections through our immune system, which is a remarkably versatile and brilliant defense system that has evolved to protect the host from a plethora of invading pathogens that bombard us on a daily basis, as well as to protect against stresses such as disease (e.g. Cancer) and injury, and to maintain homeostasis (Lee and Iwasaki, 2007; Mueller, 2010). Additionally, the immune system reacts specifically and effectively using different pathways to identify invaders and ably eliminate them. For performing all of these functions, the immune system generates an enormous array of cells and molecules which act together in a dynamic network of great complexity for the recognition and elimination of an almost limitless variety of threats, yet retaining the ability to differentiate between these insults and normal self to avoid autoimmunity (Akira et al., 2006). For many decades investigators have been struggling to increase their understanding of this amazing defense system and its strategies for mounting protection against this vast number of pathogens, and despite this so much about the immune system remains unknown.

The immune system functions as a dynamic and sophisticated network that ensures efficient response to foreign antigens and tolerance to self-antigens, so it employs defense mechanisms that are essential for fighting invading pathogens, and thus for the survival of the host (Goodnow et al., 2005). Functionally, protection by the immune system can be divided into two related activities — **recognition** and **response**. Perception of microbial intruders, nonself molecules (termed antigen), as well as altered self-molecules (e.g. modified self-proteins on transformed, damaged or stressed cells), induces immediate activation of a range of protective immune responses. Immune recognition is remarkable for its specificity, i.e., its capacity to distinguish foreign invaders from self-components. In that context, the immune system is able to selectively recognize characteristic molecular signatures that are conserved within common groups of

pathogens, as well as detect subtle chemical differences that distinguish one pathogen from another. Above all, is the exceptional property of the immune system to distinguish between foreign molecules and the body's own cells and molecules, a function which is known as **"self-nonself discrimination"**. Furthermore, the immune system in most cases is able to recognize any alterations in the host cells that may lead to cancer. Typically, pathogen recognition by the immune system triggers the activation and recruitment of various immune cells and molecules to mount an appropriate response, called an **effector response**, to eradicate or neutralize the organism. In this way the immune system is able to convert the initial recognition event into a variety of effector responses, each uniquely suited for eliminating a particular type of pathogen. Later exposure to the same foreign pathogen induces a **memory response**, characterized by a more rapid and elevated immune reaction that serves to better eliminate the pathogen and prevent disease. It is this outstanding property of "immunological memory" that protects us from recurrent infection with disease-causing pathogens. Additionally, immunological memory is the basis for vaccination, which involves educating the immune system about the nature of the pathogenic insult to prepare the host for a subsequent attack by the pathogen.

Therefore, in summary, the main functions of the immune system are the recognition with subsequent elimination of foreign antigens, formation of immunological memory, and development of tolerance to self-antigens. For performing these functions, the immune system relies on signal transduction through receptors that connect immune cells to the extracellular environment and mediate communication among the different types of immune cells. *This thesis focuses on the host's initial recognition of the pathogen and the underlying effector immune responses, which comprise what is known as the* ***"Host-Pathogen Interaction"***.

1.1.2. Innate and Adaptive Branches of the Immune System

The immune system in vertebrates is comprised of two major arms, the innate immune system and adaptive immune system, both of which are interrelated and required for the host immune defense. As a consequence of its ancestral history, the innate immune system is the ancient form of host defense passed on along the evolution from invertebrates, whereas the adaptive immune system is the more recently evolved acquired form of immunity that is uniquely present in vertebrates. In plants, primitive animals, and invertebrates, the innate immune system is the common defense system. In vertebrates, including humans, although these two systems of

immunity operate in a highly coordinated, and often interdependent synchronized fashion, they also work on very different timelines and employ different mechanisms in the removal of that which is recognized as non-self or foreign to achieve maximum protection for the organism (Kumar and Bot, 2013; Lee and Iwasaki, 2007).

The innate immune system acts as the first line of host defense against pathogens, which acts very early after infection (within minutes) for the eradication of a wide range of pathogens, through its ability to rapidly detect and respond to broad cues from these pathogens. The innate immune system includes defenses that, for the most part, are constitutively present and ready to be mobilized upon infection. Innate immune cells are able to recognize a broad range of pathogens utilizing germline-encoded innate immune receptors (will be explained extensively in the following section **1.2**) thereby providing a fast-acting broad-spectrum defense.

Adaptive immunity, on the other hand, is the second line of defense that is involved in elimination of pathogens in the late phase of infection. In contrast to innate immunity, it takes several days to become fully activated the first time it is challenged with a particular pathogen, and is considered highly specialized as it can adapt to recognize virtually any non-self molecule (antigen). Most importantly, the adaptive immune system affords protection against re-exposure to the same or closely related pathogen, through its unique ability to form immunological memory. Accordingly, the adaptive immune system unlike the innate immune system "remembers" that it has encountered an invading organism and reacts more rapidly on subsequent exposure to the same pathogen. The term **'antigen'** is actually defined as any substance that can be recognized and responded to by the adaptive immune system; therefore adaptive immunity is also referred to as "antigen-specific immunity". Microorganisms that are encountered daily in the life of a healthy individual cause disease only occasionally, as these microbes are detected and destroyed within minutes or hours by innate defense mechanisms. Furthermore, in addition to providing early defense against invading pathogens, the innate immune system plays a key indispensable role of triggering and driving adaptive immunity, in order to mount a more effective immune response against infectious agents or danger signals, which is long-lasting, antigen-specific, and has immunological memory. This key role of the innate immune system will be explained in detail in section **1.2** (Medzhitov and Janeway, 2002; Mueller, 2010).

In summary, the two arms of immunity, afford an immune response to pathogens that is

‘**biphasic’** consisting of: the initial and extremely rapid (few minutes) innate immune response, which is considered non-specific, yet remarkably effective in clearing most insulting microbes; and the later antigen-specific adaptive immune response that takes more time (days to weeks) after the initial infection to become effective, but provides the fine antigenic specificity required for complete elimination of non-destroyed pathogens and is long-lasting through its generation of immunological memory. Immunological memory, guarantees that subsequent encounters with the same pathogen will rapidly activate adaptive immunity, in contrast with the initial encounter of pathogen that usually requires one to two weeks for activation of adaptive immune responses (Iwasaki and Medzhitov, 2010; Janeway, 2013).

Effective host defense against a wide variety of pathogens in the environment requires well-coordinated innate and adaptive immune responses. Innate and adaptive immunity were originally thought to be two separate arms of the immune system, but recent evidence in the past decade, demonstrates that although each of these subdivisions of the immune system plays distinct and key roles, protective immunity against pathogens is achieved by interplay between these two systems (Geijtenbeek and Gringhuis, 2009; Gordon, 2002; Hardison and Brown, 2012; Iwasaki and Medzhitov, 2010; Kabelitz and Medzhitov, 2007). Once insulting pathogens gain hold inside the host, they require the concerted efforts of both innate and adaptive immune responses to clear them from the body. Even in these cases, the innate immune system performs a valuable delaying function, keeping pathogen numbers in check while the adaptive immune system gears up for action. In this context, both arms of the immune system are composed of cellular and soluble components (known as humoral components; explained further below), which work collaboratively as a complex integrated system to afford protective immunity to the host (Akira et al., 2006; Kumar and Bot, 2013). Cells of the innate immune system such as granulocytes, macrophages or Natural Killer (NK) cells are immediately available to fight efficiently and kill a broad range of pathogens in order to contain the infection as soon as possible, but are thought not to confer specificity or immunological memory to the host defense. In contrast, adaptive immune responses, such as the production of antibodies or the clonal expansion of specific T and B cell clones, confer specificity towards antigens, and often, lifelong protection to re-infection with the same pathogen through the generation of memory T and/or B cells. The specificity to the response against a particular pathogen and immunological memory are believed to be the main features that distinguish adaptive from innate immunity. Nonetheless,

the importance of innate immunity is highlighted by the fact that defects in its components, which are very rare, can lead to increased susceptibility to infection, even in the presence of an intact adaptive immune system (Gellert, 2002; Lee and Iwasaki, 2007; Medzhitov, 2007a; Parkin and Cohen, 2001).

1.1.2.1. The Innate Immune System

The innate immune system is the primary, or early, barrier to infectious agents, that acts immediately to eradicate these agents. The ability of the innate immune system to immediately recognize and respond to infection is reliant upon germline encoded receptors known as pattern-recognition receptors (PRRs) [explained further in section 1.2.1]. As the body's first line of defense against foreign invaders, innate immunity provides the basic defense system of general structural barriers between the environment and the interior of the host, preventing access of pathogens to potential targets inside the body. Moreover, the innate immune system is constituted of a complex mixture of multiple cell populations, in addition to humoral or soluble chemical components, that all serve to protect the host from pathogenic infection. The various cell types of the immune system, such as phagocytes, NK cells, and granulocytes, function to recognize and destroy the pathogen. The humoral effectors of innate immunity consist of families of soluble proteins such as complement and antimicrobial proteins (AMPs), which are important for the initial neutralization or elimination of a vast and diversified range of microbes and their products. Innate immunity includes four primary barriers: Anatomic, physiological, phagocytic and inflammatory. Anatomical barriers include physical barriers to pathogen entry such as skin and mucous epithelial membranes (mucosal epithelia), whereas physiological barriers include body temperature, low pH in the stomach, antimicrobial enzymes and proteins such as antimicrobial peptides (AMPs) (e.g., defensins) and lysozyme (abundant in secretions such as tears, saliva, human milk, and mucus), interferon, and complement. Both anatomical and physiological barriers act to inhibit the growth and spread of microorganisms (Parkin and Cohen, 2001; Werner et al., 2011; Wilson-Welder et al., 2009).

The first barrier encountered by invading pathogens is the anatomical barrier comprising the epithelia that line the internal and external surfaces of the body (skin and mucosal epithelia). Skin protects the host's external surface from infection through various modes of action. One critical mechanism for this self-protection is the innate production of antimicrobial peptides (AMPs) by

the epidermis, the outermost layer of skin. Keratinocytes, the predominant cells of the epidermis, are the main source of AMPs in normal human skin, but once inflamed the recruited leukocytes (white blood cells) contribute to the majority of antimicrobial activity. AMPs are major contributors to cutaneous innate immunity, and this system, combined with the unique ionic, lipid, and physical barrier of the epidermis, forms the first-line defense against microbes (Abbas, 2003; Kimbrell and Beutler, 2001).

In addition to external anatomical barriers to microbial invasion, the innate immune system involves a variety of cell types. These cells are mainly: "professional" innate immune cells such as Natural Killer (NK) cells, granulocytes, and phagocytic cells including neutrophils (granulocytes), macrophages, dendritic cells (DCs), in addition to "non-professional" somatic cells such as mucosal epithelial cells and keratinocytes. The phagocytes lie beneath all epithelial surfaces. The innate immune response is mainly mediated by these phagocytic cells, which act to engulf and digest invading microorganisms once they encounter them (Kumar and Bot, 2013; Medzhitov, 2007a).

In the situation that an infectious organism breaches the external anatomical barriers of the host, several innate immune mechanisms start acting immediately to control the infection, mainly through both cellular and humoral means. Phagocytosis is a major part of the innate cellular response, while humoral immunity, depending on the host, includes several classes of preformed soluble molecules present in blood, extracellular fluid, and epithelial secretions that can either kill the pathogen or weaken its effect. **Antimicrobial enzymes** such as lysozyme begin to digest bacterial cell walls; **antimicrobial peptides (AMPs)** such as defensins lyse bacterial cell membranes directly; and a system of plasma proteins known as the **complement system** targets pathogens both for lysis and/or for phagocytosis by cells of the innate immune system such as macrophages. In the case of phagocytosis, **complement molecules** 'opsonize' or coat the microbe in order to flag them for recognition by complement receptors, e.g., CR3 (Complement receptor 3), present on phagocytic cells, which in turn mediates the ingestion of the microbe by phagocytosis which is subsequently lysed and killed inside the cell. This process of coating the microbe is known as 'opsonization' and is mediated via the opsonic recognition of the coated microbe by specialized phagocytic receptors (Goodridge et al., 2012; Tsoni et al., 2009). Type 1 interferons have potent antiviral activity and are produced mainly by fibroblasts and monocytes as a reaction to infection (Parkin and Cohen, 2001; Wilson-Welder et al., 2009).

After infectious agents make it through the epithelium and enter the tissue, they then encounter cells of the innate immune system such as macrophages, which function to recognize and destroy them (Janeway and Medzhitov, 2002; Kumar and Bot, 2013). This tissue invasion by the pathogen also triggers inflammation, which increases blood flow, as well as vascular permeabilization into infected areas, allowing for massive influx of phagocytic leukocytes including monocytes, macrophage, neutrophils and dendritic cells from the bloodstream. A central feature of the innate reaction is recruitment and activation of neutrophils at the site of infection to eradicate pathogens; a process, which if occurs inappropriately leads to uncontrolled excessive inflammation that has detrimental effects on the host (Wilson-Welder et al., 2009).

Once innate immune cells recognize pathogens or infected cells, they can act in several different ways, both directly and indirectly, to contribute to pathogen clearance. Directly, these cells phagocytose, kill and digest foreign pathogens. Indirectly, these cells, once having made contact with foreign microorganisms, will be triggered to release a variety of soluble mediators, mainly cell signaling **cytokines**, that will provide important information to the adaptive immune system as to what type of response is required (Akira et al., 2006; Lee and Iwasaki, 2007; Takeda and Akira, 2004).

Natural Killer (NK) cells function in cell-mediated, cytotoxic innate immunity, where they destroy infected cells by releasing toxic molecules that induce cell death. Phagocytes, which include macrophages, DCs, and neutrophils, can engulf microbes or infected cells at sites of infection. Basophils stimulate vasodilatation through the release of histamine, and play a role in controlling parasitic infections. Eosinophils release free radicals and toxic proteins that can kill extracellular microbes and parasites. Moreover, all of these cells are capable of secreting cytokines and chemokines, which can further recruit or activate other nearby immune cells and trigger inflammation. **“Cytokines and chemokines”** are specific groups of signaling molecules that are used for communication between neighbouring cells of the immune system (Akira et al., 2006; Kabelitz and Medzhitov, 2007; Wilson-Welder et al., 2009).

Therefore, in summary, innate immunity functions as a first defense line that provides a primary protective effect against invading pathogens via antimicrobial activities such as phagocytosis, or secretion of microbicidal compounds that neutralize the microbes, as well as through the induction of inflammatory responses. Pathogen recognition by innate immune cells enables them

to elicit all of the above-mentioned innate immune responses, in addition to initiating and instructing the adaptive arm of immunity for the effective control or elimination of infection. Once pathogens have gained hold in the host, they require the concerted efforts of both innate and adaptive immune responses to clear them from the body. Even in these cases, the innate immune system performs a valuable delaying function, keeping pathogen numbers in check while the adaptive immune system gears up for action. Innate immune cells upon recognition of pathogens trigger adaptive immunity through their stimulation of: the synthesis and release of proinflammatory cytokines and chemokines, production of costimulatory signals, and antigen uptake and presentation (Janeway, 2013; Janeway and Medzhitov, 2002; Medzhitov, 2007a; Parkin and Cohen, 2001; Wilson-Welder et al., 2009).

1.1.2.2. The Adaptive Immune System

A great majority of animal species deals with the microbial insult relying exclusively on the innate immune system. However, vertebrates have evolved adaptive immunity as a second line of defense that is contingent upon initiation of innate immunity, and which is highly antigen-specific, long-lasting, and able to form immunological memory. Adaptive immune responses become fully activated several days or weeks after infection with the pathogen, and it could take much longer to become fully activated if it is the initial time that the host is challenged with that pathogen (Kumar et al., 2009a; Lee and Iwasaki, 2007). Nevertheless, in contrast to innate immunity, specificity is the hallmark of the acquired immune system, which includes B lymphocytes (B cells) and T lymphocytes (T cells). This unique characteristic of adaptive immunity is mediated via clonal selection from an enormous pool of antigen-specific B and T lymphocytes bearing unique antigen-specific receptors that are generated through a process known as somatic gene rearrangement. This results in a vast and diverse repertoire of somatically rearranged antigen receptors, namely T cell receptors (TCRs) and B cell receptors (BCRs), with the ability to recognize a large spectrum of antigens with fine distinctions between closely related molecules. Therefore adaptive immunity affords much greater diversity against pathogens than the innate immune system (Abbas, 2003; Pasare and Medzhitov, 2004a; Wilson-Welder et al., 2009).

All white blood cells (leukocytes) of the immune system have their origin in the bone marrow, and they include myeloid cells (such as monocytes, macrophages and dendritic cells, as well as

granulocytes: neutrophils, basophils, eosinophils, and mast cells), and lymphoid cells or lymphocytes (B lymphocyte, T lymphocyte and NKs), which differentiate along distinct pathways. B lymphocytes (B cells), and T lymphocytes (T cells) are the major cells involved in adaptive immunity. T cells (for cell-mediated, cytotoxic adaptive immunity), and B cells (for humoral, antibody-driven adaptive immunity) develop from a common lymphoid hematopoietic progenitor (stem) cell within the bone marrow. B cells remain within the marrow for the duration of their development, but for T cell development the precursor T cells must migrate at an early stage as thymocytes to the thymus where they undergo TCR gene rearrangement and differentiation into mature functional T cells. Accordingly, B and T cells derive their letter designation from their sites of maturation in the bone marrow and thymus respectively (Arstila et al., 1999; Wilson-Welder et al., 2009).

As mentioned above, specificity is the hallmark of the adaptive immune system. The immune system must recognize millions of potential antigens; nevertheless, there are fewer than 25,000 genes in the human body, so it's impossible to have one gene for every antigen. Instead, the DNA in millions of B and T cells is shuffled by controlled mutagenesis to create cells with unique B cell and T cell receptors, each of which can bind to a different antigen. This involves an unusual process of random gene rearrangement and splicing together of multiple DNA segments that code for the antigen-binding areas of the receptors (complementarity-determining regions). This process known as 'somatic gene rearrangement' or 'somatic recombination' (also known as V(D)J recombination) occurs early in the development of the B and T cells, before exposure to antigen, which leads to the production of a repertoire of over 10^8 T cell receptors and 10^{10} antibody (Ab) specificities adequate to cover the range of pathogens likely to be encountered in life (Abbas, 2003; Arstila et al., 1999; Luckheeram et al., 2012). Therefore both B and T cells are capable of generating totally novel immunoreceptors with much greater "genetic diversity" than would otherwise be possible in the mammalian host.

In the thymus, precursor T cells or thymocytes possessing both CD4 and CD8 surface molecules ($CD4^+$ $CD8^+$ thymocytes), further differentiate and mature into two well-defined populations of mature T cells: **$CD4^+$ T-helper (Th) cells**, and **$CD8^+$ T cytotoxic (Tc) cells/cytotoxic T lymphocytes (CTLs)**, which can be distinguished from one another by the characteristic expression of CD4 and CD8 membrane glycoproteins on their surfaces, respectively. A third type of T cells, called T regulatory **(Treg)** cells, also displays $CD4^+$ on its surface, but is distinguished

from other T cell types by the presence of additional cell surface markers. In the bone marrow, the BCR of mature B cells is a membrane-bound form of immunoglobulin (antibody) molecules.

Mature T and B cells that emerge from the thymus and bone marrow, respectively, despite having undergone gene rearrangement are "naïve" — i.e., they haven't previously encountered their specific antigen during an immune response. In the thymus and bone-marrow only a few of the naïve T and B cells are able to interact with their specific antigen, therefore in order to have a better opportunity to encounter foreign particles, these cells are deployed to the secondary lymphoid organs including the spleen, lymph nodes, tonsils, and mucosa-associated lymph tissue (MALT). Lymphoid tissues provide a better microenvironment for interaction with antigens. The first contact of a naïve T or B cell with its specific antigen is called 'priming' and causes activation and differentiation into effector T or B cells.

The structures recognized by TCRs and BCRs are generally protein, but despite the similarities in gene rearrangement processes, the T and B cell receptors recognize antigen differently. An epitope (antigenic determinant) is the part of the antigen recognized by the immune system, specifically by antibody or BCR, and which is capable of eliciting an immune response. BCR and antibody recognize the conformational structure of antigenic epitopes and such antigens do not require processing. Accordingly, BCR and antibodies are capable of recognizing free antigens, as well as surface antigens on extracellular pathogens. On the other hand, the T cell receptor (TCR) cannot recognize antigenic epitopes but is able to bind linear peptides usually of eight to nine amino acids, which is generally an antigen that has been broken down by intracellular processing. Additionally, these peptides are recognized only if they are bound to cell membrane glycoproteins known as **MHC (major histocompatibility complex)** molecules, which are of two major types: **Class I MHC** molecules **(MHC I)**, expressed by nearly all nucleated cells of vertebrate species, and **Class II MHC** molecules **(MHC II)** which are expressed only by antigen-presenting cells. **$CD4^+$ T-helper (Th) cells** generally recognize antigen peptides combined with class II MHC molecules (MHC II), whereas **$CD8^+$ T cytotoxic (Tc) cells** recognize antigen peptides bound to class I MHC molecules (MHC I). The processed antigen is loaded within the peptide-binding groove of the MHC molecule. Therefore TCRs, unlike BCRs, can only recognize a foreign antigen peptide that has been generated inside cells, and which is displayed on the cell surface as a membrane-bound complex in association with an MHC molecule **(peptide-MHC complex)**. This mechanism of antigen recognition by T cells is considered as a "safeguard"

against unregulated activation of T cell immune responses. When antigens are presented in this form for the very first time to inactive mature naïve T cells, this leads to **cell priming** and activation, and subsequently differentiation. This key process, where antigens are internalized, processed and displayed as a peptide-MHC complex to T cells, is termed **"antigen presentation"**, and requires specialized cells of the innate immune system known as professional **"antigen-presenting cells" (APCs)**, mainly DCs and macrophages (Reis e Sousa and Unanue, 2014; Roy and Klein, 2012; Wilson-Welder et al., 2009). APCs continuously capture antigens, process them, and present them to naïve T cells. Antigen-presentation, together with additional cues provided by APCs (explained in **section 1.2.2** below), eventually leads to clonal selection and expansion of T cells with the TCR most specific for the microbial-derived products, and ultimately memory cells that could target the pathogen with very high specificity in future attacks. Despite the remarkable specificity of TCRs and BCRs, evasion of a particular T or B cell clone's receptor could occur, which involves mutation at only one site for the pathogen (Luckheeram et al., 2012; Pasare and Medzhitov, 2004c; Reis e Sousa, 2004a; Wilson-Welder et al., 2009).

Priming and activation of naïve B cells and T cells occurs after they encounter their respective antigens in the periphery within the specialized environment of secondary lymphoid organs, which leads to their proliferation and differentiation into various functional effector cells that eventually leave the lymphoid tissue and home to the disease site in order to drive targeted effector responses. Priming of naïve CD8$^+$ T cells generates antigen-specific cytotoxic T lymphocytes **(CTLs)** that can directly kill infected cells, which forms a powerful component of cell-mediated adaptive immunity. CTLs play a vital role in monitoring body cells and killing any that display foreign antigen (complexed with class I MHC molecules), such as virus-infected cells, tumour cells, and cells of a foreign graft. On the other hand the activation of B cells leads to their differentiation into plasma cells, which secrete antibodies that constitute the humoral arm of adaptive immunity. CD4$^+$ naïve T cells usually differentiate into distinct functional subsets of effector T-helper (Th) cells. Th cells are effector T cells that provide help or activating signals to other immune cells through the production and secretion of specific cytokines, in order to assist in the processes of cell-mediated and humoral immunity (Pasare and Medzhitov, 2004b; Reis e Sousa, 2004a; Stetson and Medzhitov, 2007; Stetson et al., 2004). CD4$^+$ T cells are generally treated as having a pre-defined role as T-helper cells within the immune system. For example, when an antigen-presenting cell expresses an antigen on MHC class II, a CD4$^+$ cell will aid those

cells through a combination of cell-to-cell interactions (e.g. CD40 and CD40L) and through the production of certain cytokines. Nevertheless, there are rare exceptions; for example, sub-groups of regulatory T cells (Tregs), natural killer T cells (NKTs), and cytotoxic T cells (CTLs) express CD4, and all of these $CD4^+$ T cell groups are not considered T helper cells.

The main functional subsets of $CD4^+$ effector T cells are **Th1**, **Th2** and **Th17** or inducible regulatory T cells **(iTreg cells)**, which will be explained in detail in section **(1.2.2)**. Briefly, Th cells are essential for effective immunity against a wide range of pathogens, i.e., extracellular versus intracellular. Th1 cells help in protection against intracellular pathogens (viruses and bacteria) via aiding the activation of cell-mediated immunity performed by macrophages and Natural Killer cells, as well as assisting the differentiation of $CD8^+$ pre-cytotoxic cells into CTLs. On the other hand, Th2 cells help in immunity against extracellular parasites and pathogens, and aid naïve B cells to differentiate into plasma cells that secrete antibodies. Th17 cells help in immunity against fungi and extracellular bacteria.

It is of great importance for the immune system to employ mechanisms for shutting down immune responses after they have successfully eliminated invading organisms, and also for preventing autoimmunity. **Regulatory T cells (Tregs)**, are a subpopulation of T cells that play a key role in controlling immune responses elicited by foreign pathogens or danger signals. Tregs perform this vital function by negatively regulating the responses of other T cells, thereby maintaining tolerance to self-antigens, and abrogating autoimmune disease. This is an important built-in "self-check" of the immune system to prevent excessive immune reactions such as autoimmunity (Diveu et al., 2008).

When a naïve B cell first binds to the antigen that matches the BCR on its surface, it rapidly proliferates and its progeny differentiate into memory B cells and effector B cells called **plasma cells**. Memory B cells display the same BCR as the parent naïve cell but have a longer life span, whereas plasma cells are the activated effector form of B cells that produce antibodies. Antibodies are a family of proteins collectively known as immunoglobulins (Igs), and they are the secreted form of the corresponding BCR with similar antigen specificity. Thus the antigen that activated a specific B cell becomes the target of the antibodies produced by the cell's progeny. Secreted antibodies are the major effector molecules of humoral adaptive immunity, and they are able to mediate a broad range of protective effector functions via several mechanisms.

They serve to neutralize toxins **(neutralizing Abs)**, prevent microrganisms from adhering to mucosal surfaces, activate complement, opsonize bacteria for phagocytosis **(opsonizing Abs)**, and sensitize tumour and infected cells as targets for antibody-dependent cytotoxic attack by Natural killer (NK) cells. Pathogens opsonized by Abs are targets of Fc receptors that are present on the surface of phagocytic cells, thereby mediating microbial uptake and elimination. Therefore, from the above listed functions it is clear that antibodies are key humoural components of innate immunity that act to enhance various elements of the innate immune system (Iwasaki and Medzhitov, 2010; Parkin and Cohen, 2001; Wuthrich et al., 2012). Additionally, there are several classes of antibodies (Igs) that are adapted to function at different locations of the body. Each Ig class has class-specific structural and functional properties generated via class switching that yields antibodies with distinguished amino acid sequences in the heavy chain constant region. The main classes of Abs are IgA, IgG, IgM and IgE.

The conclusion of adaptive immunity is the expansion of antigen-specific lymphocytes that target the pathogen specifically, as well as the formation of memory cells that provide long-lasting specific immunity to mount a stronger response upon the next exposure to the pathogen (Iwasaki and Medzhitov, 2010).

1.2. Pattern Recognition Receptors (PRRs) and Antigen-presenting Cells (APCs): Key Players Linking Innate Immunity to Adaptive Immunity

For decades investigators have been dedicated to increase our understanding of the immune system and its strategies for mounting protection against an enormous numbers of pathogens. Before the discovery of the so-called Pattern Recognition Receptors (PRRs), innate immunity was seen as a crude and unsophisticated part of the immune system. The molecular basis for innate immunity was not known, and it was particularly unclear how innate immune agents such as the cytokines interleukin-1 (IL-1), tumour necrosis factor (TNF), IL-6, and antiviral interferons (IFNs) were induced. An increasing amount of research in the past two decades has shown key links between the innate and adaptive arms of the immune system. Although the main function of innate immunity is to provide a primary and immediate protective effect for rapid elimination of the pathogen, it has been demonstrated through a huge wealth of research in the past 15 years that the innate immune system is also indispensible for the activation of adaptive immune responses. The first step of this key function of the innate immune system is now known to be mediated through the recognition of pathogens by pattern recognition receptors (PRRs) present in innate

immune cells (Janeway, 2013; Kabelitz and Medzhitov, 2007; Kaisho and Akira, 2001; Kerrigan and Brown, 2011b).

Charles A. Janeway, Jr. (1943 – 2003), a Professor of Immunobiology at Yale University School of Medicine, was one of the leading immunologists of his generation. He was famously dubbed the **“Father of Innate Immunity”** because of his renowned work in the field of innate immunity. It was originally thought by T cell and B cell immunologists that innate immune recognition depends solely on specific clones of B and T cell receptors (BCRs and TCRs). However Charles Janeway Jr. revealed that these immunologists had what he called “The Immunologist’s Dirty Little Secret.” They knew that T cells and B cells, the main drivers of adaptive immunity, could recognize proteins, but nevertheless they are incapable of mounting an immune response on their own. Injection of purified proteins and antigens doesn’t lead to immunity, or activation of T cells and B cells. Pure antigen by itself is not capable to elicit the adaptive immune response without the help of special additional substances known as “immunological adjuvants” (Janeway, 1989, 2013; Janeway and Medzhitov, 2002). Adjuvants are substances that generally enhance *in vivo* immunogenicity, i.e., antigen-specific responses and are absolutely necessary to get immune activation with pure proteins. Therefore, adjuvants are essential components of vaccines that are included to promote the recipient’s immune response to the supplied antigen with which they are co-administered. They are generally immunological or chemical agents, and include inorganic (aluminum hydroxide) and/or organic chemicals (paraffin oil), macromolecules or entire cells of certain killed bacteria. This key role of the adjuvant component of vaccines in the activation of the immune system is the so-called “immunologist’s dirty little secret” as originally described by Janeway (O'Hagan and Singh, 2003; O'Hagan and Valiante, 2003; Paul and Germain, 2003). Janeway had a theory in mind for the explanation of this so-called “secret” or the mechanism behind the activation of immunity by adjuvants. In the preceding years, scientists had discovered that in addition to the signal T cells received through their main receptors (TCRs), they required a second signal, or “co-stimulation.” Other researchers had independently shown that certain microbial products like LPS (lipopolysaccharide), a component of bacterial cell walls, had strong adjuvant activities. Janeway’s insight was that the 2nd signal for T cell and B cell activation was linked to recognition of particular molecular microbial patterns associated with the pathogen. With brilliant insight, Janeway predicted, in his opening lecture at the 1989 Cold Spring Harbor Symposium, that specialized receptors (which he termed **pattern recognition receptors**, or

PRRs), which exist in innate immune cells, mainly antigen-presenting cells (APCs), would mediate the host's ability to recognize invasion by microorganisms through the detection of evolutionarily conserved features of microbes (which he called **pathogen-associated molecular patterns** or **PAMPs**). Janeway's brilliant introduction of the concept of "**Pattern recognition"** including PAMPs and PRRs, has been key to Innate Immunology, especially to the recent field of 'Host-Pathogen Interactions', and was an especially prescient aspect of his hypothesis (Janeway, 1989, 2013; Janeway and Medzhitov, 2002; O'Hagan and Singh, 2003; O'Hagan and Valiante, 2003; Paul and Germain, 2003).

2014 marks the 25th anniversary of Charles Janeway's striking hypothesis that receptors on innate immune cells can detect microbial pathogens. Before his remarkable prediction, immunologists were mainly interested in studying T and B lymphocytes, which express highly specific antigen receptors, but Janeway's prediction that direct microbial detection by immune cells other than T and B lymphocytes precedes and is required for subsequent lymphocyte activation, gave birth to a modern era of **"Innate Immune Research**". His hypothesis stimulated new interest by immunologists into this emerging field of Immunology (Gleeson, 2014). Research over the last 25 years has been extraordinarily fruitful and has witnessed major advances in our understanding of Innate Immunity, specifically mechanisms of innate immune recognition and underlying immune responses. Several discoveries, especially in the late 90's have marked the reawakening of the field, where numerous pattern recognition receptors (PRRs) have been characterized, as well as their signaling pathways underlying microbial recognition and how they control the generation of adaptive immune responses (Medhitzov, 2010). Janeway's predictions provided a conceptual framework for our current understanding of innate immune recognition and its fundamental role in the activation of adaptive immunity. It is currently well established in the field of Immunology that innate immune recognition of pathogens is essential to instruct the adaptive arm of the immune response via production of cytokines and chemokines, and key costimulatory signals, as will be explained in further detail below. Therefore, Charles Janeway laid out the major theory that unifies the principles of innate and adaptive immunity and indeed deserved the title "The Father of Innate Immunity" (Finlay and Medzhitov, 2007; Germain, 2004; Rudensky and Chervonsky, 2003; Wagner, 2012).

The early concept that the innate immune system nonspecifically recognizes microbes was greatly challenged by Janeways's proposal of pattern recognition, which has been experimentally

validated by key discoveries in the past two decades, most significantly by the identification, in the late 90's, of the PRR family of Toll-like receptors (TLRs) and their signaling pathways in insects and vertebrates (Akira et al., 2006; Janeway, 1989; Medzhitov et al., 1997). The key discovery of TLRs added considerable weight to Janeway's hypothesis, and specifically enhanced our understanding of how pathogens are recognized by the innate immune system and how this sensing is translated into signaling events that control immune responses. Extensive research on TLR members and their ligands demonstrated that pathogen recognition by the innate immune system is actually specific to broad classes of pathogens, and relies on germline-encoded pattern-recognition receptors (PRRs) that have evolved to detect characteristic microbial components (PAMPs).

In 1996, Jules Hoffmann and coworkers including Bruno Lemaitre, discovered that the gene product '**Toll**' is an essential receptor for host defense against fungal infection in *Drosophila* adult flies (Lemaitre et al., 1996). One year later a mammalian homolog of Toll, human TLR, was discovered by Janeway and Ruslan Medzhitov (a then post-Doctoral fellow in Dr. Janeway's laboratory), and was shown to induce expression of genes (including genes for the transcription factor NF-κB) involved in inflammatory responses (Medzhitov et al., 1997). This human homolog of *Drosophila* Toll is now known as TLR4. Moreover in 1998, Bruce Beutler and colleagues discovered that TLR4 is required for recognition of LPS, the major glycolipid constituent of the cell wall of Gram-negative bacteria (Poltorak et al., 1998). Extensive research over the past two decades has led to the discovery of an increasing number of TLR and non-TLR members of the PRR family and their respective PAMPs, as well as characterization of their underlying signaling pathways. All of these exciting discoveries in the field of microbial recognition by PRRs have provided evidence for the importance of innate recognition of pathogens in the initiation of adaptive immune responses and have culminated in the verification of Janeway's striking theory of pattern recognition (Akira, 2001; Janeway, 2013; Kato et al., 2005; Kerrigan and Brown, 2011b; Medzhitov, 2007a).

In 1973, Ralph Steinmann, together with Zanvil Cohn, discovered a new type of cells that he then named "dendritic cells". This turned out to be a significant discovery that greatly revolutionized our understanding of how innate immunity is activated, and how it eventually triggers an adaptive immune response (Steinman and Cohn, 1973). A huge wealth of research efforts following Steinman's discovery of DCs have established them as the major antigen-

presenting cells (APCs) necessary for activation of adaptive immunity (explained further below in section **1.2.2**)

As described above, the past two decades or more have witnessed rapid progress and major advances in our understanding of microbial recognition by the innate immune system and its key role in host defense. Accordingly, the **2011 Nobel Prize in Physiology/Medicine** was awarded to Ralph Steinmann, Jules Hoffmann, and Bruce Beutler in acknowledgement of their ground-breaking discoveries in the field of Immunology that led to a revolutionary paradigm shift in our understanding of innate immunity, and its impact on the initiation and regulation of adaptive immunity. This Nobel Prize received intensive media attention and highlighted the initial discoveries of Toll's role in immunity in flies, Toll-like receptors in mammals, and the establishment of dendritic cells and PRRs as the key initiators of adaptive immunity (Gleeson, 2014; Lemaitre et al., 1996; Poltorak et al., 1998; Steinman and Cohn, 1973; Wagner, 2012).

1.2.1. Pattern Recognition Receptors (PRRs): Sentinels for Innate Recognition of Microbes and Initiation of Adaptive immunity

Although the innate immune system is very effective with dealing with a vast majority of pathogens, the **"dogma"** that had been blindly accepted for many years in the field of Immunology was that innate immunity was 'non-selective' in the nature of its immune responses, as well as 'non-specific', and 'rather primitive', in the detection of pathogens with specificity being conferred only to the acquired arm of immunity through antigen-specific receptors on B and T lymphocytes (formed by somatic gene rearrangement). It had also long been believed that the innate immune system functions as a temporal defense system until the adaptive system comes into action. However, this simplistic model, in which innate immunity performs only simple 'ingest and destroy' tasks, could not explain how innate immune cells recognize microbial pathogens as 'non-self', or why different classes of microorganisms trigger different immune responses (Janeway and Medzhitov, 2002; Netea et al., 2008). Only in the past two decades it become clear that the innate immune system not only specifically recognizes various classes of microorganisms, but also initiates and mounts subsequent adaptive immune responses (Geijtenbeek and Gringhuis, 2009; Janeway and Medzhitov, 2002; Netea et al., 2008; Roy and Klein, 2012; Takeda and Akira, 2001). This dogma of the "non-selective" nature of innate immunity has been dramatically challenged specifically by substantial research that led to the

discovery of the class of innate immune receptors known as pattern recognition receptors **PRRs**. The innate immune system utilizes these receptors for a defense strategy designed to detect a wide range of pathogens by recognizing broad and evolutionary conserved patterns, which differ between the microorganisms and their multicellular hosts Figure 1: Microbial PAMPs and Pattern Recognition Receptors Involved in Innate Immune RecognitionFor this task of non-antigen-specific detection of pathogens and initiation of appropriate host responses innate immune cells are equipped with an arsenal of germline encoded receptors PRRs that form a diverse and evolutionary conserved family of innate immune receptors (Figure 1)(Janeway, 2002; Medzhitov, 2007a). PRRs recognize invariant molecular structures that are unique to the microbe and not present in the host. These microbial components are structural motifs that comprise characteristic molecular signatures of the pathogen, and which are highly conserved and commonly shared among related groups of microorganisms, and therefore are collectively referred to as **pathogen-associated molecular patterns** or **PAMPs** (Figure 1) (Akira, 2011; Kumar and Bot, 2013; Kumar et al., 2009a; Medzhitov, 2007b).

1.2.1.1. PAMPs

PAMPs are structurally and chemically diverse compounds that share the common features of being highly conserved, critical for microbe survival, and uniquely present in pathogens but absent from the host. The term **"PAMP"** as coined by Charles Janeway in 1989 was conceived as a structural constituent of a pathogen with some critical function such that it could not be mutated without loss of viability or pathogenicity (Janeway and Medzhitov, 2002). Indeed, PAMPs are generally microbial structural components that are essential for the survival and life cycle of the pathogen, and which cannot change without affecting or totally losing microbial viability, pathogenicity or virulence. Accordingly PAMPs are difficult for the microbe to alter and are very stable against mutational variations. This immutability allowed sufficient time for mammalian immunity to evolve germline-encoded receptors to recognize them (PRRs) (Finlay and Medzhitov, 2007; Janeway and Medzhitov, 2002). PAMPs are conserved products of biosynthetic pathways specific to the pathogen and not used by the host, and the product of these pathways are usually key structural components of entire classes of microbes that include specific molecular motifs present within microbial carbohydrates, lipids, proteins, or nucleic acids (Kawai and Akira, 2010; Medzhitov, 2007a). Bacterial PAMPs are often components of the cell wall, such as peptidoglycan, cell wall lipoproteins, lipopolysaccharide (LPS) [a cell wall glycolipid

from gram-negative bacteria], and lipoteichoic acid (LTA) [a major constituent of the cell wall of gram-positive bacteria]. Two important fungal PAMPs are β-glucan and mannans, which are carbohydrate components of the fungal cell wall (Figure 1) (Geijtenbeek et al., 2004; Takeda and Akira, 2004; Uematsu and Akira, 2008). However, because all viral components are synthesized within host cells, the main targets of innate immune recognition in this case are viral nucleic acids. Discrimination between self (host) and viral nucleic acids occurs on the basis of specific chemical modifications and structural features that are unique to viral RNA and DNA, and not present in the host. From the above description of PAMPs, they are well suited to innate immune recognition for three main reasons: **1)** PAMPs are invariant among microorganisms of a given class; **2)** they are products of pathways that are unique to microorganisms, allowing discrimination between self and non-self molecules; and **3)** they have essential roles in microbial physiology, limiting the ability of the microorganisms to evade innate immune recognition through adaptive evolution of these molecules (Janeway, 1989; Rifkin et al., 2005; Romani, 2011).

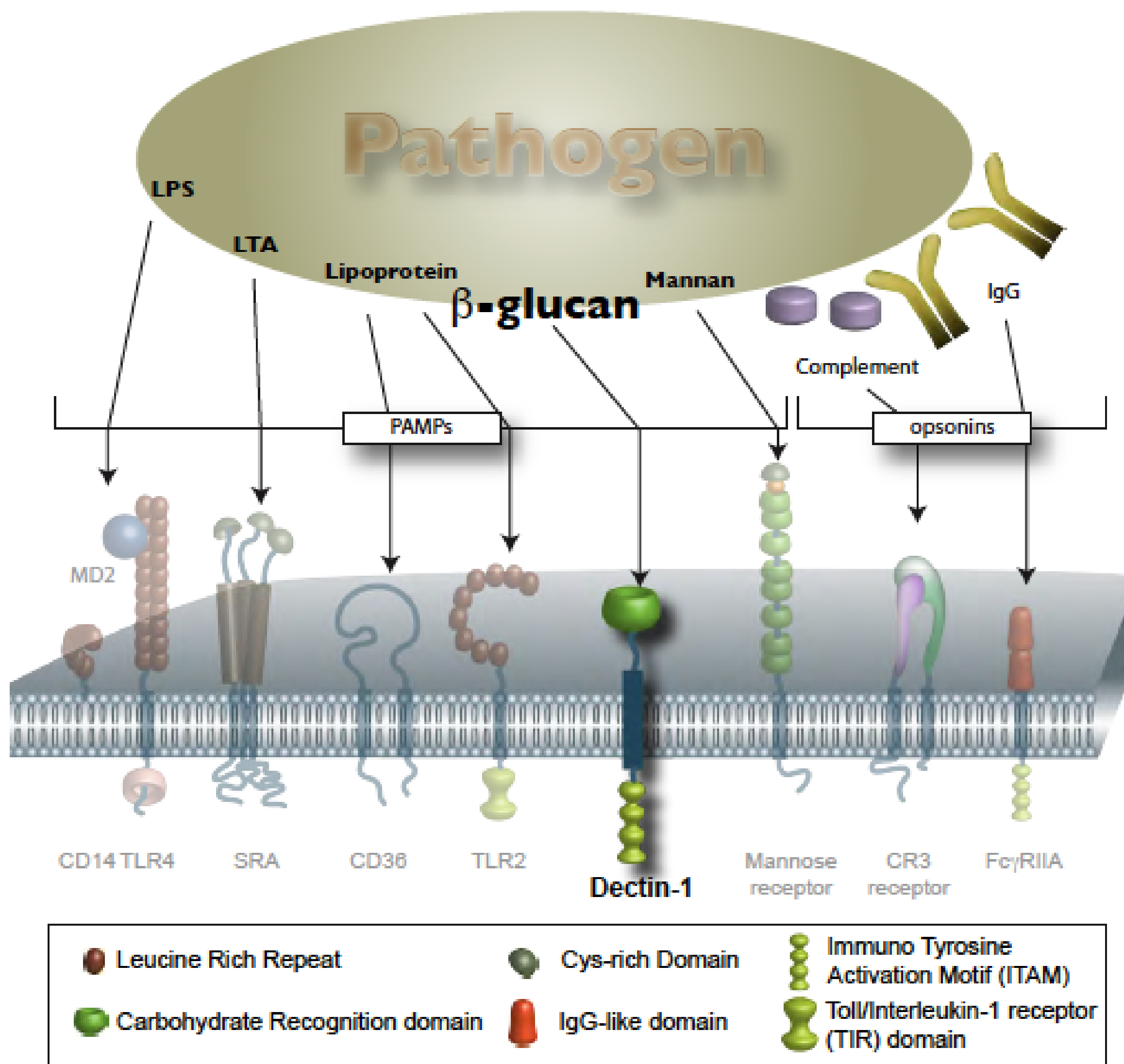

Figure 1: Microbial PAMPs and Pattern Recognition Receptors Involved in Innate Immune Recognition

Cells of the innate immune system are equipped with an arsenal of receptors for either direct recognition of unique pathogen associated molecular patterns (PAMPs) by pattern recognition receptors PRRs or indirect recognition of opsonin-coated (opsonized) pathogens by opsonic receptors including Fc receptor (FcγRIIA) and complement receptor (CR3). Fc receptor (FcγRIIA) detects antibody-coated pathogens, and complement receptor (CR3) detects complement-coated microbes. TLR4 (Toll-like receptor 4), SRA (Scavenger Receptor A), CD36, TLR2 (Toll-like receptor 2), Dectin-1, and mannose receptor are PRRs that detect specific microbial PAMPs that are illustrated in figure: LPS (lipopolysaccharide), LTA (Lipoteichoic acid), lipoprotein, β-glucans and mannan, respectively. CD14 acts as a co-receptor along with the TLR 4 for the detection of LPS.

1.2.1.2. Host-Pathogen Interactions

Entry of a pathogen into the host initiates complex interactions between **1)** an array of pathogen-derived molecules represented by **PAMPs**, and **2)** a myriad of 'host microbial sensors' represented by **PRRs.** These interactions constitute what is known as "**Host–pathogen**

interactions”. The goal of these key interactions is to trigger a battery of immune responses through dynamic networks of innate immune cells and their mediator molecules, for effective eradication of the pathogen and the ultimate production of appropriate pathogen-specific adaptive immunity (Akira et al., 2006; Janeway and Medzhitov, 2002). For that purpose, professional APCs, mainly DCs, are especially equipped with an abundant variety of PRRs that detect microbe-specific PAMPs present on a wide and diverse range of pathogens (Hajishengallis and Lambris, 2011; Janeway and Medzhitov, 2002; Kumar and Bot, 2013).

The PAMP-PRR system of pattern recognition employed by the innate immune system, is exceptional in that each host PRR receptor, unlike antigen-specific B cell and T cell receptors, has a broad specificity and can potentially bind to a large number of molecules that have a common structural motif or pattern (O'Hagan and Valiante, 2003; Olive, 2012). This mechanism of host-pathogen recognition allows a small set of receptors to recognize a wide variety of microbes and microbial products. This broad recognition strategy of pattern recognition allows the innate immune system to respond rapidly to infection. As such, the key advantage of an integrated system of pattern recognition (via PRRs and PAMPs) and antigen specific responses (via BCR and TCR) is that the immune system divides the responsibility of recognizing and eliminating infectious agents by fulfilling two mutually exclusive goals of the primary immune response: **‘speed and specificity’.** (Hajishengallis and Lambris, 2011; Janeway and Medzhitov, 2002; Kawai and Akira, 2005; Kumar and Bot, 2013; Medzhitov, 2007a).

So it is evident from the above description that the **‘PAMP’** and **‘PRR’** concepts introduced by Janeway, have been extremely useful in terms of our modern understanding of “Host-pathogen Interactions” and the crucial role that they play in initiating and establishing immune responses, both innate and adaptive.

1.2.1.3. The PRR Family of Innate Immune Receptors

In the case of a pathogenic attack, it is essential for the innate immune system, as the first line of defense, to be able to recognize a wide range of pathogens to contain infection until the subsequent adaptive immune response is fully activated (Dorhoi et al., 2010). For those key functions innate immune cells including macrophages, dendritic cells and neutrophils act as the front-line of defense against pathogens and are equipped with an arsenal of pattern recognition receptors (PRRs) that comprise a large group of innate immune receptors responsible for innate

immune responsiveness to pathogens (Kabelitz and Medzhitov, 2007). **PRRs** are expressed in innate immune cells as diverse arrays of membrane-bound and soluble receptors, thereby serving as **'sentinels'** for the fast-acting detection and defense against a broad spectrum of pathogens through their recognition of **PAMP** motifs specific to the foreign microbe and shared among common classes of microbes related to the detected pathogen (Dorhoi et al., 2010). This system of pattern recognition of microbes by PRRs serves to detect broad categories of pathogens and microbial products for two crucial functions: activation of immediate innate immune responses against the pathogen to restrict the infection at the very beginning, as well as mounting and shaping the subsequent adaptive immune responses essential for complete and effective clearance of pathogens that are not completely eliminated by the innate immune system (Janeway, 2002; Medzhitov, 2007). PRRs are well suited for these key functions: **1)** They are widely distributed on various innate immune cells as diverse and distinct sets of receptors, where each receptor has a broad specificity for a particular class of pathogens, and **2)** they are strategically located in different cellular compartments: extracellular (secreted PRRs), membrane (cell membrane and endosomes) and cytoplasmic. This diverse distribution of PRRs enable them to effectively monitor the extracellular environment, endosomal compartments, or cytosol for the presence of microbes, by sensing various classes of types of PAMPs including proteins, lipids, carbohydrates, and nucleic acids. Furthermore this diverse distribution of PRRs is of special importance for enhancing ligand accessibility especially in situations where microbes evade recognition by cell surface PRRs and become hidden or compartmentalized inside the host cell. Additionally, PRRs are also expressed in non-immune cell types such as fibroblasts and epithelial cells to enhance recognition of microbes in the epithelia (Abbas, 2003; Akira et al., 2006; Ferwerda et al., 2009; Hajishengallis and Lambris, 2011; Kabelitz and Medzhitov, 2007).

The function of the immune system to protect the host against infectious pathogens greatly relies on signal-transduction that connects immune cells to the extracellular environment, as well as mediates communication among the different cell types. In this context innate immune cells are constantly involved in immune surveillance for foreign intruders and receive a variety of 'input messages' from their environment, including those communicated by **PRRs** that act as **pathogen-sensing "sentinels"**. The cell needs to appropriately process and integrate this information, which is relayed intracellularly through signal-transduction machinery. For that purpose PRRs are connected to intricate signaling networks that translate the detection of the

pathogen to orchestrated immune responses appropriate for effective clearance of the particular type of pathogen detected (Gordon, 2002; Lowell, 2011). Effective sensing of PAMPs by PRRs elicits specific intracellular signaling cascades that involve key effector and adaptor signaling molecules, and which culminate in the activation of several cellular processes that eventually lead to a specific immune defense program tailored to the microbial PAMP recognized by the host. The various cellular responses include, maturation of APCs, (discussed further below in section **1.2.2)**, internalization of microbial PAMPs, antigen processing and presentation, as well as phagocytosis and production of a respiratory burst that involves the production of reactive oxygen species (ROS) in order to destroy the pathogen (Abbas, 2003). Most importantly, downstream signaling from PRRs triggers the production of a variety of transcription factors, such as NF-κB and AP-1 (consisting of c-Jun and c-Fos), leading to the expression of key effector molecules such as: MHC and costimulatory molecules, that are important for activation of naïve T cells (explained further below in section **1.2.2**); as well as, cytokines, chemokines, and anti-viral type I interferons that culminate in the generation of antimicrobial inflammatory responses (Lee and Kim, 2007). The ultimate output of these signaling and cellular responses is the mobilization and instruction of pathogen-specific adaptive immunity aimed to restrict multiplication of the pathogen and destroy pathogens incompletely eliminated by the innate immune system, and therefore inhibit dissemination of the infection, (Medzhitov, 2007a; Olive, 2012; Parkin and Cohen, 2001; Reis e Sousa, 2004a). Therefore PRRs function as key mediators between innate and adaptive immunity. The details of this coupling of innate immune recognition by PRRs to adaptive immunity will be further discussed below in section **1.2.2**.

Although the basic machineries underlying innate immune recognition are highly conserved among species, different PRRs activate specific signaling pathways leading to distinct anti-pathogen responses. Nonetheless, to fine-tune immunity individual PRRs produce distinct but interrelated signaling pathways that collaborate for optimal cellular responses. In fact, each type of pathogen expresses a set of different PAMPs, and the combination of these multiple types of PAMPs on each pathogen serves as a fingerprint that triggers a specific array of PRRs, leading to the integration of different signaling pathways to tailor the immune response against that specific pathogen. The key role of PRRs in producing optimal immune responses against a particular class of microbes, and which is required for protective immunity against specific pathogens, has attracted researchers to exploit different PRRs as potential targets in the development of a new

generation of vaccine adjuvants (Akira, 2011; Iwasaki and Medzhitov, 2010; O'Hagan and Valiante, 2003). It is also noteworthy that dysregulation of PRR-triggered signal activation could lead to pathologic inflammatory responses. In this regard, it has been shown that many of the 'auto-inflammatory diseases', have putatively causative mutations in the genes that encode PRRs or their signaling mediators (Kumar and Bot, 2013).

Since the discovery of the of TLR family of pattern recognition receptors in the late 90's, different classes and multiple members of the PRR family have been identified. Several structurally and functionally distinct classes of PRRs evolved to recognize PAMPs and to induce various host defense pathways. PRRs, although a diverse family of receptors, possess common characteristics. **Firstly,** each PRR has a 'broad specificity' for a specific microbial PAMP that is collectively shared among a common class of microrganisms, which is unlike the exact specificity for a particular pathogen conferred by antigen-specific BCRs and TCRs. **Secondly,** PRRs unlike receptors of the adaptive immune system (BCRs and TCRs), are germline-encoded (do not undergo somatic recombination), 'nonclonal', expressed constitutively in the host, and are independent of immunological memory (Medzhitov, 2007b; Uematsu and Akira, 2008). **Thirdly,** PRRs are expressed on, but not limited to, cells of the innate immune system and, unlike BCRs and TCRs, exist on all cells of a given type.

Although PRRs largely exist as transmembrane receptors on the cell surface they can be broadly categorized into secreted (extracellular), membrane-bound (endosomal and cell membrane), and cytosolic classes. Secreted PRRs (including collectins, ficolins, and pentraxins) bind to microbial cell surfaces, activate the complement system, and coat or 'opsonize' pathogens for phagocytosis. To date, the major classes of membrane-bound and cytosolic PRRs that have been identified include: Toll-like receptors **(TLRs)** and C-type lectin receptors (**CLRs)**, which are mainly transmembrane; and the cytosolic PRRs, nucleotide-binding oligomerization domain (NOD)-like receptors **(NLRs)**, and retinoic acid-inducible gene-I (RIG-1)-like receptors **(RLRs)**. These PRR subfamilies are at the forefront of both extracellular and intracellular pathogen recognition and can sense various PAMPs including proteins, lipids, carbohydrates and nucleic acids of the microbe. CLRs and the TLRs are so far the most important and most extensively studied of these classes, where numerous studies have highlighted their key role in host defense. In contrast, cytosolic NLRs and RLRs have been less characterized, yet they have been currently attracting increased attention (Finlay and Medzhitov, 2007; Kerrigan and Brown, 2011b; Kumar and Bot,

2013; Takeda and Akira, 2005). Figure 1, shows the main classes of PRRs and their corresponding ligands/PAMPs.

Toll-like Receptors (TLRs)

The **TLR subfamily** is the archetypical and best-characterized class of the PRR family. TLRs are evolutionarily conserved from the worm *C. elegans* to mammals. Toll, the founding member of the TLR family, was initially identified as a gene product essential for embryonic development in Drosophila, and later it was also shown to play a critical role in the antifungal response of flies (Lemaitre et al., 1996; Uematsu and Akira, 2008). To date, 13 members of the TLR family have been discovered in mammals, and most of the ligands for these TLRs have been identified (Table 1) (Akira et al., 2006). TLRs are transmembrane proteins comprised of an extracellular or lumenal N-terminal leucine-rich repeat (LRR) domain that mediates recognition of PAMPs (ligands); a transmembrane domain; and a cytoplasmic domain containing a signaling motif homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain (Figure 1) (Bowie and O'Neill, 2000).

Mammalian TLRs can be roughly divided based on their subcellular localization into ‘cell surface TLRs’, that are expressed mainly in the plasma membrane (TLR1, 2, 4, 5, 6, and 11), and are involved in detecting extracellular pathogens; and ‘intracellular TLRs’ that are found exclusively in vesicular compartments, primarily endosomes (TLR3, 7, 8 and 9), and are responsible for detecting intracellular pathogens (Table 1). As seen in Table 1, TLRs recognize a wide variety of microbes, such as bacteria, viruses, fungi and parasites, through recognition of microbial PAMPS or ‘ligands’ including lipids, lipoproteins, proteins and nucleic acid. Endosomal TLRs detect intracellular pathogens, mainly viruses, through recognition of microbial nucleic acid species that need to be internalized to the endosome to induce signaling (Table 1) (Barton, 2007; Ishii and Akira, 2005). For example, TLR9 can detect unmethylated CpG motifs of bacterial and viral DNA, and TLR3 can detect double-stranded (ds) RNA of viruses. Cell-surface TLRs recognize conserved microbial components that are accessible to the cell surface, such as lipids, lipoproteins and lipopolysaccharide. These PAMPs include: (LPS) of Gram-negative bacteria (TLR4), lipoteichoic acid (LTA) and lipoproteins of Gram-positive bacteria (TLR2), bacterial lipoprotein (TLR1), bacterial and viral lipoproteins (TLR6), and flagellin (TLR5). Expression of TLRs is cell-type specific, allowing allocation of recognition responsibilities to various cell types (Akira et al., 2006; Ishii and Akira, 2005; Kato et al., 2005). Some TLRs form functional

heterodimers for further broadening of PAMP recognition. For instance TLR2 forms heterodimers with either TLR1 or TLR6, for the discrimination between, triacyl lipoproteins (TLR1/TLR2) and diacyl lipoproteins (TLR1/TLR6), respectively (Table 1). TLRs also form heterodimers with other PRRs, e.g., Dectin-1 (Dendritic Cell-associated C-type Lectin) from the CLR family, to produce collaborative pathogen recognition and signaling (Kaisho and Akira, 2006).

Ligand binding to TLRs leads to the recruitment of TIR-domain containing adaptor proteins, such as MyD88 (Myeloid differentiation factor 28), TRIF (Toll-interleukin-1 receptor domain-containing adaptor for induction of type I IFNs), and TRAM (TRIF-related adaptor molecule), to the cytoplasmic TIR domain of TLR, which initiates intracellular signaling. Most TLRs use MyD88 (except TLR3 that only uses TRIF) as a key adaptor molecule central to TLR signaling. TLR4 also uses the adaptor molecule TRIF for the induction of type I interferons. Regardless of the adaptor proteins used, all of the signaling pathways triggered by TLRs commonly activate the transcription factor NF-κB to induce the production of inflammatory cytokines (Kawai and Akira, 2006). Furthermore, binding of microbial nucleic acids to endosomal TLRs 3,7, 8 and 9, as well as to TLR4, activates interferon regulatory factor 3 (IRF3) and/or IRF7, to induce the production of high levels of type I IFNs such as IFNβ (Koga et al., 2006). Ligation of TLRs on the surface antigen-presenting cells also induces the expression of costimulatory molecules, and specific cytokine profiles, which in turn drive the activation and differentiation of naïve $CD4^+$ T cells into functional effectors. The ultimate outcome of these signaling pathways is the initiation of adaptive immunity and effective antimicrobial immunity (Medzhitov, 2007b).

Table 1. PRRs and Their Ligands

PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5'triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi

Table 1: Different Families of PRRs and Their Respective PAMPs

The above table shows different classes or subfamilies of PRRs: TLRs, RLRs, NLRs and CLRs. Each class has several members, only some examples of which are shown here. Each PRR recognizes a specific microbial component or PAMP present in a certain class of pathogens (bacteria, virus, protozoa and fungi). Also shown in the table is the subcellular localization of each PRR member (plasma membrane or endosomal/lysosomal). G-D-glutamyl-meso diaminopimelic acid (iE-DAP), and muramyl dipeptide (MDP) are structures of bacterial peptidoglycans. Ss: single-stranded. Ds: double-stranded; CpG: unmethylated "Cytosine —phosphate — Guanine" motif; SAP130: spliceosome-associated protein 130, which is a product of necrotic host cells. Adapted with permission from (Takeuchi and Akira, 2010).

C-type lectin receptors (CLRs) (explained in further detail in section 1.3.1)

C-type lectins receptors comprise a large superfamily of proteins that can recognize carbohydrates moieties of exogenous/endogenous molecules. The term 'lectin' refers to a 'carbohydrate-binding protein', and the 'C-type' designation is classically derived from their calcium-dependency for ligand binding (Zelensky and Gready, 2005). Nevertheless, the term 'C-type' is a misnomer for this protein family because numerous research studies have demonstrated that not all CLRs require calcium for ligand binding (Zelensky and Gready, 2003, 2005). They comprise a large superfamily of proteins that share a carbohydrate recognition domain (CRD), which determines the carbohydrate specificity of the receptor. Historically, before the discovery of PRRs and CLRs, receptors such as the mannose receptor (MR), and the scavenger receptor SRA, (Scavenger receptor class A), have been known to bind to the surface glycoproteins of

pathogens and mediate phagocytosis and elimination of such pathogens (Geijtenbeek et al., 2004; Gordon, 2002) (Figure 1). Currently it is well-documented that most CLRs function as endocytic and/or phagocytic receptors that mediate antigen-uptake, but of interest here is the role of many CLRs as PRRs that serve to recognize and capture a wide variety of microbes such as bacteria, mycobacteria, and fungi by binding to carbohydrate-based PAMPs present in these microbes.

As PRRs, CLRs detect and ingest pathogens for microbial clearance and antigen presentation of antigenic-peptides of the microbe to naïve T cells in order to initiate appropriate adaptive immunity against the invading pathogen (Figure 2) (explained further in section 1.2.2). In addition to their function as antigen-uptake receptors that internalize microbes, many CLRs are key PRRs for the induction of intracellular signaling (Figure 2). These CLRs have the capacity to induce intracellular signaling, either on their own (CLRs with 'intrinsic' signaling capacity), e.g., Dectin-1, or with the help of adaptor proteins or other receptors, e.g., DC-SIGN (**D**endritic **C**ell-**S**pecific **I**ntercellular adhesion molecule-3-**G**rabbing **N**on-integrin) (CLRs with 'dependent' signaling capacity). Upon pathogen recognition or binding to carbohydrate ligands/PAMPS, CLRs induce multiple signaling cascades, which lead to key cellular processes including cellular maturation, uptake of pathogens/ligands as well as antigen presentation. Downstream signaling from CLRs also activates several transcription factors e.g., NF-κB and NFAT (Nuclear factor of activated T cells) to induce inflammatory responses that lead to the activation of antimicrobial mechanisms and triggering of acquired immunity (Gordon, 2002; Lowell, 2011). Interestingly, it is becoming clear that CLRs also induce signaling pathways that affect TLR signaling, thereby tailoring the adaptive immune response to specific pathogens (Figure 2) (van Kooyk, 2008; van Vliet et al., 2008). Another, important physiological function of CLRs is the recognition of glycosylated self-antigens for homeostatic control. In this context, CLRs mediate uptake of various endogenous glycoproteins for the purpose of clearance, maintenance of constant levels of endogenous glycoprotein, and antigen presentation for the immune system to check whether the detected proteins are self, altered-self, or non-self proteins (van Kooyk, 2008). For instance, the macrophage C-type lectin 'MINCLE' is responsible for the detection of an endogenous glycoprotein, spliceosome-associated protein 130 (SAP130), which is produced by necrotic host cells (Table 1). Accordingly, a huge variety of CLRs are expressed on the surface of antigen-presenting cells (APCs), especially DCs, to enable these cells to perform the vital functions mentioned above (Figure 2) (Geijtenbeek et al., 2004; Gordon, 2002).

Although, CLRs recognize carbohydrates on different microorganisms including bacteria, viruses, and fungi, multiple members of the CLR family have been shown to be of special importance in the recognition of fungi through their detection of various fungal PAMPs, and therefore play significant roles in antifungal immunity (Table 1). For example, Dectin-1 and Dectin-2 are CLRs that play key roles in antifungal immunity against diverse pathogenic fungi, by detecting mannan and β-glucans, respectively, on fungal cell walls (Table 1) (Gordon, 2002; Lowell, 2011). Also, the CLR 'MINCLE' senses infection by fungal species such as Malassezia and Candida (Yamasaki et al., 2009).

Cytosolic PRRs (RLRs and NLRs)

In addition to transmembrane PRRs, recent studies have also revealed the importance of non-TLR cytosolic PRRs in pathogen recognition in the cytoplasm including NOD-like receptors (NLRs) and RIG-1 receptors (RLRs). Because double-stranded (ds) RNA, single-stranded (ss) RNA and CpG-DNA are normally not found in the cytosol of mammalian cells, these structures are recognized as (PAMPs) by the respective cytosolic PRRs (Kumar and Bot, 2013). The activation of cytosolic PRRs activates interferon regulatory factor (IRF) 3 and the transcription of the type I interferon (IFN) genes in the infected cells (Finlay and Medzhitov, 2007; Honda and Taniguchi, 2006).

RLRs are cytoplasmic PRRs that mainly detect viral nucleic acids, specifically dsRNA from RNA viruses, and have been shown to be required for the recognition of infection by these viruses (Table 1). There are three receptors in the RLR family: **RIG-I, MDA5** and **LGP2** RIG-I and MDA5, but not LGP2, contain a caspase-associated recruitment domain (CARD) that is thought to interact with other CARD-containing adaptor proteins to initiate intracellular signaling (Table 1). RLR signaling culminates in the activation of NF-kB and IRF3, leading to the production of antiviral type I interferons (Foy et al., 2005; Loo and Gale, 2011).

The **NLRs** comprise a large family of cytoplasmic pathogen sensors. Members of the NLR family share a common domain architecture composed of a central nucleotide-binding domain (NBD) and C-terminal leucine-rich repeats (LRR) (Inohara et al., 2005). NLRs respond to various PAMPs and stress molecules to trigger proinflammatory response. The best-characterized NLRs are NOD1 and NOD2. NOD1 and NOD2 sense structures of bacterial peptidoglycans, g-D-glutamyl-mesodiaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively

(Table 1). Ligation of NOD1 and NOD2 results in the activation of NF-κB, IRF3, and IRF7 to induce the production of proinflammatory cytokines and type 1interferons (Foy et al., 2005; Kato et al., 2005; Yoneyama et al., 2004). As TLRs also recognize bacterial peptidoglycan components, TLRs and NODs synergistically activate proinflammatory cytokine production (Mueller, 2010). NLR family members such as NLRP1, NLRP3, and NLRC4 harbor a 'pyrin' domain or a 'BIR' domain in their N-terminus, and are not involved in transcriptional activation of inflammatory mediators, but instead form components of 'inflammasomes', which are protein complexes that regulate caspase-1 activation. Activated caspases (particularly, caspase-1) play a key role in the proteolytic processing of proinflammatory cytokines from an inactive (e.g., pro-IL-1β) to an active form (IL-1β). In this way, NLRs play a key role in host defense by mediating both the synthesis of proinflammatory cytokines e.g. Il-1β via NF-κB activation, as well as the processing of these cytokines via inflammasome-mediated caspase activation (Elinav et al., 2011; Inohara et al., 2005).

1.2.2. Dendritic Cells: Professional Antigen-presenting Cells (APCs) Bridging Innate and Adaptive immunity

One function of innate immunity is to provide a primary and immediate protective effect against invading pathogens via direct antimicrobial activities such as phagocytosis or secretion of microbicidal compounds that neutralize the microbe. Nevertheless, in some cases the innate immune system is unable to deal with the infection, so activation of the adaptive immunity becomes necessary. Therefore another vital and indispensible role of innate immunity is to mount an adaptive immune response. Specialized innate immune cells known as "professional" antigen-presenting cells (APCs), mainly macrophages and DCs, perform this pivotal function of the innate immune system. They have the capacity to instruct the adaptive arm of the immune response via antigen uptake and presentation, production of soluble mediators including cytokines and chemokines, as well as upregulation of costimulatory molecules [mainly B7 molecules: B7-1 (CD80), and B7-2 (CD86)], which are all biological processes necessary for driving the full activation and differentiation of naïve T cells into functional effectors (Figure 2). The first step towards these crucial processes is the innate immune recognition of pathogens or foreign antigens by PRRs present on the surface of these APCs, which in turn induces intracellular signaling pathways that gives rise to these cellular responses. Detection of the

pathogens by PRRs is accompanied by internalization of the pathogen via phagocytosis, macropinocytosis of soluble antigens (also known as fluid uptake or cell drinking), or receptor mediated endocytosis (Figure 2). This internalization step is then followed by **antigen presentation**, which is the ability of APCs to present antigens on their surface as antigenic-peptide–MHC II complexes for the priming of naïve T cells into activated effector cells (Figure 2) (Mansour and Levitz, 2002; Roy and Klein, 2012).

Dendritic cells (DCs) and macrophages are professional APCs that are distinguished by their unique ability to express high levels of MHC molecules, as well as deliver costimulatory signals to T cells (Figure 2) (van Kooyk, 2008; van Vliet et al., 2008). Professional APCs including DCs and macrophages line all peripheral tissues, where they 'screen' their environment for pathogens or changes in immune homeostasis. APCs have unique plasticity: they are capable of adopting various functional phenotypes depending on the environmental conditions and the external stimuli they receive. They are instrumental in initiating adaptive immunity and pathogen clearance; however, they also actively control several processes involved in tissue repair, clearance of apoptotic cells and maintenance of tolerance to nonharmful self-antigens (van Vliet et al., 2008). Several studies have identified the DC as the principal, most potent, and effective of the professional APCs, owing to its enhanced ability to uptake, process and present antigens as compared with other APCs. Additionally, immature DCs constitutively express high levels of MHC molecules whereas macrophages must be activated by antigen uptake before they express MHC molecules (Akira, 2011; Erbacher et al., 2009; Huang et al., 2001; Iwasaki, 2007).

Owing to its superior capacity of "antigen presentation", the **DC** is considered the **most potent APC** and the key regulator of adaptive immunity. The late Nobel laureate Ralph Steinman was awarded the **2011 Nobel Prize in Physiology and Medicine** for his groundbreaking discovery of DCs in 1973 (together with the late Zanvil Cohen), a discovery that revolutionized our understanding of the immune system. Later on after Steinman's discovery, DCs were established as the main connectors of the innate and adaptive immune systems as will be discussed below (Wagner, 2012).

1.2.2.1. Activation and Maturation of DCs to Initiate Adaptive Immunity

DCs are efficient phagocytic cells, yet their main role is not to primarily destroy pathogens but to serve as highly specialized "professional antigen-presenting cells" that effectively link innate recognition of invading pathogens to the generation of appropriate types of adaptive immune

responses. The DC is therefore more famously ‘coined’ as the most effective of the so-called APCs, very much at the interface between innate and adaptive immunity (Kumar and Bot, 2013).

As mentioned above, DCs although highly phagocytic, don’t primarily serve to merely ingest and destroy pathogens, but to sense and capture pathogen antigens for efficient antigen presentation and production of additional signals essential for the activation of adaptive immunity. For that purpose DCs express an abundant variety of PRRs that are able to recognize a wide range of seemingly unrelated pathogens through the detection of pathogen-specific microbial PAMPs. In their immature state, DCs are well distributed throughout the body, particularly at the portals of pathogen encounter such as the skin, mucosal surfaces, and peripheral tissues, to act as ‘sentinels’ of the immune system for sampling, sensing and capturing almost any invading pathogen (Soloff and Barratt-Boyes, 2010). As guard cells involved in immune surveillance, DCs constantly monitor the extracellular space for foreign antigens, and with high efficiency acquire these antigens, and target them for degradation in lysosomal compartments in order to process them into antigenic peptides that are subsequently loaded onto MHC molecules for antigen presentation. Typically, these MHC molecules are rapidly recycled and reloaded with more recently generated peptides (Figure 2) (Iwasaki, 2007; Lee and Iwasaki, 2007; Wevers et al., 2013).

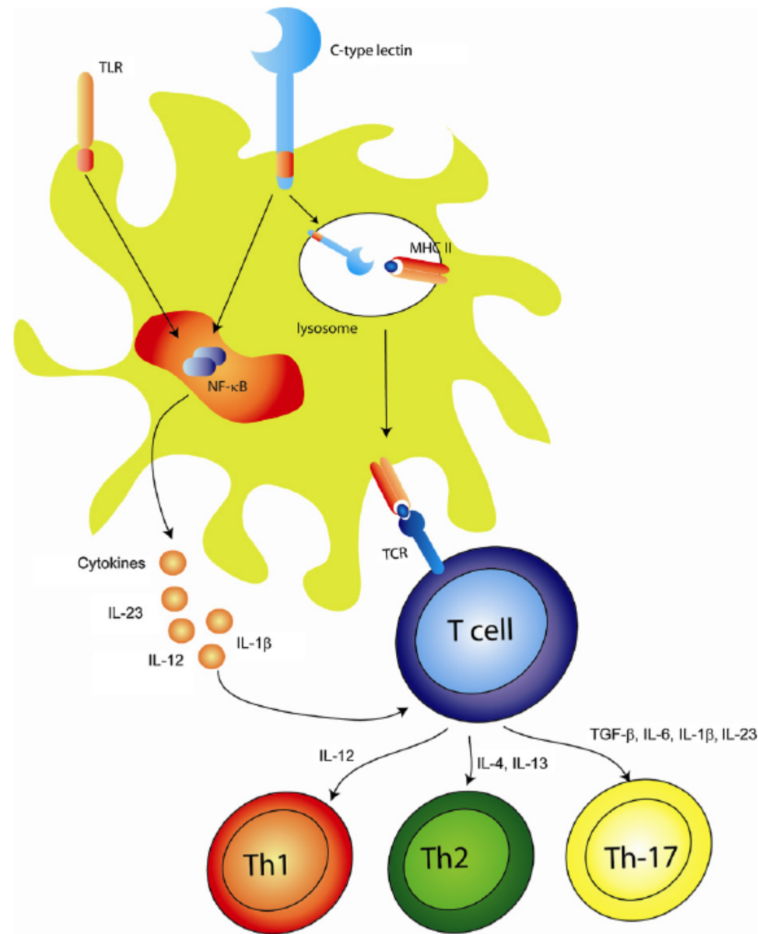

Figure 2: Role of Antigen Presentation and PRR Signaling in the Induction and Shaping of Adaptive Immune Responses

Antigen-presenting Cells (APCs), mainly DCs, express different PRRs, such as Toll-like receptors (TLRs) and C-type lectins, which interact with the pathogen. TLRs induce NF-κB activation that leads to DC maturation and expression of cytokines. C-type lectins capture pathogens for internalization and efficient antigen presentation. However, it is becoming clear that C-type lectins also induce signaling pathways that affect TLR signaling, thereby tailoring the adaptive immune response to specific pathogens. As depicted in figure, the expression of different cytokines by DCs is essential in the induction and instruction of T cell differentiation therby shaping adaptive immune responses: IL-12 induces the development of Th1 cells, IL-4, IL-13 and IL-10 induce Th2 development. Combinations of various cytokines including TGFβ, IL-21, IL-1β, IL-6 and IL-23 promote Th17 development from naïve T cells. Adapted with permission from (den Dunnen et al., 2010).

The initial recognition of pathogenic or foreign antigens at the infection site by cell surface PRR on DCs induces intracellular signaling pathways, which eventually lead to the activation of DCs. Upon activation, DCs undergo a transitional process of maturation where they switch from an "antigen-capturing" phenotype to one that supports "antigen presentation" to T cells. As they mature, DCs lose their antigen/pathogen internalization capacity, and upregulate expression of MHC II molecules and costimulatory molecules (mainly CD80/B7-1 and CD86/ B7-2), as well as

cytokines and the chemokine receptor CCR7 (facilitates migration of DCs) (Figure 2). The cell surface density of MHC II is increased on mature DCs and remains on the surface for several hours (Akira, 2011; McLellan et al., 1995; Medzhitov, 2007a). DCs then abandon residency in peripheral tissues, enter the lymphatic circulation and migrate towards the T cell areas of lymphoid organs, mainly in lymph nodes, in order to present the "antigenic peptide-MHC complex" to naïve T cells and provide them with the appropriate signals required for their activation (Erbacher et al., 2009).

1.2.2.2. Signals Provided by APCs for Priming and Differentiation of Naïve T cells

The development of adaptive immunity is shaped by DCs through three key functions of DCs: **antigen presentation, secretion of specific cytokine patterns, as well as upregulation of costimulatory molecules** These three functions of DCs are necessary for providing crucial signals required for naïve T cell activation and differentiation into functional effectors of the immune system. This pivotal role of DCs highlights them as key regulators of the immune system that bridge innate immunity to adaptive immunity.

In section **1.1.2.2,** I have mentioned that there are two major subpopulations of T cells **CD4$^+$ T-helper (Th) cells**, and **CD8$^+$ T cytotoxic (Tc) cells** characterized by the expression of CD4 and CD8 surface glycoproteins, respectively. CD4 and CD8 act as coreceptors that associate with the TCR to form a receptor complex. As illustrated in Figure 3, TCR binds to the antigenic peptide in the antigen-MHC complex displayed on the surface of APCS. On the other hand, the extracellular domains of CD4 and CD8 bind to conserved regions of the MHC molecule in the antigen-MHC complex on APCs. **CD4** on **Th cells** preferentially bind to **MHC class II** molecules, whereas **CD8** on **Tc cells** binds to **MHC class I** molecules (Figure 3).

Engagement of a specific antigenic peptide-MHC complex on the APC with its cognate TCR on the naïve T cell is not sufficient for activating the T cell. As depicted in Figure 3, the activation of naïve T cells requires more than one signal for activation and subsequent proliferation into effector cells, these signals are of three types namely, **signal 1, signal 2,** and **signal 3**. I will specifically focus in this section on explaining the activation and differentiation of **CD4$^+$ Th cells** by APCs (Luckheeram et al., 2012; Parkin and Cohen, 2001; Pasare and Medzhitov, 2004c; Reis e Sousa, 2004a, 2006; Soloff and Barratt-Boyes, 2010; Wilson-Welder et al., 2009).

The initial step of activation **(Signal 1)** is the antigenic stimulation generated by the intimate and high affinity interaction of the antigenic peptide-MHC II complex on APCs with its cognate TCR-CD4 receptor complex on naïve T cells. Signal 1 activates T cell intracellular signaling pathways that are essential for T cell activation, and thus represents the first and primary pro-activation signal in a T cell. However this signal on its own does not initiate T cell proliferation and differentiation into distinct subsets of effector cells, i.e., it is not sufficient for the amplification of specific T cell responses. The antigen specific clonal expansion of a naïve T cell requires two more additional or "costimulatory" signals that are generally provided by the same antigen-presenting cell. As illustrated in Figure 3**,** these subsequent **costimulatory signals** are mainly divided into signals that are essential for promoting survival and expansion of the T cells **(Signal 2)**, and those that are primarily involved in directing T cell differentiation into the different subsets of effector T-helper (Th) cells **(Signal 3)** (Luckheeram et al., 2012; Pasare and Medzhitov, 2004c; Reis e Sousa, 2004a; Soloff and Barratt-Boyes, 2010; Wilson-Welder et al., 2009)

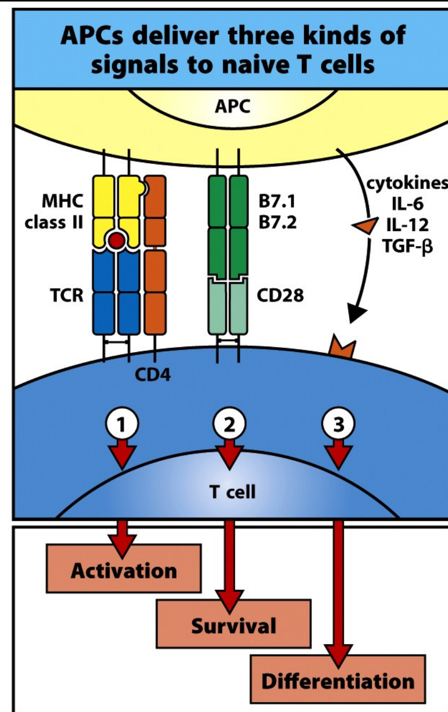

Figure 3: The Three Major Signals Provided by APCs for T cell Priming

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Having received the first signal **(Signal 1)**, the naïve T cell must receive a second independent

signal known as *Signal 2.* Signal 2 is an "antigen-nonspecific" costimulatory signal provided by **costimulatory molecules** expressed on the surface of APCs. As mentioned above these are key surface molecules, which are upregulated upon activation of APCs. The best-characterized costimulatory molecules that deliver *signal 2* are the two related B7 molecules: **B7-1 (CD80)** and **B7-2 (CD86)**, which belong to the immunoglobulin superfamily. As shown in Figure 3, the receptor for B7 molecules (CD80 and CD86) on T cells is **CD28**, which is a heterodimeric membrane protein expressed by all naïve T cells. Ligation of CD28 by B7 molecules induces positive costimulatory signaling, essential for the optimal survival, clonal expansion and proliferation of naïve T cells into effector cells. Activation of PRRs on specialized antigen-presenting cells (APCs), such as dendritic cells, plays a critical role in the priming and activation of T cell adaptive immune responses. Triggering of PRRs on these cells induces the expression of costimulatory molecules (*Signal 2*) on the cell surface, which is as discussed above completely necessary for the activation of naïve T cells specific for antigenic-peptide–MHC II complexes expressed on the same APC. Because the costimulatory molecules are induced by PAMPs, their expression on APCs flags the antigenic peptides presented by the same APC as being of microbial origin and activates antigen-specific T cells (Medzhitov, 2007b; Medzhitov and Janeway, 2002). Therefore *signal 2* is considered as a verification step, which is a protective measure to ensure that a T cell is responding to a foreign antigen. If this second signal is not present during initial antigen exposure, the T cell presumes that it is auto-reactive. This results in the cell becoming 'anergic', i.e., unable to mount an immune response against the encountered antigen. Anergic cells will not respond to any antigen in the future, even if both signals are present later on. These cells are generally believed to circulate throughout the body with no value until they undergo apoptosis.

Finally, *signal 3* is responsible for lineage-specific differentiation of naïve $CD4^+$ T cells into distinct subsets of effector T cells mainly T-helper (Ths1, Th2 and Th17) and Treg cells. This final costimulatory signal **(Signal 3)** is largely provided through the cytokine milieu of the local microenvironment, which is formed by the specific profile of cytokines released by mature activated APCs (mainly DCs) during the initial period of their 'priming' (activation) of naïve T cells (Akira, 2011; Diveu et al., 2008; Drummond and Brown, 2013; Erbacher et al., 2009; Parkin and Cohen, 2001; Pasare and Medzhitov, 2004c; Reis e Sousa, 2006). $CD4^+$ Th cells play an important role in the initiation of immune responses tailored for a particular class of pathogens by

providing help to other cells and by taking on a variety of effector functions specific to the nature of the pathogenic infection. Accordingly, optimal polarization of naïve T cells into specific types of effector T cells is critical for effective immunity against a wide variety of infectious agents in the environment. The mechanism by which an adaptive immune response is skewed towards a certain subset of effector T cells specific to a particular class of pathogens relies greatly on the ability of PRRs on antigen-presenting cells (APCs) to detect certain microbial PAMPs of that pathogen, thereby stimulating the same APC to produce a characteristic pattern of cytokines required for the differentiation of naïve $CD4^+$ T cells into an effector Th subset appropriate for immunity against that pathogen (Figure 4 & Figure 5). Therefore, the cytokine profile produced by the APC largely depends on the characteristic signaling pathways triggered by PRRs specific for the PAMPs of the pathogen (Akira, 2011; Iwasaki and Medzhitov, 2010).

Activated effector $CD4^+$ T cells are key players of adaptive immunity. From a functional perspective, $CD4^+$ effector T cells are subdivided into: **T-helper subsets** that primarily help other immune effector cells in their role to combat infectious pathogens, and **T regulatory (Treg)** that show immunosuppressive activity. Each subset is characterized by the production of distinct signature cytokines and effector functions. An important aspect of antimicrobial activity is the choice of the appropriate weaponry to eradicate a specific type of pathogen, which is facilitated by different subsets of $CD4^+$ T that help in mediating immune characteristic effector functions specific to the invading pathogen (e.g., bacteria, fungi, or parasite) (Geijtenbeek and Gringhuis, 2009; Kurts, 2008).

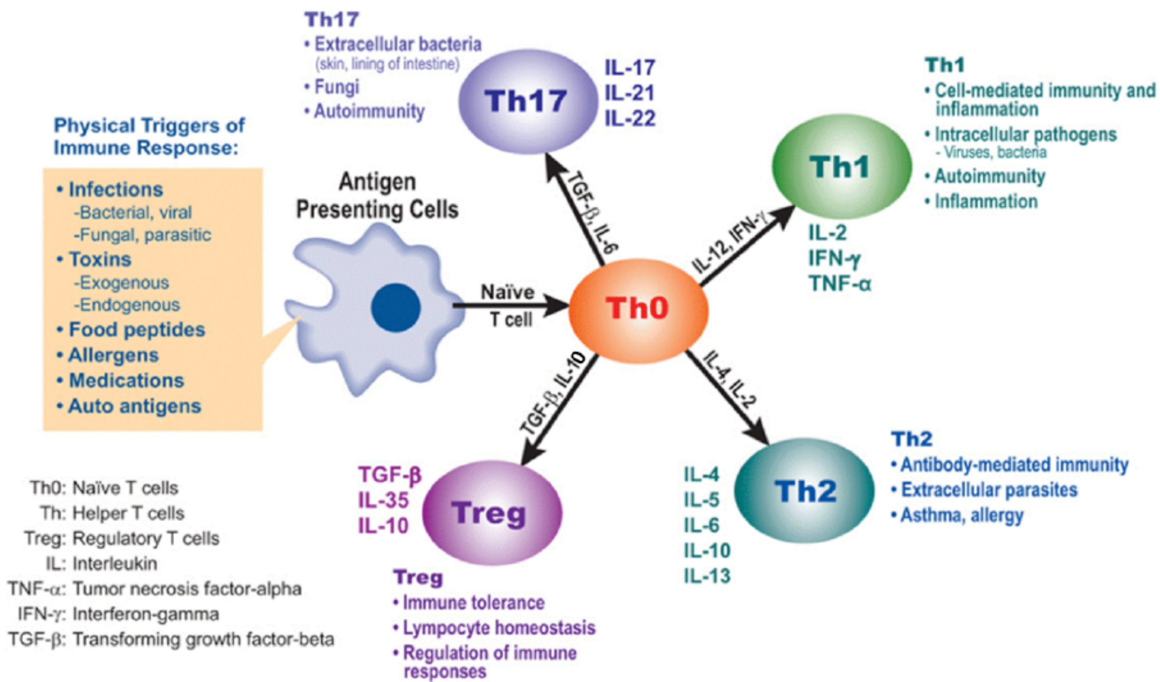


Figure 4: Major Subsets of T-helper (Th) cells: Main Functions and Signature Cytokines

Above Figure shows key cytokines involved in the differentiation of naïve T cells into respective effector T-helper (Th) cell subsets. Also shown are the signature cytokines secreted by each subtype and main functions performed by each subset. The functional differentiation of Th cells is instructed by antigen presenting cells (APCs) of the innate immune system, which provides costimulatory molecules to allow for optimal T cell activation and proliferation, and cytokine production, which mediates the development of naïve T cells into distinct effector Th cell lineages that produce immune responses appropriate for the specific type of invading pathogen. The expression of different cytokine profiles by APCs is essential for the induction of T cell differentiation: IL-12 induces the development of Th1 cells, which activate cell-mediated immunity against intracellular pathogens. Th2 development is mainly induced by IL-4 and mainly mediates humoral (Ab-mediated) immunity to extracellular pathogens and parasites. Combination of the cytokines TGF-β, and IL-6 promote Th17 development from naïve T cells. Th17 cells mediate immunity against bacterial and fungal pathogens, especially at the mucosa. IL-10 and TGF-β promote the induction of Treg cells, which regulate immune responses by limiting collateral damage during immune responses to pathogens. Adapted from http://varuncnmicro.blogspot.ca/2014/04/helpers-available.html

Th1 cells are a key subset of effector Th cells that are primarily made in response to intracellular viruses and microbes that infect or activate macrophages and Natural Killer cells. IL-12 (Interleukin-12) and IFN-γ (Interferon γ) secreted by APCs form the cytokine milieu that favours the differentiation of $CD4^{+}$ Th cells into Th1. Th1-type cytokines tend to produce proinflammatory responses responsible for defense against intracellular pathogens (intracellular bacteria, protozoa and viruses) and for perpetuating autoimmune responses (Figure 4) (Abbas, 2003; Kurts, 2008; Nelms et al., 1999). The signature effector cytokine mainly produced by Th1 cells is IFN-γ, but Th1 can produce other key cytokines such as TNF-α/β and IL-2 (Figure 4).

Th1 cells stimulate cell-mediated immunity, which involves the activation of immune cells including macrophages and NK cells, in order to trigger protection against intracellular pathogens. IL-2 licenses CTL for killing virally infected cells. Finally, Th1 cells could produce excessive proinflammatory responses, which in turn leads to uncontrolled inflammation and tissue damage, which are greatly harmful to the host. In this regard, Th1-cell responses have been associated with autoimmunity (Stetson and Medzhitov, 2007; Stetson et al., 2004; Zhu and Paul, 2008) (Figure 4).

As for **Th2** cells, the cytokines IL-4 and IL-2 provide *signal 3* that stimulates the differentiation of T cells into Th2 effector cells. Th2 cells help in mounting an immune response, specifically humoral immunity, against extracellular parasites, including helminthes (parasitic worms). The key effector cytokines secreted by Th2 cells include IL-4, IL-5 and IL-13, which all control the function of eosinophils, basophils, and mucosal epithelia (Stetson et al., 2004) (Figure 4). IL-4 is considered the signature cytokine produced by Th2 cells. IL-4 plays a major role in promoting humoral immunity by instructing B cells to produce Abs, especially of the IgE class, which are important in defense against parasites through their stimulatory effects on mast cell and basophil activation. On the other hand, Th2 cells also secrete IL-10, which has more of an anti-inflammatory response, and therefore Th2 could counteract Th1-mediated antimicrobial effects to some extent (Luckheeram et al., 2012; Medzhitov, 2007a; Nelms et al., 1999). Th2 effects are also made in response to allergens and toxins. Accordingly, uncontrolled Th2 responses, as well as downstream cytokines IL-4, IL-5, and IL-13, and excessive IgE production have been linked to development of allergic and atopic diseases such as asthma (Cohn et al., 2004; Glimcher and Murphy, 2000; Nelms et al., 1999; Zhu et al., 2004).

Th17 is a recently discovered subset of T-helper cells, characterized by the secretion of IL-17A, IL-17F, IL-22, and IL-21 (Khader et al., 2009; Korn et al., 2009). Th17 cells are a T-helper subset with highly proinflammatory properties. They were initially implicated by their dysregulated appearance in murine models of inflammatory diseases. Nevertheless, Th17 have recently gained favour because of numerous studies that demonstrate their capacity to afford protection against infection with an increasingly broad variety of pathogenic fungi and extracellular bacteria, as well as viruses, especially at mucosal surfaces. Th17 cells are particularly important effector cells in host defense against fungi. However, Th17 cells are not only instrumental in resistance to fungal pathogens and essential for mucosal immunity, but they

are also implicated in various types of organ-specific autoimmunity, a function previously attributed primarily to Th1 cells (Conti and Gaffen, 2010; Korn et al., 2009). In mouse and humans, only the cytokine combination of **TGF-β** plus **IL-6/IL-21**, but neither of them alone, induces differentiation of naïve T cells into Th17 cells associated with robust production of IL-17. Notably, although IL-23 is not an inductive cytokine for the Th17 subset, it is nonetheless required for the stabilization, expansion and maintenance of the Th17 phenotype, which is further amplified by IL-21 and IL-1β (Figure 4) (Awasthi and Kuchroo, 2009; Bar et al., 2014). It is notable that the presence of TGF-β alone, without IL-6, in the cytokine milieu will lead to the differentiation of $CD4^+$ T cells into Tregs instead of Th17 cells (Awasthi and Kuchroo, 2009; Conti and Gaffen, 2010; Khader et al., 2009; Korn et al., 2009). IL-17 (IL-17A) is the signature effector cytokine produced by Th17 cells. Cytokines released by Th17, especially IL-17 and IL-22, are important for the host defense against infection by augmenting neutrophil function and recruitment to sites of tissue inflammation, in addition to inducing strong proinflammatory responses, which are all responses essential for effective bacterial clearance and antifungal defense (Khader et al., 2009). Some of these cytokines, mainly IL-22 can also mediate immune responses appropriate and essential for mucosa-specific immunity. These responses include the maintenance of epithelial cell barrier function, as well as the production of antimicrobial proteins (primarily β-defensins) by epithelial and stromal cells, which can directly kill fungi and other extracellular pathogens (Dorhoi et al., 2010; Romani, 2011). However broad Th17 proinflammatory responses lead to massive tissue inflammation causing tissue destruction (Korn et al., 2009). In this regard, excessive uncontrolled Th17 responses have been shown to play a prominent role in tissue damage associated with mouse models of autoimmune diseases, such as arthritis, multiple sclerosis and colitis; as well as with inflammatory syndromes in humans, including asthma and inflammatory bowel disease (Awasthi and Kuchroo, 2009; Diveu et al., 2008). In the mouse, these inflammatory diseases seem to arise when Th17 cells get activated in closed spaces, such as in joints and in the brain, where neutrophils cannot be so readily disposed of (Khader et al., 2009; Korn et al., 2009; Mansour and Levitz, 2002).

Tregs (T regulatory cells) are $CD4^+ CD25^+$ T cells whose main function is to sustain tolerance to self-antigens and dampen excessive immune responses. They mediate this key function by inducing immunosuppression of responses mediated by the various Th effector cell subtypes. TGF-β is the main cytokine involved in the differentiation of T cells into Tregs, however, in the

presence of TGF-β plus IL-6 or IL-21, the Treg developmental pathway is abrogated, and instead T cells differentiate into Th17 cells. Treg differentiation could also occur in conjunction with other cytokines mainly IL-10. The signature cytokine produced by Tregs is **IL-10**, which is an anti-inflammatory cytokine. These IL-10–producing Treg cells play a central role in suppressing inflammation and autoimmune responses. This is mainly because IL-10 is an important inhibitory cytokine that limits proinfammatory responses and accordingly tissue damage by excessive inflammation. Also, Tregs secrete TGF-β that together with IL-10 supresses IgE antibody production, and thereby Tregs play an important function in attenuating allergic inflammation.

Finally, taken together, it is clear from the above discussion covered in this section **1.2**, that there is significant cross-talk between innate and adaptive immunity, and that they complement one another for providing effective immunity against the pathogen, with DCs playing a key role in bridging these two arms of immunity.

The key events involved in host-pathogen interaction and initiation of host defense are summarized in (Figure 5). Pattern recognition of microbes by PRRs expressed in APCs (mainly DCs), provides the foundation to develop an adaptive immune response, which involves the first key step of binding of microbial PAMPs to PRRs on the surface of APCs. This initiates signaling cascades leading to the maturation of DCs to perform key functions including antigen presentation, and production of costimulatory molecules and cytokines that ultimately generate antigen-specific adaptive immunity and life-long memory against pathogens (Figure 5) (Akira et al., 2006; Ardavin et al., 2004; Arstila et al., 1999; Drummond and Brown, 2013; Geijtenbeek and Gringhuis, 2009; Kabelitz and Medzhitov, 2007; Medzhitov, 2007b).

DCs are uniquely adept at decoding pathogen-associated information, and translating it into qualitatively different adaptive T cell immune responses depending on the nature of the pathogenic infection. They have the ability to distinguish among a wide variety of pathogens via distinct PRRs on their surface that are specific for certain classes or modules (PAMPs) of microbes. This allows DCs to direct tailored adaptive immune responses against the specific module of the invading pathogen by the secretion of a ‘characteristic cocktail’ of cytokines appropriate for effective protection against the class of pathogen detected (Olive, 2012). (Engering et al., 2002a; Erbacher et al., 2009; Geijtenbeek et al., 2009; Geijtenbeek and Gringhuis, 2009; Gordon, 2002; Hardison and Brown, 2012; Wevers et al., 2013; Wuthrich et al.,

2012). This ability of DCs to shape the T cell effector response depending on the nature of the pathogen, make these cells essential gatekeepers that determine how the immune system responds to the presence of infectious agents (Akira, 2011; Iwasaki and Medzhitov, 2010).

Therefore, DCs not only act as sentinels involved in immune surveillance, but also act as key cells at the crossroad between innate and adaptive immunity with the unique ability to control the fate of the immune response. This makes DCs both central to balancing immunity, and a prime target for vaccine development against specific pathogens. In this regard, the distinct PRRs expressed on the cell surface of DCs have been increasingly attracting attention as targets of new vaccine adjuvants (O'Hagan and Valiante, 2003; Ouyang et al., 2008).

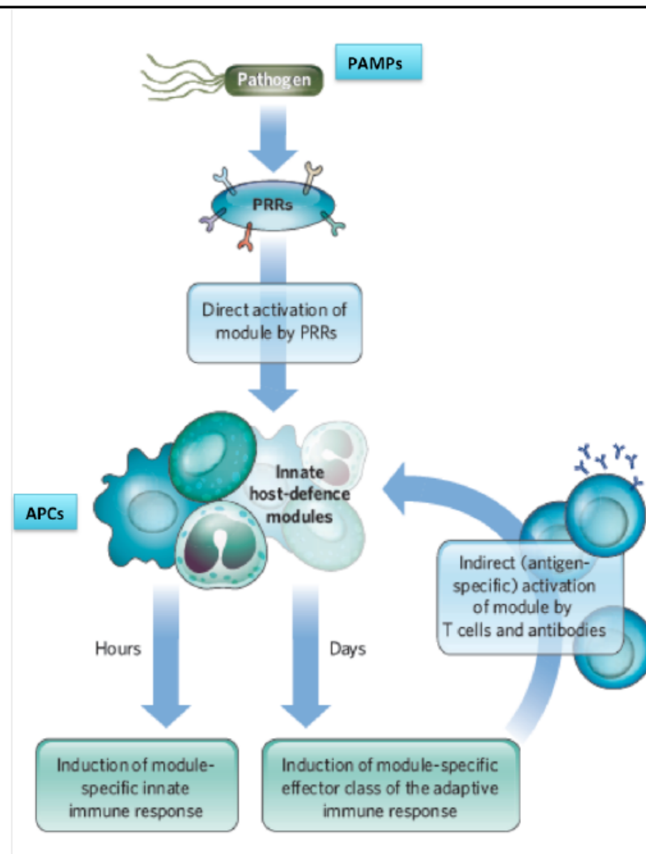


Figure 5: Host-Pathogen Interactions and Host Defense Mechanisms

Host–pathogen interactions begin with the initial recognition of a PAMP (pathogen-associated molecular pattern) module specific to the class of the invading pathogen by PRRs (pattern recognition receptors) present in innate immune cells including antigen-presenting cells (dendritic cells and macrophages). This initiates two phases of module-specific host defense mechanisms: innate and adaptive. Each module of pathogens recognized by the host triggers distinct antimicrobial defense mechanisms and can instruct the adaptive immune system to mount a response involving a module-specific effector class. The first phase of the immune response is specific to the class of the pathogen recognized, and is produced by the innate immune system within hours of the infection. The innate recognition of pathogens by innate antigen-presenting cells (DCs and macrophages) also triggers the second phase of immunity within days of infection, which is antigen-specific adaptive immunity that mediates effector responses

specific to the module of the pathogen. Briefly, detection of PAMPs by PRRs triggers pathogen uptake by APCs that in turn present antigenic peptides of the pathogen to T cells of the adaptive immune system. This key step of antigen presentation, in addition to costimulatory signals and cytokines provided by APCs, leads to the priming and differentiation of naïve T cells into a pathogen module-specific effector type of T-helper cells (Th1, Th2, Th17). Additionally, the adaptive immune response activates B cells to produce antibodies specific to antigens of the pathogen. After an adaptive immune response has been initiated, it results in antigen-specific activation of the same innate immune module that instructed the adaptive immune response. For example, macrophages can be activated either directly by PRRs or indirectly by IFN-γ secreted by Th1 cells, and on the other hand eosinophils (a granulocyte innate immune cell) can be activated by cytokines released from Th2 cells. In combination, innate and adaptive immunity complement one another for providing effective host defense. Adapted with permission from (Medzhitov, 2007).

1.3. C-type lectin Receptors (CLRs) as Pattern Recognition Receptors (PRRs)

1.3.1. The Superfamily of C-type lectin Receptors (CLRs)

As mentioned above in section **1.2.1.3,** the term 'C-type lectin' was originally used to differentiate a group of Ca^{2+}-dependent (and hence the name 'C-type') carbohydrate-binding (lectin) proteins from the rest of the animal lectins (carbohydrate-binding proteins). Later, it became evident through numerous studies that not all C-type lectins bind to carbohydrates exclusively, and that for some, carbohydrate-binding does not require calcium (Zelensky and Gready, 2005). Therefore the term 'C-type' is currently considered a misnomer for this protein family.

The carbohydrate binding properties of C-type lectins were found to reside within a highly conserved compact protein region with a unique structural fold that became known as the "C-type carbohydrate recognition domain (CRD)" or "C-type lectin domain" (Weis and Drickamer, 1996). The CRD contains highly conserved residues that are characteristic of this domain, and sequence homology led to the identification of C-type CRDs in many additional proteins, including ones that did not necessarily bind any carbohydrate structures or calcium. Therefore, although the hallmark of classical CLRs is the dependence on Ca^{2+} for carbohydrate recognition, surprisingly some of the CRD-containing proteins, such as Dectin-1, were found to display carbohydrate recognition that is independent of calcium binding and many other of these proteins have been characterized that preferentially bind in a calcium independent manner to proteins, lipids, or inorganic ligands rather than to carbohydrates (Osorio and Reis e Sousa, 2011; van Kooyk, 2008). All of these non-classical C-type lectin proteins were found to contain large parts of the classical CRD fold, but lack the residues required for coordination of Ca^{2+} ions and/or carbohydrate binding function (Sancho and Reis e Sousa, 2012; Zelensky and Gready, 2005). This contradiction was recently resolved by introducing the designation of 'C-type lectin-like domain'

(CTLD) as a more general term to refer to this more common protein fold, classically known as CRD, (Figure 6). Also to avoid any ambiguity the C-type lectin receptors (CLRs), both classical and non-classical, have been collectively re-named "C-type lectin-like receptors" **(CTLRs)** (Kogelberg and Feizi, 2001; Zelensky and Gready, 2005). The CTLD is now considered a structural motif in protein databases and the terms "C-type lectin" and "C-type-lectin-like" are still used interchangeably in the literature for describing any protein possessing one or more CTLD, regardless of its carbohydrate or calcium binding ability (Sancho and Reis e Sousa, 2012; Xie, 2012). Crystallographic analysis has revealed that the CTLD is a distinct and highly conserved protein structural fold that is normally involved in ligand recognition, and which consists of two protein loops stabilized by three disulphide bridges formed between two highly conserved cysteines at the base of each loop (Figure 6) (Weis et al., 1991a; Weis et al., 1991b). These six cysteine residues are the most conserved CTLD residues (Figure 6) (Kerrigan and Brown, 2009; Osorio and Reis e Sousa, 2011). For the purpose of simplicity and avoiding possible confusion during the context of this thesis, 'CTLD-containing proteins' will be collectively referred to as 'C-type lectin receptors' **(CLRs)** [instead of C-type-lectin-like receptors (CTLRs)], and the 'CTLD' domain will be mostly referred to as **'CRD'**, where 'CLR' and 'CRD' are the terms more commonly used in the literature (Figure 6) (Osorio and Reis e Sousa, 2011; Sancho and Reis e Sousa, 2012; van Kooyk, 2008).

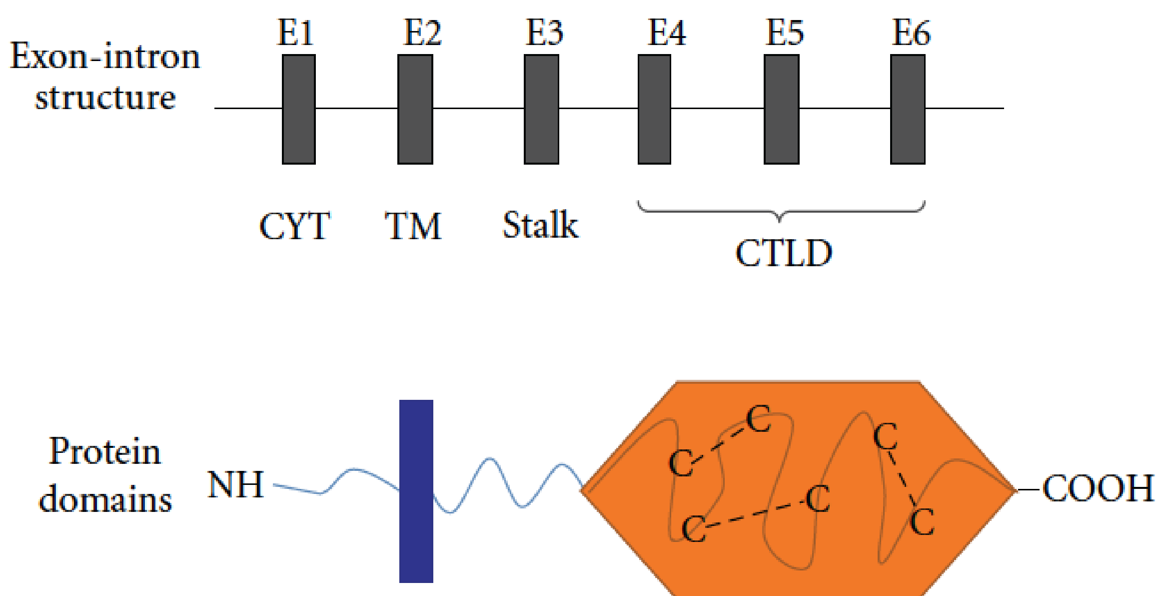


Figure 6: Schematic Representation of the Primary Structure of C-type lectin-like receptors (CLRs)

The above figure depicts the common exon-intron structure of C-type lectin-like receptors (CLRs) and the corresponding protein domains. CLRs are generally transmembrane proteins that are comprised of 4 protein domains: an intracellular cytoplasmic domain (CYT), transmembrane domain (TM), and a stalk region (each of these sections is encoded by one exon denoted as 'E'), and a extracellular C-type lectin-like domain (CTLD) or carbohydrate recognition domain (CRD), which is encoded by three exons (E1, E2 and E3). As depicted in figure, disulfide-bridged bonds between six highly conserved cysteines in the extracellular CTLD stabilize the CTLD proteins fold. Adapted with permission from (Sattler et al., 2012).

Currently, CLRs constitute a diverse and large superfamily of soluble [e.g., mannose binding lectin (MBL)] and transmembrane proteins/receptors [e.g., DC-SIGN and mannose receptor (MR)] characterized by the presence of at least one carbohydrate recognition domain (CRD) [or in the broader sense, a C-type lectin-like domain (CTLD)]. The large superfamily of C-type lectins encompasses more than 1000 members, and includes collectins, selectins, endocytic receptors, and proteoglycans. Despite the presence of a highly conserved CRD domain, members of this family are functionally diverse and have been implicated in various processes such as: cell adhesion, complement activation, tissue remodeling, platelet activation, endocytosis, phagocytosis, pathogen recognition and innate immunity (Osorio and Reis e Sousa, 2011; Weis and Drickamer, 1996). The CLR superfamily is subdivided into 17 groups based on phylogeny (which depends on CRD sequence homology) and domain organization/structure (Table 2) (Zelensky and Gready, 2005). The general protein structure common to CLRs is illustrated in Figure 6. A CLR is mainly a membrane protein that typically consists of four components: an extracellular carbohydrate-recognition domain (CRD/CTLD), a stalk region that links the CRD domain to a transmembrane region (TM), which is followed by an intracellular cytoplasmic domain/tail (CYT) (Figure 6) (Osorio and Reis e Sousa, 2011; Sattler et al., 2012; van den Berg et al., 2012). CLRs possess different numbers of CRDs ranging from a single CRD domain [e.g. DC-SIGN, Dectin-1, and Dectin-2] to eight or ten different CRDs [e.g. MR (mannose receptor)] (Figure 7). Accordingly, membrane-bound CLRs have also been classically divided into two major types based on the number of CRDs and their cellular localization: **Type I** CLRs are type I transmembrane proteins (which have their N terminus pointing outwards) with multiple CRDs, e.g., the mannose receptor (MR) and; **Type II** CLRs are type II transmembrane proteins (which have their N terminus pointing into the cytoplasm of the cell) with a single CRD, e.g. Dectin-1, Dectin-2, and DC-SIGN (Table 2) (Figure 7). This classification is still conceptually being used to date (Table 2) (Hollmig et al., 2009; van den Berg et al., 2012).

C-Lectin-like receptors, their classifications, and ligands

C-Lectin-like receptors	Type[a]	Group[b]	Pathogen-associated molecular patterns
Mannose receptor	I	VI	Mannose, fucose, *N*-acetylglucosamine
DC-SIGN	II	II	Mannin
Langerin	II	II	Mannose, fucose, *N*-acetylglucosamine, mannin
MGL	II	II	GalNAc
ASGP-R	II	II	Galactose
Collectins	II	III	Mannose, fucose, *N*-acetylglucosamine
Dectin-1	II	V	β-Glucan
Dectin-2	II	VI	High-mannose oligosaccharide

Table 2: Representative Examples of CLRs, Their Classifications (Groups and Types), and Ligands

a: "Type" refers to 'carbohydrate recognition domain' (CRD) type. CLRs have been classically divided into two major 'types' based on the number of CRDs and their cellular localization. 'Type I' receptors contain multiple CRDs, (e.g. Mannose Receptor) whereas 'Type II' receptors consist of a single CRD (e.g. Dectin-1 and Dectin-2). **b:** "Group" refers to domain organization and phylogeny as described by Zelensky and Gready (Zelensky and Gready, 2005). There are at least 17 subgroups of mammalian CLRs, which are classified by their phylogenetic relationships and domain structures. CLR Groups II, V, and VI are expressed on myeloid immune cells. Groups II and V usually form receptor oligomers (di-, tri-, or tetramers) on the cell surface, mainly via the formation of disulphide bridges between conserved cysteine residues in the stalk region. Langerin and DC-SIGN belong to the Group II CLRs, whereas Dectin-1 belongs to the Group V NK receptor group. Adapted with permission from (Hollmig, 2009).

1.3.2. Ligand Specificity of CLRs and Oligomerization/Multimerization/Clustering of CLRs

Many CLRs are able to bind to different classes of ligands and have the capacity to recognize both exogenous and endogenous ligands. Although they share structural homology, C-type lectins usually differ significantly in the types of glycans that they recognize with high affinity. Some CLRs recognize N-linked glycans/polysaccharides, whereas others interact specifically with O-linked glycans (Table 2). O-linked structures are often exposed on collagens, mucins and some pathogens, whereas N-linked structures are present on the vast majority of glycoproteins in the body as well as on pathogens, such as retroviruses, that use the glycosylation machinery of the host for their survival and spread. Although some CLRs recognize monosaccharides, such as mannose, fucose or galactose, others recognize more complex sugar moieties present in glycoproteins and glycolipids (Table 2) (Figdor et al., 2002; Robinson et al., 2006). Some CLRs can even bind non-glycosylated lipids and proteins. This multitude of C-type lectin ligands is due

to the versatility of the CTLD fold that has been demonstrated by structural studies, and which allowed divergent evolution away from carbohydrate binding (Zelensky and Gready, 2005).

The specificity of CLRs to carbohydrate structures is primarily determined by the amino acid sequence of the CRD fold, however, it also appears to be strongly influenced by the multimerization/oligomerization state of the receptor, the pattern and degree of branching of the carbohydrate chains, the spacing of carbohydrates on the ligand, and the backbone of the glycoprotein that exposes the carbohydrate structure (Table 2) (Geijtenbeek et al., 2004; van Kooyk, 2008). CLRs can exist both as monomers and oligomers. Nevertheless, many CLRs often tend to oligomerize/multimerize into homodimers, homotrimers, and higher-ordered oligomers/multimers, in order to increase their avidity of binding to multivalent ligands. The presence of single cysteine residues in the stalk/neck region is usually associated with homo- and hetero-dimer formation due to covalent disulfide bonding between these cysteines. Interestingly, the NK 'Group V' of CLR transmembrane proteins consist of an extracellular CRD and a variable-length neck region that in many cases contains cysteine residues involved in covalent homo- or heterodimerization (see section **1.3.5**). The oligomerization of CRDs on the CLRs alters the affinity and specificity of carbohydrate recognition and increases the avidity of binding (Geijtenbeek et al., 2004). Accordingly, some CLRs form oligomers within the cell membrane to strengthen and limit binding to a specific structure with a certain carbohydrate density and spacing (van Kooyk, 2008; van Vliet et al., 2008).

Binding of monosaccharide residues in specific orientations to the CRD domain is sometimes further stabilized by hydrophobic interactions. On the other hand, binding of oligosaccharide ligands with high affinity results from extension of the binding site within a single CRD or from multivalent interactions of multi-antennary glycans with clusters of CRDs (van Kooyk, 2008; van Vliet et al., 2008). In this regard, some CLRs, such as DC-SIGN (Group II CLRs; see Table 2), increase their avidity of ligand binding by clustering or aggregating into multimeric structures on the cell surface. These clusters or multimers of CLRs do not necessarily involve physical interaction or bonding between individual receptors, i.e., these clusters are not necessarily created due to observable formation of physical dimers or oligomers (de Bakker et al., 2007; Gringhuis et al., 2009a; Itano et al., 2011; Neumann et al., 2008). In this regard, Dectin-1, a member of the NK 'Group V' of CLRs, although lacking cysteine residues in its stalk region that are normally required for dimerization of members of this group, has been recently shown to cluster in

response to particulate β-glucan polysaccharide ligands (see section **1.5** & *Chapter 3* below)(Goodridge et al., 2011). Strikingly, on the other hand, DC-SIGN has been shown to form physical tetramers that aggregate into clusters (de Bakker et al., 2007; Gringhuis et al., 2009a; Itano et al., 2011; Neumann et al., 2008). DC-SIGN belongs to 'Type II' CLRs that have a single C-terminal CRD, which is separated from the transmembrane domain by a neck region. In some cases the neck is short, but it is often extended with heptad repeats that mediate trimer- or tetramerization of the polypeptide through the formation of a coiled-coil of alpha helices (Dodd and Drickamer, 2001). Interestingly, some CLRs can occur as multiple homotrimeric units that associate via disulphide bond formation to form supramolecular complexes. The MR (mannose receptor) has also been shown to form physical trimers in the plasma membrane (Mitchell et al., 2001; Neumann et al., 2008). Other C-type lectins that occur as trimers include the trimeric MBP-A (Mannose Binding Protein-A), SP-A and SP-D (Surfactant Protein (SP)-A and –D) of the 'collectin' Group III of soluble/secreted CLRs, which are 'Type II' CLRs with multiple CRDs (see Table 2). In this group of CLRs, the collectin monomer has an N-terminal collagen-like region, which is followed by a section of heptad repeats in the stalk/neck domain that mediate trimerization, and a C-terminal CRD. The CRDs in the collectins, bind mannose-type sugars using a simple binding site, which interacts only with terminal residues. Collectin monomers assemble into characteristic homotrimeric units, each of which has a collagen-like triple helical tail, a coiled-coil neck region, and a rigid cluster of CRDs. The CRD of trimeric lectins is angled to the side of the stalk/neck domain through which the protein associates to form the trimer. The CRDs are at the top of the trimer and can function to enhance multivalent interactions with carbohydrate ligands. Accordingly, these collectin oligomers can bind with high affinity and specificity to arrays of structurally complex and diverse glycans found on pathogen surfaces, due to the multivalent nature of the collectin complexes and the fixed geometry of the CRDs. Similar to collectins, the neck region of the Tetranectin 'Group IX' of CLRs also mediates homotrimerization through the formation of a coiled-coil of alpha helices.

The above discussion indicates that C-type lectins, which have similar basic carbohydrate specificities, can still interact with a very diverse set of ligands, and that clustering/oligomerization/multimerization of the CLR maximizes its chances of binding to a broader range of pathogens and microrganisms.

1.3.3. Functions of C-type lectin receptors (CLRs)

CLRs were originally defined by their carbohydrate-binding function, which enables them to bind to complex oligosaccharides displayed on various biological structures such as cell surfaces, circulating proteins, and extracellular matrices. By binding to these carbohydrate structures, C-type lectins mediate diverse biological processes including a variety of crucial cellular processes such as cell adhesion, serum glycoprotein turnover and quick innate-type immune responses to potential pathogens (van den Berg et al., 2012; Weis et al., 1998). Although many CLRs function as adhesion and signaling receptors in many immune functions such as inflammation and immunity to tumor and virally infected cells, CLRs are better known for their function as internalization receptors (endocytic or phagocytic) for antigen uptake and presentation. CLRs such as DC-SIGN and Dectin-1 (Table 2) are capable of internalizing glycosylated antigens and efficiently targeting these antigens for loading onto MHC class I and class II molecules (Wells et al., 2008). Nevertheless, extensive research has revealed that CLRs also play important roles in the innate immune response through their recognition of microbial saccharides (microbial sugars). Many CLRs, especially those of Group II (e.g., DC-SIGN, Langerin), VI (e.g., Dectin-2 & MR), and V (e.g., Dectin-1) (see Table 2), serve as key PRRs on immune cells not only for microbial recognition and ingestion, but also for the induction of intracellular signaling and immune responses (Table 2). In this context, many transmembrane CLRs are expressed prominently on the surface of innate immune cells, especially myeloid cells (monocytes, macrophages, granulocytes and DCs) to act as PRRs that mediate specific interactions with different pathogens (Geijtenbeek and Gringhuis, 2009; Robinson et al., 2006). Microbial and viral signatures or 'PAMPs' (section **1.2.1.1**) are often made up of carbohydrates, therefore CLRs on innate immune cells can interact with a wide range of pathogens primarily through recognition of distinct carbohydrates, such as mannose structures, fucose GalNAc (*N*-Acetylgalactosamine), or glucans (Table 2) (van Kooyk, 2008; van Vliet et al., 2008). Accordingly, carbohydrate recognition by these CLRs enables the cells to recognize the major pathogen classes that challenge our species; mannose specificity allows recognition of some viruses, fungi and mycobacteria, whereas fucose structures are more specifically expressed on the surface of helminthes (parasitic worms) and particular bacteria, and glucan structures prominently exist on cell walls of mycobacteria and fungi. Pathogen Recognition by CLRs is important for internalization of pathogens for innate protection and efficient antigen presentation in order to initiate adaptive immunity against the

invading pathogen (section **1.2**), therefore, it is not surprising that most CLRs, which have been shown to act as PRRs, play key roles in host defense (Figure 2). CLRs, such as MR, Langerin, DC-SIGN, Dectin-1, Dectin-2, and Mincle (Macrophage-inducible C-type lectin) have been shown to recognize specific glycans exposed on pathogens or pathogenic structures (Table 2) (van Kooyk, 2008; van Vliet et al., 2008; Vautier et al., 2010). DC-SIGN recognizes a broad range of pathogens ranging from viruses, such as HIV (Human Immunodeficiency Virus), HCV (Hepatitis C virus), to bacteria, such as *Mycobacterium tuberculosis (M. tuberculosis*,) as well as components of parasites (Erbacher et al., 2009; van den Berg et al., 2012). Langerin, which has a more restricted glycan specificity, has been found to bind to HIV-1 and *M. leprae* (Stoitzner and Romani, 2011). MR has been demonstrated to recognize HIV-1, *M. tuberculosis* and *C. albicans*, whereas Dectin-1 binds to β-glucan, a polysaccharide exposed on fungi and yeast, such as *C. albicans*. The CLR Mincle binds a range of carbohydrate structures, predominantly containing glucose or mannose, and has been shown to interact with the fungal species *C. albicans* and *Malassezia,* as well as with the mycobacterial cord factor, trehalose-6,6'-dimycolate (TDM), a glycolipid on *M. tuberculosis* cell wall (van Vliet et al., 2008). As seen above some CLRs recognize fungal pathogens (explained in further detail in section **1.4.3.3**), and these CLRs play important roles in antifungal immunity (Ferwerda et al., 2010). Also it is clear from the above discussion that some pathogens e.g., *C. albicans* are recognized by more than one specific CLR, which indicates that there is significant redundancy. However, *in vivo* CLRs are often expressed on specific subsets of DCs. For instance, the MR is highly expressed on macrophages, whereas Langerin expression is restricted to Langerhans cells, and DC-SIGN is widely expressed on DCs in lymph nodes and mucosal tissues. This means that *in vivo* each subset of DCs is specialized in handling pathogens in a certain way depending on which type of CLRs are expressed on its surface.

Additionally, many extracellular soluble C-type lectins also act as PRRs that contribute to host defense. For instance the defensin RegIIIg is a C-type lectin produced in the gut, which exerts direct microbicidal activity. Also, the mannose binding lectin (MBL), a collectin, functions as an opsonin that that binds to carbohydrates on microbial surfaces to mediate phagocytosis of the microbe and activation of the complement cascade (Pyz et al., 2006; van den Berg et al., 2012).

1.3.4. CLR Signaling

CLRs such as MR, DEC-205, and Langerin were among the first CLRs shown to bind pathogens, as well as mediate endocytosis of ligands/PAMPs, and allow for antigen processing and presentation, and such properties make these CLRs interesting candidates for antigen delivery to dendritic cells (DCs) in immunotherapy (Osorio and Reis e Sousa, 2011). However, although these CLRs can recognize PAMPs on pathogens and modulate cell activation, there is limited evidence to date that signals from these receptors alone are sufficient to elicit microbicidal effector functions in cells, e.g., respiratory burst or, importantly, induce the gene transcription modules that lead to production of immunity to infection. Therefore these CLRs cannot directly induce immune responses on their own and usually collaborate with other PRRs to mediate signaling. However, it has become clearly evident that the interaction of many other C-type lectins with pathogens, akin to the TLR family members, can also self-sufficiently elicit intracellular signaling cascades that propagate into the cell to stimulate gene transcription, which in turn induces innate microbicidal and inflammatory responses and directs adaptive immunity appropriate to the nature of the infection. Thus, many CLRs do not function merely as PRRs for antigen/pathogen uptake; they also facilitate and shape adaptive and innate immune responses against the pathogen (Figure 2). Dectin-1 and Mincle are two examples of such “self-sufficient” CLRs that have been shown to autonomously (independent of other PRRs) couple pathogen recognition to signaling pathways that induce gene transcription programs essential for the activation of host defense (Figure 2). The intracellular signaling pathways induced by these signaling CLRs can either activate, dampen, modulate, or fine-tune both innate and adaptive immune responses for the production of an immune response tailored to the specific class of the invading pathogen (as explained in section 1.2) (Figure 2) (van Vliet et al., 2008). Furthermore, “self-sufficient” CLRs, although capable of inducing signaling pathways and exerting cellular functions independent of other PRRs, can also modulate the responses of other PRRs, primarily TLRs.

It is currently recognized that CLRs can mediate cellular responses by triggering intracellular signaling pathways either directly, through integral signaling domains, or indirectly, by associating with signaling adaptor molecules (Kerscher et al., 2013). CLRs are expressed as transmembrane proteins with or without signaling motifs in their intracellular cytoplasmic domains (Figure 7). Some CLRs, e.g., Dectin-1 and CLEC-2 (C-type lectin-like receptor-2),

possess intrinsic signaling properties and can directly trigger intracellular signaling through integral signaling motifs present in their cytoplasmic tails, usually 'immunoreceptor tyrosine-based activation (ITAM)-like motifs' known as **hemITAMs** (explained below) (Figure 7). On the other hand, CLRs, e.g., Dectin-2 and Mincle, can induce signaling indirectly through association or coupling with signaling chains/adaptor molecules, such as the Fc receptor γ (FcRγ) chain, and the adaptor molecules DAP12 or DAP10 (DNAX activation protein of 12 kDa or 10 kDa, respectively) that usually harbour classical 'immunoreceptor tyrosine-based activation motifs' **(ITAMs)** (Figure 7). Stimulation of these **ITAM-** or **hemITAM-bearing** CLRs produces activatory signaling, which initiates cell activation and cellular responses, including the induction of gene expression, cell maturation, and the production of a respiratory burst, upregulation of costimulatory molecules and inflammatory cytokines (Robinson et al., 2006). In contrast, other signaling CLRs, e.g., DCIR [Dendritic Cell (DC) Immunoreceptor], contain 'immunoreceptor tyrosine-based inhibitory motifs' **(ITIMs)**, and suppress cellular function (Figure 7) (Sancho and Reis e Sousa, 2012). Inhibitory signaling from **ITIM-bearing** CLRs leads to inhibition or downregulation of cellular responses and gene transcription. This illustrates potential immuno-suppressive or –activation functions of CLRs, where they can dampen or alter cell activation thereby modulating and shaping the outcome of the subsequent immune response (Geijtenbeek et al., 2009; Geijtenbeek and Gringhuis, 2009; van Vliet et al., 2008).

As mentioned above the signaling pathways elicited upon CLR engagement depend on tyrosine–based signaling motifs in their cytoplasmic domains or in associated adaptor proteins. Accordingly, signaling CLRs have been grouped into three broad categories according to their signaling potential in relation to intracellular tyrosine-based signaling motifs (1) **SYK-coupled CLRs (hemITAM-** and **ITAM- dependent CLR signaling)**; (2) **CLRs with ITIM domains (ITIM-dependent CLR signaling)**; and (3) **CLRs without ITAM or ITIM domains (tyrosine -independent CLR signaling)** (Figure 7) (Kerrigan and Brown, 2011b; Osorio and Reis e Sousa, 2011; Sancho and Reis e Sousa, 2012).

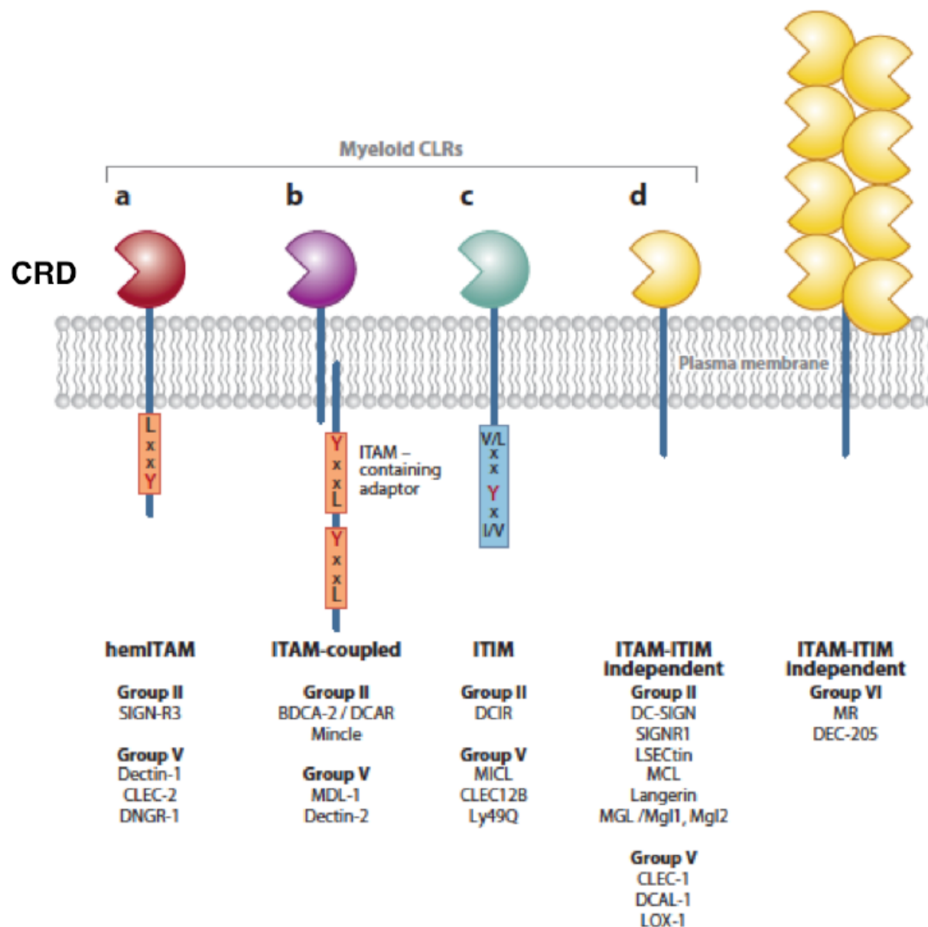


Figure 7: Classification of CLRs on the Basis of Cytoplasmic Tyrosine-based Signaling Motifs

Signaling families of myeloid CLRs. Myeloid CLRs can be grouped—independently of structure—into four groups based on cytoplasmic signaling motifs and the binding of early adaptors, kinases, or phosphatases. Roman numerals II, V, and VI refer to CLR Groups as demonstrated in Table 2. (*a*) hemITAM-coupled CLRs signal via SYK through a single tyrosine-based motif in their tail; (*b*) ITAM-coupled CLRs signal via SYK through association with ITAM-bearing adaptors as FcRγ chain or DAP12; (*c*) ITIM-containing CLRs possess an ITIM motif that can recruit tyrosine phosphatases SHP-1 and SHP-2; (*d*) ITAM-ITIM-independent CLRs do not signal through SYK nor phosphatases, although they may contain tyrosine-based motifs involved in endocytosis. Significant abbreviations: CRD, C-type carbohydrate recognition domain; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; SHP-1 & SHP-2 (SH2-containing ubiquitously expressed tyrosine-specific protein phosphatase-1 & -2). Adapted with permission from (Sancho, 2012).

The ITAM motif (immunoreceptor tyrosine-based activation motif) was initially recognized as a common sequence in the cytoplasmic tails of the signaling chains associated with the immune receptors, T cell and B cell antigen receptors (TCR and BCR), and certain Fc receptors such as Fc γ receptors (FcγR) e.g. FcγRIIA (Figure 8). Now it is known to exist in the cytoplasmic domain of a number of immune receptors and membrane-associated adaptor proteins. The classical ITAM motif typically consists of the short consensus amino acid sequence [Yxx(L/I)$x_{6\text{-}12}$Yxx(L/I)],

where ‘Y’ is tyrosine, ‘L’ is leucine, ‘I’ is isoleucine, ‘x’ is any residue, and the slashes indicate alternative amino acid residues (L or I). The tyrosine and the L/I residues are conserved in this sequence, and the spacing between them is critical for the signaling function of an ITAM. Clearly, the classical ITAM sequence is considered a duplicate of the canonical sequence ‘YxxL/I’, with six to twelve intervening residues. On the other hand, a single tyrosine-based activation motif, known as the **‘ITAM-like motif’** or **‘hemITAM’**, was first discovered in the cytoplasmic tail of the CLR Dectin-1, and was interestingly found to be different from the classical ITAM motif which typically contains two tyrosines in the context of ‘YxxL/I’ motifs (Figure 8) (Brown, 2006a). The hemITAM motif is now known to exist in in a small group of CLRs for initiating intracellular signaling from these receptors. The hemITAM resembles the classical ITAM in that it also contains two tyrosines, which are appropriately spaced and positioned in an arrangement similar to the traditional ITAM (Figure 8). However, the hemITAM possesses only one of the canonical ‘YxxL/I’ motifs present in the classical ITAM sequence (Figure 8). Therefore the hemITAM sequence does not conform exactly to the consensus sequence of the conventional ITAM, and uses only one tyrosine in the context of ‘YxxL/I’ for signal transduction (Figure 8) (explained further in section **1.5.4**) (Humphrey et al., 2005; Osorio and Reis e Sousa, 2011).

Signaling by classical ITAMs is instigated by ligand binding, which usually induces receptor clustering that in turn leads to the phosphorylation of the two tyrosines of the ITAM by Src family kinases (SFKs) to trigger signaling. These phosphotyrosines located in the ITAM motif create docking sites for the recruitment of SYK (Spleen Tyrosine Kinase) via its two SH2 (Src homology 2) domains (e.g., in the case of FcR and BCR) (Figure 8), or ZAP-70 (in the case of TCR). SYK is considered one of the most interesting biological targets of the last decade as a result of the potential capacity to block its action via inhibitors and abrogate excessive inflammation (Riccaboni et al., 2010). SYK kinase is expressed ubiquitously by hematopoietic cells and associates with different receptors on immune cells, including B cells, mast cells, macrophages, and neutrophils. It is a key mediator of ITAM- and hemITAM-based immune receptor signaling, where it is a major tyrosine kinase involved in upstream signaling from these receptors (Mocsai et al., 2010). SYK is considered not only a protein tyrosine kinase, but also an adaptor protein, as it possesses a linker domain with several tyrosine residues, which upon phosphorylation recruit several signaling proteins that contain SH2 domains.

SYK contains two tandem SH2 (Src homology 2) domains that are each capable of binding to a

phosphorylated tyrosine, and which are essential for SYK activation (Figure 8) (Liu et al., 2012; Sancho and Reis e Sousa, 2012). The binding of both of these two SH2 domains of SYK in *trans* to the dual phosphotyrosines of the ITAM motif is necessary and sufficient to cause a conformational change in SYK that promotes its autophosphorylation and activation (Figure 8) (Mocsai et al., 2010). SYK is phosphorylated on several residues (e.g., Tyr352 in the linker domain, and Tyr525 in the catalytic kinase domain, of human SYK), which consequently provide docking sites for other signaling molecules, e.g., PKC (Protein Kinase C), PLCγ1 (Phospholipase C, gamma1), and VAV1 [a guanine nucleotide exchange factor (GEF)]. This stabilizes the active conformation of SYK and causes it to phosphorylate the signaling molecules bound to it, which in turn initiates downstream signaling (Mocsai et al., 2010).

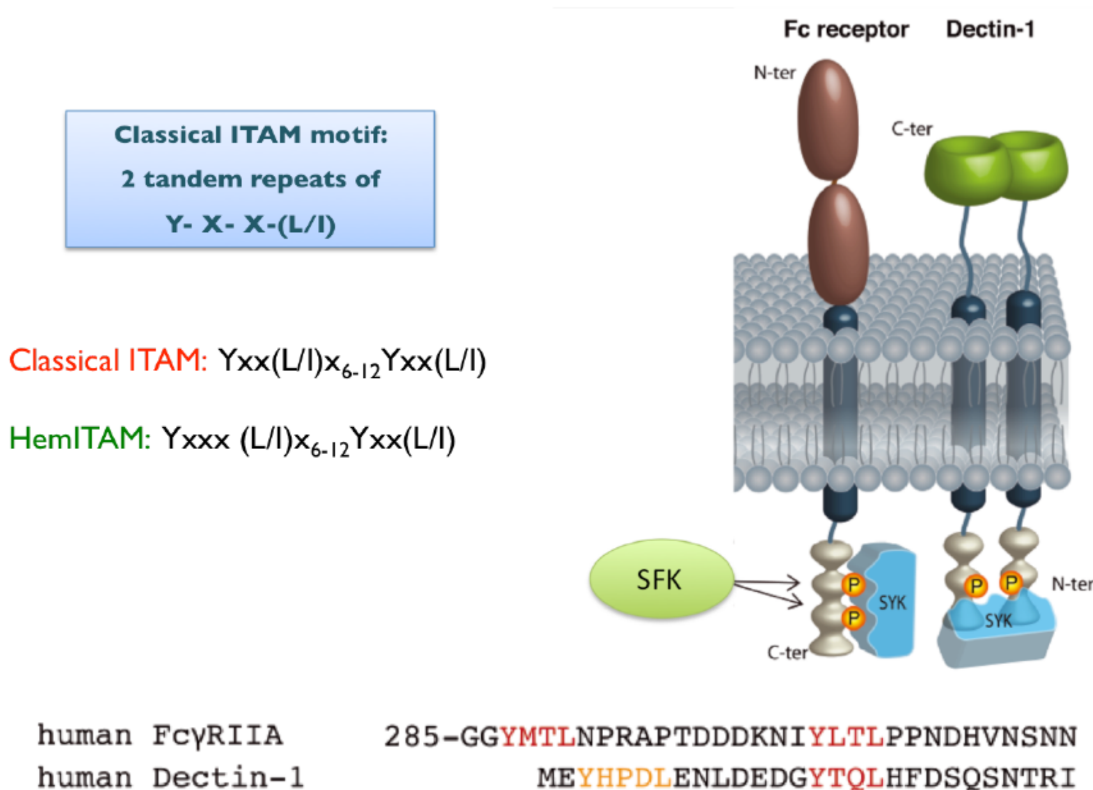


Figure 8: ITAM & HemITAM (ITAM-like) Motifs and SYK recruitment

The above figure shows the consensus sequences and 'YxxL/I' motifs of the classical ITAM and non-conventional

hemITAM motifs of the FcγRIIA and the C-type lectin receptor Dectin-1, respectively. The figure also demonstrates the recruitment of SYK (Spleen Tyrosine Kinase) to each of these motifs. Ligand binding induces receptor clustering which permits activation of Src family kinases (SFKs) family kinases in order to phosphorylate the two tyrosines found in the Fc γ receptor (FcγR) ITAMs or the single tyrosine found in the Dectin-1 hemITAM. The phosphorylated tyrosines form docking sites for the recruitment and activation of SYK kinase. In the case of Fc γ receptors (FcγR), e.g. FcγRIIA, SYK binds in *trans* to the two phosphotyrosines in the consensus sequence of a single classical ITAM motif present in only one Fc receptor. In contrast, SYK binds in *cis* to two individual phosphotyrosine residues on separate hemITAMs of two closely spaced Dectin-1 molecules. 'Y': tyrosine, 'L': leucine, 'I': isoleucine, 'x': any residue; slashes indicate alternative amino acid residues (L or I).

Though the mechanism is still not very clear, it has been suggested that SYK in hemITAM signaling is activated by binding of its two SH2 domains in *cis* to two individual phosphotyrosine residues present in the hemITAMs of two separate and closely spaced receptors (Brown, 2006a; Humphrey et al., 2005; Strasser et al., 2012). Indeed, SYK has been demonstrated to bind directly to phosphotyrosines on the hemITAM motif (Hughes et al., 2010b; Underhill et al., 2005). It has therefore been proposed that receptor clustering brings receptors in close proximity to facilitate such binding of SYK to hemITAM motifs (Brown, 2006a; Hughes et al., 2010b; Watson et al., 2009).

SYK-coupled CLRs either possess intracellular hemITAM domains or associate with ITAM-containing adaptor proteins, and mainly utilize SYK as their major membrane-proximal adaptor and key tyrosine kinase involved in upstream signaling (Osorio and Reis e Sousa, 2011). **SYK-coupled CLRs** are 'self-sufficient' PRRs (i.e., they signal independent of other CLRs and PRRs and) and ligation of these receptors leads to the phosphorylation and activation of SYK, which consequently binds to and induces the phosphorylation of numerous signaling substrates thereby eliciting a SYK-dependent signaling cascade (Mocsai et al., 2010). This coupling of CLRs to SYK activation can either be indirect (e.g Mincle), via association of the CLR in *trans* with adaptors (e.g FcγR or DAP12) that contain SYK-recruiting classical ITAMS, or direct through a small group of CLRs, which have the capacity to bind to and activate SYK directly via single tyrosine-based hemITAMs in their cytoplasmic tails (e.g. Dectin-1 and CLEC-2) (Kerrigan and Brown, 2011b). Engagement of SYK with SYK-coupled CLRs activates kinases including PKC (Protein Kinase C) and MAPKs (MAP kinases), as well as the transcription factors NFAT, and NF-κB that is activated through the adaptor protein CARD9. As a result, inflammatory cytokines are produced (Kerrigan and Brown, 2011b; Marakalala et al., 2010). Furthermore, CLR activation by ITAMs and hemITAMs is kept in check by another separate group of self-sufficient **CLRs that contain ITIMs** (immunoreceptor tyrosine-based inhibitory motifs) in their cytoplasmic tails. Analogous to hemITAMs, the 'ITIM' motif is a single tyrosine-based motif with the sequence:

S/I/V/LxYxxI/V/L (where ‘S’ is Serine, ‘V’ is Valine, and ‘x’ is any residue). Nevertheless, this domain antagonizes SYK activity by recruiting phosphatases, e.g., SHP-1, SHP-2, rather than kinases, and thereby negatively regulates signaling from SYK-coupled CLRs as well as other PRRs (Kerrigan and Brown, 2010; Sancho and Reis e Sousa, 2012). These inhibitory CLRs, e.g. DCIR and MICL, don’t trigger any activity per se but will modulate cell activation when triggered with other activatory receptors. Finally, a distinct group of CLRs that include receptors such as MR, DC-SIGN and Langerin, are **without ITAM or ITIM domains,** and can signal independent of ITAM and ITIM and do not recruit SYK or SHP phosphatases for signaling. These CLRs do not possess any tyrosine–based signaling motifs, and therefore utilize tyrosine-independent signaling pathways that also impact innate and adaptive immune responses (Figure 7). For instance, some these CLRs such as DC-SIGN, have been shown to activate the serine/threonine kinase Raf-1 instead of SYK (Geijtenbeek et al., 2009; Sancho and Reis e Sousa, 2012). Furthermore, these receptors can engage the endocytic machinery and mediate the capture of antigenic cargo for processing and presentation to T cells (Engering et al., 2002b). However, this group of CLRs, unlike SYK-coupled and ITIM-based CLRs, is not self-sufficient and cannot in isolation induce obvious signs of cell activation (Geijtenbeek and Gringhuis, 2009). They rather act to regulate and fine-tune cell activation and the signaling outcome of other CLRs or PRRs. For some of the CLRs in this group, e.g. DC-SIGN, the signaling pathway has been characterized, but for most it is unknown (Osorio and Reis e Sousa, 2011).

Finally, as clear from the above discussion, CLRs can influence signaling pathways, and in turn shape immune responses, produced by other PRRs (Geijtenbeek and Gringhuis, 2009; Wevers et al., 2013). This is mediated by collaborative and manipulative crosstalk between signaling pathways induced by these PRRs and CLRs. For instance, CLRs can enhance, inhibit or modulate the production of proinflammatory cytokines generated by TLR signaling (Geijtenbeek and Gringhuis, 2009). This leads to the production of distinct sets of cytokine profiles, which in turn tailor the differentiation of naïve T cells into certain effector subsets that ultimately generate an adaptive immune response specific to the invading pathogen (Geijtenbeek and Gringhuis, 2009; Hardison and Brown, 2012). Thus pathogens sometimes manipulate these signaling pathways to skew the immune response in favour of survival. Indeed, several pathogens that target CLRs seem to subvert the function of these CLRs, either by inhibition of antigen presentation or by modification of the underlying signaling pathways that are required for shaping the adaptive

immune response towards the pathogen (Aarnoudse et al., 2008; Brown and Gordon, 2003). Most importantly, the shaping of adaptive immune responses by CLRs could be exploited in vaccine development to tailor specific adaptive immune responses against certain types of infections and also according to the patient's needs (Geijtenbeek and Gringhuis, 2009; O'Hagan and Valiante, 2003).

1.3.5. 'Group V' NK-like C-type lectin-like Receptors (NKCLs) and the Dectin-1 Cluster

The NK cell receptor group includes true NK cell receptors as well as structurally related proteins, which have other functions. Inhibitory NK receptors block NK cell cytolytic activity upon recognition of markers of healthy self-cells, predominantly major histocompatibility (MHC)-related molecules. In contrast, activating receptors stimulate cytolytic activity upon recognition of infected or transformed cells.

An interesting subgroup of the CLR family is 'Group V' that consists of the 'NK (natural killer)-like C-type lectin-like receptors' or **'NKCLs'**, which are related to the NK cell receptor group (described in the above paragraph), but additionally contain the CTLD/CRD domain common to CLRs. Although 'Group V' NKCLs are part of the CLR superfamily, they do not generally recognize carbohydrates, and their CRD domain lacks the conserved residues associated with this activity that is found in their classical C-type lectin counterparts. In fact, NKCLs are more structurally and functionally related to NK cell receptors that are traditionally associated with the control of cellular cytotoxicity and the ability to distinguish self cells from non-self, through recognition of MHC class I molecules, MHC-related molecules, and other proteinaceous ligands including endogenous ligands. Accordingly, Group V CLRs are classified as 'non-classical' CLRs that have evolved to recognize non-sugar ligands including proteins, such as MHC class I or related molecules, but nevertheless, some of these receptors are also able to recognize carbohydrates, probably, via alternative mechanisms (Pyz and Brown, 2011).

NKCLs consist of several subfamilies of related molecules, which are mostly encoded by a single genomic region, known as the NK complex (NKC), located on the chromosome 12 in humans and chromosome 6 in mouse. Several of the NKCLs are functionally related to proteins of the NKC complex, where they similarly regulate leukocyte function, but their specific roles are often unclear [e.g., Killer cell lectin-like receptor (KLR) F1, CD69, Myeloid DAP12-associating lectin

(MDL)-1, C-type lectin-like receptor-1 (CLEC)-1]. Although many of these receptors are exclusively expressed by NK-cell and T cell subsets, a growing number of these receptors have now been identified on myeloid cells and other cells. Myeloid-expressed NKCLs appear to have far more divergent functions and ligands than their NK-counterparts and play important roles in both innate and adaptive immunity through their ability to modulate cell functions [e.g. Dectin-1 (Dendritic cell-associated C-type lectin-1) and Dectin-2]. Some of these receptors, e.g. Lox-1 (lectin-like oxidized low-density lipoprotein (LDL) receptor-1) and CLEC-2, are expressed on endothelial cells and platlets, respectively, and perform roles in maintaining regular homeostasis. For instance, Lox-1 is an endocytic receptor on vascular endothelial cells for oxidized low-density lipoprotein, and has been implicated in the development of atherosclerosis.

The NKCL group of CLRs has around 20 members in human and more in mouse. Similar to proteins of the CLR super-family, members of the NKCL group are usually encoded by six exons and share a common structure. They are type II transmembrane proteins consisting of a single extracellular carbohydrate-recognition domain CRD and a variable-length neck/stalk region that in many cases contains cysteine residues involved in covalent homo- or heterodimerization (through the formation of disulphide bridges between these cysteines) (Brown, 2006a). The stalk region is followed by a cytoplasmic tail, which also varies in length and commonly contains internalization motifs, tyrosine-based motifs involved in signalling, or binding sites for cytosolic proteins (Robinson et al., 2006). The NKCL C-type lectins that do not have signaling motifs in their cytoplasmic tails can associate with other adaptor signaling molecules through charged residues in their transmembrane domains (Robinson et al., 2006). Members of the NKCL subfamily are often alternatively spliced yielding variable isoforms that encode receptors, which could lack the stalk or transmembrane regions. Alternative splicing may also give rise to isoforms with different cytoplasmic tails, influencing receptor functions.

As mentioned above, many of the genes of the ‘Group V’ NKCL receptors are clustered within a single genetic locus, the ‘natural killer complex’ (NKC) (Sancho and Reis e Sousa, 2012). Many of these genes are further grouped into several distinct ‘receptor clusters’ within the NKC complex. Of particular interest for this thesis, is the NKCL receptor cluster known as the **‘Dectin-1 cluster’** whose genes are clustered together in the NK complex (Plato et al., 2013). Members of the ‘Dectin-1 cluster’ have functions that span many areas of immunity and homeostasis and include receptors such as: Dectin-1, LOX-1, CLEC-1, CLEC-2 and MICL

(Myeloid inhibitory C-type lectin-like receptor) (Xie, 2012). Receptors of this cluster are primarily expressed by myeloid and/or endothelial cells and seem to have a more diverse range of ligands and cellular functions compared with the classical NKCLs. Dectin-1 (gene symbol Clec7a), CLEC-2 and LOX-1 have been the most extensively investigated of this cluster, however, many of the natural ligands of other receptors in this subgroup are unknown, but they all have potential signaling motifs (similar to the ones discussed above in section **1.3.4**) in their cytoplasmic tails and might function as PRRs (Brown, 2006a; Pyz and Brown, 2011).

In addition to the 'Dectin-1 cluster' the NK complex contains a structurally separate subgroup of CLRs termed the **'Dectin-2 cluster'**, which includes genes for the CLRs: Dectin-2 (gene symbol Clec4n) and Mincle (Macrophage-inducible C-type lectin). Despite their related names Dectin-2, is only 20% homologous to Dectin-1 (Kerscher et al., 2013). Members of the Dectin-2 subgroup possess a single extracellular CRD and have short cytoplasmic tails, and are capable of mediating intracellular signaling indirectly (by associating with adaptor molecules), except for DCIR (Dendritic cell immunoreceptor) that has a longer cytoplasmic tail containing an integral inhibitory signaling ITIM motif. These CLRs also function as PRRs for several classes of pathogens including fungi, bacteria and parasites, driving both innate and adaptive immunity. The C-type lectin NKCL receptor Dectin-1, will be covered in detail in section **1.5**.

1.4. Fungal Infections in Human Health

1.4.1. Fungal Pathogens and the Fungal Disease Burden

We live on a moldy planet — it is estimated that there are well over one million species of fungi. The challenge of defending against fungal invasion is formidable given the frequent exposure to environmental fungi (Hohl, 2014). Strong innate and adaptive host defenses exist to protect animals against fungal invasion, and therefore in people with healthy immune systems, serious fungal diseases are rare (Brown et al., 2012b). Humans encounter, ingest, and inhale fungi daily. Although only several hundred (<600) of the estimated 10^5 fungal species cause human disease, the incidence of clinically relevant fungal infections has risen substantially in the past 3–4 decades. This increase in invasive fungal infections by opportunistic fungal pathogens is becoming increasingly prevalent globally, and has emerged as a public health concern and a major threat to human health, especially in immunocompromised patients. The global rise in fungal disease burden is largely owing to an increase in the population of immunocompromised

patients due to the advent of the global AIDS/HIV (Acquired Immunodeficiency Syndrome/Human Immunodeficiency Virus infection) pandemic, and the increased impact of modern medical interventions including: organ transplantation surgery, vascular access, immuno-suppressive therapies and chemotherapy for autoimmune diseases and cancer, respectively (Brown et al., 2012a; LeibundGut-Landmann et al., 2012). The incidences of both superficial and invasive fungal infections are rapidly growing throughout the world ever year not only due to the increasing numbers of at-risk immunocompromised patients, but also due to the emergence of strains resistant to antimycotic drugs, with *Candida albicans* and *Aspergillus fumigatus* being the two most common causes of fungal disease (Kabelitz and Medzhitov, 2007; Romani, 2011; Wuthrich et al., 2012). For instance, invasive infections with *Candida albicans* (*C. albican*s) constitute a major threat to patients who are immunosuppressed and those who have undergone major surgical procedures, with mortality reaching 30-40%, despite the availability of antifungal drugs (Gudlaugsson et al.; Wisplinghoff et al., 2004). Therefore, understanding the mechanisms through which the host immune system recognizes and eliminates fungal pathogens has become increasingly important due to a new resurgence of fungal infectious diseases (Brown et al., 2012a). In this regard, it is essential to understand host-fungus interactions in order to develop new therapies for fungal infections, such as adjunctive immunotherapy as well as novel antifungal vaccines, which are promising yet, unfulfilled treatment and preventive strategies for fungal infections (see section **1.4.3**). Much research has been done in the past decade to elucidate such mechanisms of the host defense against fungal pathogens, including the discovery of fungal PAMPs and PRRs involved in host-fungus interactions and antifungal immunity. This section covers a brief overview of medically important fungal pathogens involved in fungal infections to the human host.

Fungi are eukaryotic, saprophytic organisms with a rigid cell wall. Yeasts form round, oval, or spherical cells that usually divide asexually by budding. Molds form asexual spores (conidia) that are dispersed in the environment and germinate into tubular filaments, termed hyphae. Dimorphic fungi (e.g. Blastomyces dermatitidis, Histoplasma capsulatum, and Paracoccidioides brasiliensis) can exist as yeast cells (in human tissues) or as hyphae (in soil) with environmental conditions (e.g. temperature) guiding the transition between morphologic states. Figure 9 illustrates the medically relevant fungal pathogens, syndromes and common sites of fungal disease in humans. As seen in Figure 9, fungi cause a broad range of diseases with syndromes that involve

superficial, tissue-invasive, and allergenic manifestations (Figure 9). Route of acquisition, tissue tropism, and nutritional requirements all contribute to fungal pathogenesis (LeibundGut-Landmann et al., 2012). The majority of fungi associated with life-threatening infections are inhaled as infectious propagules, invade sinopulmonary tissues, and in specific instances, disseminate to extrapulmonary sites. *Candida species* are the major exception to this rule and reside in the gastrointestinal tract as commensal organisms in humans. Of the estimated 2 million annual life-threatening invasive infections, 90% are caused by *Candida, Cryptococcus, Aspergillus, and Pneumocystis species* (Brown et al., 2012b). *Candida* and *Aspergillus* species are considered the most serious fungal pathogens that infect humans (Drummond and Brown, 2011). Infections with these two opportunistic and medically important fungal species represent an increasing problem and a major threat to public health, where they are associated with high incidence of mortality and morbidity (Romani, 2004).

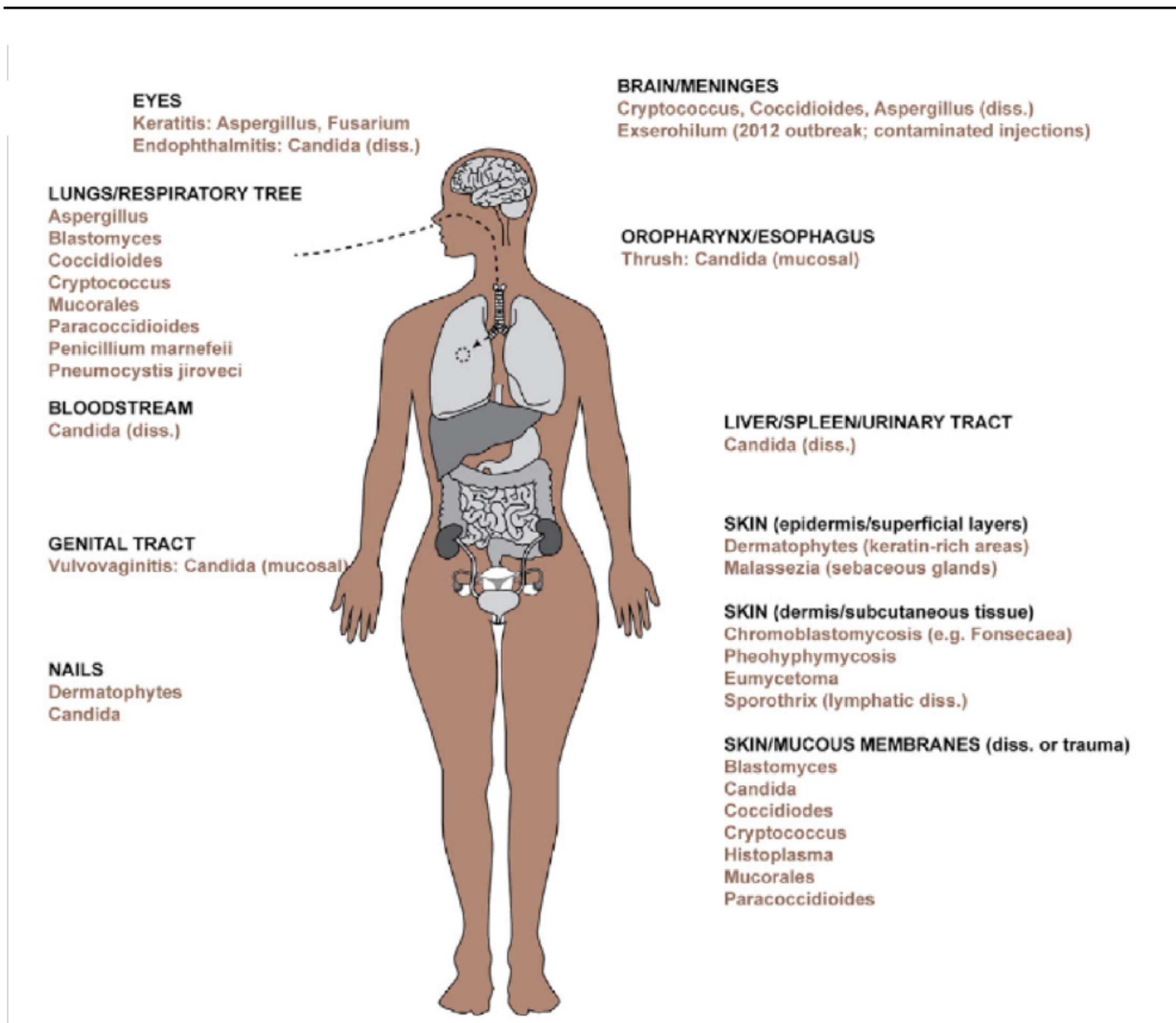


Figure 9: Medically Relevant Fungi, Syndromes, and Common Sites of Infection in Human

The above schematic depicts the primary anatomic sites commonly affected by medically relevant fungal diseases in humans. Most fungal diseases are acquired when infectious particles are inhaled, inoculated via trauma, or penetrate breaches in mucosal integrity. Common sites of fungal dissemination (diss.) are indicated. The figure also shows examples of organ involvement following fungal dissemination from the primary site of infection. Disseminated fungal disease occurs typically in severely immune compromised patients, for example, disseminated cryptococcosis in patients with $CD4^{+}$ T cell dysfunction. Adapted with permission from (Hohl, 2014).

As seen in Figure 9, fungal microbes are capable of causing a wide variety of infections, ranging from superficial skin and nail infections through to life-threatening invasive diseases. Skin and nail infections are associated with a number of dermatophytes, including *Trichophyton* spp., *Microsporum* spp. and *Epidermophyton* spp., causing infections such as athlete's foot and ringworm (Havlickova et al., 2008; Romani, 2004). Human commensal fungi can also cause skin infections, e.g., *Candida* spp., particularly *Candida albicans*, are linked with intertrigo

(inflammation of body folds, such as between the digits) and *Malassezia* spp are associated with dandruff and pityriasis versicolor (skin rash and pigment changes) (Figure 9) (Romani, 2011). *Candida* spp. are also associated with oral and vaginal thrush and with chronic mucocutaneous candidiasis, where individuals suffer from recurrent skin, nail and/or mucosal infections (Figure 9) (Brown et al., 2012a; Netea et al., 2006a).

Deep-seated mycoses develop from inhalation of infectious propagules into the airways of susceptible hosts (e.g. *Aspergillus fumigatus, Cryptococcus neoformans, Pneumocystis jiroveci*) or penetration of mucosal breaches by commensal organisms such as *Candida albicans* (Figure 9). Disruption of host microbial communities (e.g.vulvovaginal candidiasis ensuing antibiotic therapy) or metabolic disorders (e.g. mucormycosis in diabetic ketoacidosis, (LeibundGut-Landmann et al., 2012) can predispose to superficial and invasive fungal disease. *Candida albicans* is a human commensal of the mucosa, which, under circumstances when immune defenses are impaired, can become pathogenic and invade host tissues (Figure 9). Seventy five percent (75%) of invasive fungal infections are attributed to this, otherwise, commensal fungi (*C. albicans*), which results in 40%–50% mortality (De Rosa et al., 2009). Candidemia is typically observed in severely ill patients with breaches in gastrointestinal or mucocutaneous barrier function (e.g. indwelling vascular access catheters or following abdominal surgeries). The pathogenesis of invasive candidiasis typically involves colonization of the gastrointestinal tract or mucosal surface, followed by translocation across damaged barrier structures and into internal organs and compartments such as the bloodstream, mesenteric lymph nodes, spleen, and liver, often in the setting of neutropenia (Figure 9). *Candida* species are the fourth most common cause of nosocomial bloodstream infections in the United States Hohl, 2014 #1003}, and fungus-attributable mortality rates for nosocomial candidemia are approximately 40%. The increase in invasive medical procedures, the use of broad-spectrum antibiotics, the advent of intensive care units, and the use of medical therapies that cause damage to barrier function (i.e. mucositis) and neutropenia have all contributed to the emergence of and to the clinical risk of developing invasive candidiasis in modern medical practice.

Aspergillus fumigatus primarily affects patients undergoing solid organ transplants and those receiving immunosuppressive drugs (Said-Sadier et al., 2010), which can result in invasive pulmonary *aspergillosis* (IPA) (Figure 9) (Werner et al., 2011). *Pneumocystis carinii* is a clinically important fungal pathogen that is a frequent cause of pneumonia in HIV-positive

individuals (Figure 9) (Steele et al., 2003). Another opportunistic and medically important fungus is *Cryptococcus neoformans,* a cause of morbidity and mortality in AIDS patients (Figure 9) (Romani, 2011). *Coccidioidomycosis* is an endemic fungal disease primarily caused by *Coccidioides immitus* and *Coccidioides posadasii,* depending on geographical location (Figure 9). The pathogenesis of *coccidioidomycosis* is complex with patients displaying a wide range of symptoms, from asymptomatic to extra-pulmonary dissemination (Romani, 2011). Environmental fungi are the major causative agents of fungal lung infections, which are generally self-limiting. However, in immunocompromised individuals these infections can disseminate, leading to more serious consequences. The major fungal species associated with lung infections are *Aspergillus* species (particularly *Aspergillus fumigatus*), *Cryptococcus neoformans* and *Pneumocystis jirovecii.* In specific regions of North, Central and South America, endemic fungi are also major causes of fungal lung disease (Figure 9) (LeibundGut-Landmann et al., 2012; Vautier et al., 2010).

The most serious fungal infections are invasive infections, which occur in immunocompromised and severely ill individuals and are associated with high mortality rates (Brown et al., 2012a; Morace and Borghi, 2010). Fungi are the causative agents in ~20% of all invasive infections in ICU (intensive care unit) patients with 90% of infections caused by *C. albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis* and *Candida krusei* (Morace and Borghi, 2010; Vincent et al., 2009; Wisplinghoff et al., 2004). Pulmonary infections can also be the source of invasive fungal infection in immunocompromised individuals (Brown et al., 2012a; Netea and Brown, 2012).

From the above discussion, it is evident that most fungal infections are opportunistic; therefore, it is vital that the immune system is capable of recognizing fungal cells and spores that enter the body and that it mounts an appropriate immune response to deal with the potential threat (Bourgeois et al., 2010; van de Veerdonk et al., 2008). Moreover, invasive fungal infections have recently emerged as an increasing clinical problem and the relatively new appreciation of this problem has highlighted deficiencies in current antifungal therapies and, thus, the need for new therapeutic approaches as well as better knowledge of the underlying mechanisms by which normal immune responses control fungal infections. In a recent review by Brown et al. (2012), human fungal infections are currently considered ‘hidden killers (Brown et al., 2012a). Understanding the initial interaction between fungi and the host offers potential for development

of new drugs or vaccines (see section 1.4.3). In this context, it has recently been recognized that like other pathogens, fungi initially interact with the innate immune system via binding between fungus-specific chemical signatures (pattern-associated molecular patterns or PAMPs) and pattern recognition receptors (PRRs) on mononuclear phagocytes. Fungal PAMPs are restricted to complex carbohydrates in the cell wall, including mannoproteins, phospholipomannan, β-glucans and possibly chitin. These PAMPs bind specifically to two main classes of PRR in phagocyte membranes, TLRs and CLRs, through which they initiate signaling responses that culminate in release of pro- and anti-inflammatory cytokines, linking the innate immune response with the adaptive immune response and initiating phagocytosis and intracellular killing. Isolated PAMPs have been used to dissect phagocyte responses *in vitro* and have revealed mechanisms by which host cells can tailor innate immune responses to individual pathogens. The interactions are complex and are yet to be translated into a clear understanding of the roles of the respective PAMPs and PRRs *in vivo.* The next two sections discuss in further detail the different fungal PAMPS and PRRs involved in host-fungus interactions.

1.4.2. Fungal PAMPS and the Fungal Cell Wall

Fungi and animals are members of the eukaryotic kingdom and on a cellular level have more similarities than differences. The major difference, which is exploited by the innate immune system, is the presence of cell walls on fungi. While there is much interspecies and even intraspecies variability in fungal cell wall composition, cell walls of virtually all medically important fungi have significant amounts of three polysaccharides: **β-glucan**, **chitin**, and **mannan** (Netea et al., 2008) (Figure 10). β-(1,3)-glucans containing β-(1,6)-linked side branches, and chitin form the core structural component (Figure 10). Mannoproteins are attached to this skeleton (Figure 10). Both mammalian and fungal cells have glycosylated proteins linked via N- and O-linkages. However, whereas mammalian proteins rarely have exposed mannose residues, fungi utilize mannose as their preferred sugar (Levitz and Specht, 2006). These highly mannosylated polysaccharides are referred to as mannans, and in the cell wall of *Candida albicans*, chains comprised of up to 200 mannose groups can be found (Levitz and Specht, 2006; Netea et al., 2008). Importantly, mannans tend to be on the outer fungal cell wall, whereas β-glucans are largely on the inside (Figure 10). However, some surface exposure of β-glucans does take place, particularly in areas where yeast cells bud.

The cell wall composes an appreciable part of fungal cell mass; in yeasts it represents between 15% and 25% of total cell mass. Research on the cell wall of different fungal species does not lead to a straightforward model of its structure (Netea et al., 2008; Romani, 2011). According to Stratford (Stratford, 1994), the yeast cell wall resembles reinforced concrete. An armature, representing about 35% of cell wall mass and formed by fibrils of alkali insoluble β-(1,3)-glucan, is dipped into mannoproteins (about 25–35% of wall mass) and bound to the armature through amorphous β-glucan and chitin (Figure 10). The cell wall of other 'fungi' is constructed in a similar way. Essentially, the same model of the fungal cell wall was published by Selitrennikoff et al. (Selitrennikoff, 2001). Though different models of the fungal cell wall differ somewhat, they agree that β-glucan is not located on the surface of the wall but is more or less immersed in the wall material (Figure 10). These models also agree that the fungal cell-wall components chitin (a polymer of *N*-acetylglucosamine), α- and β-glucans (polymers of glucose), and mannans (chains of *N*- or *O*-linked mannose molecules) are the three major PAMPs that are unique to fungi and distinguish them from the mammalian host (Figure 10).

Despite the growing number of PRRs implicated in antifungal immunity, relatively few fungal PAMPs have been identified and nearly all of these appear to be carbohydrate-based structures found in the fungal cell wall (Willment and Brown, 2008). β-Glucan, however, is one of the few fungal PAMPs, which is well characterized, and can comprise up to 50% of the dry weight of the cell wall. These polymers are classified as biological response modifiers and have been shown to induce antimicrobial, fungicidal, radioprotective, antioxidant and anti-tumour properties *in vivo* (section **1.6.3**) (Brown, 2006a; Brown and Gordon, 2003). The mechanisms whereby these molecules induce protection have not been strictly defined, but the activity of these polysaccharides appears to depend on their structural properties, including degree of branching, tertiary structure and molecular weight (see section **1.6**).

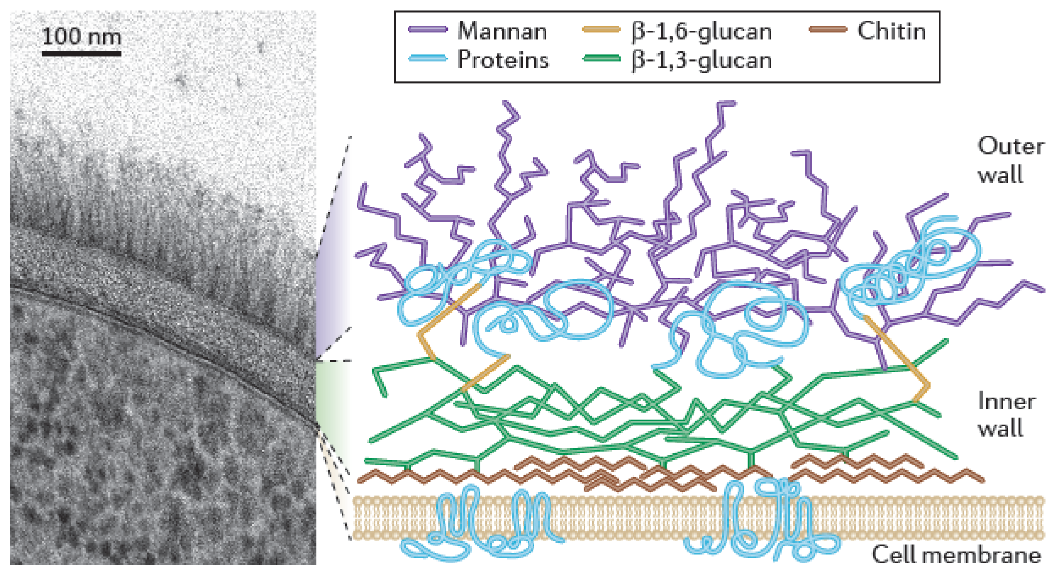


Figure 10: Structure of the Fungal Cell wall

Two layers can be distinguished in the fungal cell wall. The most external layer is highly enriched with O-and N-linked mannose polymers (mannans) that are covalently associated with proteins to form glycoproteins, whereas the more internal layer contains chitin and β-(1,3)-glucan. Chitin and β-(1,3)-glucan present in the inner layer are considered skeletal polysaccharides, which confer strength and cell shape. The outer cell wall proteins are attached to this inner wall framework predominantly by glycosylphosphatidylinositol (GPI) remnants that are linked to the skeleton through a more flexible β-(1,6)-glucan. Adapted with permission from (Neil A. R. Gow, 2011).

1.4.3. Fungal PRRs and Mechanisms of Antifungal Immunity

1.4.3.1. Mechanisms of Host Defense Against Fungal Infection

Opportunistic fungal pathogens have a huge impact on our society. As described above, invasive infections with these fungal microbes, especially *Candida* and *Aspergillus* species, are an increasing problem and are associated with a high incidence of mortality due to modern medical practices and a significant rise in the numbers of immunocompromised individuals (Brown et al., 2012a). The relatively new appreciation of this problem has highlighted deficiencies in current antifungal therapies and in the scientific knowledge of the underlying mechanisms employed by the host in antifungal immunity (Romani, 2011; Wuthrich et al., 2012).

Successful host defense against fungal microbes requires an effective innate and adaptive immune response. In healthy individuals, the host control of fungal infection is largely mediated by phagocytic cells of the innate immune system, including macrophages, dendritic cells (DCs), monocytes and neutrophils (Romani, 2011; Wuthrich et al., 2012). One function of these innate

immune cells is to provide a primary protective effect via direct antifungal activities such as phagocytosis or secretion of microbicidal compounds that neutralize fungal particles. Indeed, in the initial response to fungal infection, phagocytic cells, mainly neutrophils and macrophages, phagocytose invading pathogens. Accordingly, neutrophils and macrophages are thought to be the most crucial in host defense to fungi. A myriad of receptor-ligand interactions occur following fungal challenge through the innate immune recognition of the fungal cell wall, which is largely achieved by pattern-recognition receptors (PRRs) on innate immune cells, mainly macrophages and DCs (Figure 17) (Netea et al., 2008). Opsonization or coating of fungi with complement and antibody results in fungal recognition by complement and Fc receptors, respectively, which are present on the surface of phagocytic cells. Furthermore, signaling cascades initiated during this process activate the phagocyte, leading to the killing of the pathogen via the respiratory burst that involves the production of microbicidal ROS (reactive oxygen species), and the presentation of fungal antigen(s) to the adaptive immune system.

The initiation of all key immune responses involved in effective protection against fungal infection relies on the innate immune recognition of fungal PAMPs by PRRs. Infection studies in knockout mice and polymorphisms identified in humans have highlighted the involvement of several fungal-specific PRRs in host antifungal immunity. Indeed, a wide gamut of membrane-bound and soluble/secreted PRRs recognize fungi (Figure 17) [reviewed in (Brown, 2011)] (further described below in the next two sections). As shown in Figure 17, PRRs that are best described for the recognition of fungi and induction of soluble mediators are C-type lectin receptors (CLRs) and TLRs. Signaling pathways triggered via these PRRs have been shown to induce the release of numerous immune-activating cytokines (e.g. IL-1β, IL-12, IL-17, IL-23, and TNF-α) that are important in directing innate and adaptive immune responses to fungal pathogens (Vautier et al., 2012). The initial proinflammatory cytokines that are released in response to fungal recognition by these PRRs act to mobilize the innate immune system (as described in section **1.2**). The cytokines IL-6 and G-CSF promote neutrophil production (granulopoiesis) and function, and mice deficienct in these two cytokines are neutropenic and therefore susceptible to *Candida* infections (Sambatakou et al., 2006). The proinflammatory cytokine TNF-α also plays an important role in fungal immunity. Indeed, $TNF^{-/-}$ mice have increased fungal burdens due to reduced activation of neutrophils (Netea et al., 1999). The protective nature of TNF-α has also been demonstrated in humans, where individuals who show increased TNF-α levels are more

resistant to infection with *Aspergillosis* (Sambatakou et al., 2006).

Once activated, the innate immune system, via antigen presenting cells (APCs), initiates, sustains and regulates the adaptive immune response, thereby controlling microbial growth and facilitating microbial clearance. It is well established that the adaptive immune response, in particular that of T cells, plays a pivotal role in antifungal host defense (Romani, 2011; Wuthrich et al., 2012). $CD4^+$ Th responses are critical for the control of many fungal infections, including mucocutaneous *candidiasis* (Conti and Gaffen, 2010; Vautier et al., 2010). To induce antifungal T cells, innate PRRs on APCs sample fungal PAMPs and induce a cocktail of signature cytokines and costimulatory molecules that determine the differentiation of naïve $CD4^+$ T cells into distinct Th subsets (Kabelitz and Medzhitov, 2007). A balanced Th1/Th17 response, rather than either a Th1 or Th17 response, is believed to be the most effective for antifungal defense (Vautier et al., 2012; Vautier et al., 2010). Indeed upon recognition of fungi by fungal-specific PRRs, signaling pathways engaged on APCs induce a myriad of cytokines that influence Th differentiation, mainy into Th1 and Th17 $CD4^+$ T cells. These two subsets of Th cells are of particular interest for antifungal immunity because activation of these cells further aids fungal killing by inducing effectors such as the respiratory burst, as well as inflammatory responses that further activate and recruit other cells to the site of infection (Brown, 2011). In this regard, Th1 and Th17 cells (so named because they secrete IL-17) produce proinflammatory cytokines such as IFN-γ and IL-17, respectively, which are known to recruit and activate phagocytes to kill fungi (Kabelitz and Medzhitov, 2007; Romani, 2011; Wuthrich et al., 2012). Accordingly, neutrophils and macrophages are thought to be crucial in host antifungal defense, not only because of their capacity to kill the invading microbe via phagocytosis, but also due to their ability to induce Th1 responses (Brown, 2011; Romani, 2011). Interestingly, the recognition of *C. albicans* by Dectin-1, a CLR key to antifungal immunity, induces both Th1 and Th17 immune responses by DCs (see section **1.5.3**) (Gringhuis et al., 2009b; Gross et al., 2006; LeibundGut-Landmann et al., 2007). It was previously thought that Th1 cells are the main effectors of the T helper response against fungal pathogens, however, recent evidence in the past few years points to Th17 cells as key effector cells in the response to fungal infections, including *C. albicans* (Conti et al., 2009; LeibundGut-Landmann et al., 2007; Vautier et al., 2010). Th17 have been demonstrated by several studies to be integral to antifungal defense, primarily by mobilizing neutrophils (Vautier et al., 2010).

Historically, inflammatory cytokines and Th1 responses were considered to provide protection against fungal infections, while immunosuppressive cytokines and Th2 responses were thought to contribute to susceptibility. This concept was supported by both human patients and animal models in which deficiencies in Th1 or inflammatory cytokines (such as IFN γ or TNF, for example), or upregulation of Th2 or immunosuppressive cytokines (e.g., IL-4 and IL-10) led to enhanced susceptibility to infections with various fungal pathogens (Romani, 2011). However, this distinction was not always clear-cut; for example, nonprotective cytokines, such as IL-10, are required to limit inflammatory pathology, in part by promoting the development of Tregs cells, whereas some level of IL-4 is needed to induce protective immunity. Furthermore, deficiencies in Th1 responses did not always correlate to susceptibility, especially for mucocutaneous fungal infections. This latter paradox was solved recently following the identification of Th17 adaptive immune responses, which were originally linked to autoimmunity. In the past years several studies have highlighted a central role of Th17 cells in host defense against several pathogenic fungi, specifically in protection against mucocutaneous fungal infections (Conti and Gaffen, 2010). Interestingly, Th17 responses including the generation of Th17-related cytokines IL-17 and IL-22 appear to be primarily responsible for protection against fungal infections at the mucosa (mucosal-specific immunity) (Brown and Netea, 2012; Vautier et al., 2010; Zelante et al., 2011). In humans, genetic studies of inherited conditions known to predispose individuals to mycoses (fungal infections), usually *Candida* infections, have demonstrated an important role for Th17 responses (Vautier et al., 2012). Th17 responses may also be partially required for the control of systemic infections caused by some (but not all) fungal pathogens, including *Candida*, *Aspergillus*, *and Cryptococcus* (Wuthrich et al., 2012). The importance of Th17 responses in *C. albicans* immunity is evident in IL-23p19 and IL-17A-deficient mice, which show increased susceptibility to systemic *C. albicans* infection, indicating the importance of IL-17A for protection against this pathogen (Conti et al., 2009; Saijo et al., 2007). Defects in Th17 immunity have been directly linked to susceptibility to mucocutaneous fungal infections in humans. The most common infection observed in these patients is chronic mucocutaneous candidiasis (CMC). Indeed, Hyper-IgE syndrome patients, in which Th17 cell differentiation is suppressed, are associated with CMC, highlighting the importance of Th17 cells for antifungal defense in humans (Milner et al., 2008; Puel et al., 2010). Furthermore, people with an autosomal dominant mutation

in the IL-17F gene, as well as with a recessive mutation in the IL-17RA gene show CMC (Puel et al., 2011). Th17 cells have also been demonstrated to play a central role in resistance to oropharyngeal candidiasis (OPC; thrush), a mucosal infection cause by the commensal pathogen *C.albicans*. The protective mechanisms induced by Th17 for host defense against fungal infection have not been clarified and are still being elucidated, but appear to be mediated by neutrophil activation and recruitment [through chemotactic chemokines and granulopoietic cytokines, e.g., G-CSF and IL-6, and chemokines e.g, CXCL1, CXCL (MIP-2) & CXCL5], and the production of antimicrobial peptides (such as β-defensins) at the site of infection (Brown et al., 2012a; Palm and Medzhitov, 2007).

The requirement for neutrophils in host defense against disseminated candidiasis is well described (Concia et al., 2009; Koh et al., 2008). Actually, decreased neutrophil recruitment to the peritoneal cavity was observed in Dectin-1-deficient mice upon intraperitoneal infection with *C. albicans* (Taylor et al., 2007). Because IL-17A is important for the generation and recruitment of neutrophils to inflammatory sites, and neutrophils are important in antifungal defense, this function of IL-17A may be related to the protective activity (van de Veerdonk et al., 2009a; Vautier et al., 2010). Neutrophil recruitment is markedly attenuated in mice defective in Il-17 signaling. Strikingly, recent reports of single gene defects in humans resulting in chronic mucocutaneous candidiasis (CMC) cluster in the IL-17 pathway, including mutations in IL-17RA, IL-17F (Huppler et al., 2012; Huppler et al., 2014). Additionally, because IL-17A is also important for the activation of T cells and B cells, it is possible that IL-17A is involved in fungal eradication through activation of acquired immunity (Brown and Netea, 2012). Although IL-17A and IL-17F can induce innate antimicrobial peptides such as β-defensins, the roles of β-defensins in systemic candidiasis are not clear at present. IL-22, one of the Th17 cytokines, is also suggested to be involved in the host defense against fungal infection. Blocking *Candida*-mannan/ β-glucan-induced prostaglandin E2 production with non-steroidal anti-inflammatory drugs suppressed IL-17A and IL-22 secretion in human PBMCs, causing increased susceptibility to infection with *Candida* (Smeekens et al., 2010). IL-22 mediates protection against *C. albicans* in IL-17RA–deficient mice by controlling the growth of infecting yeasts as well as by contributing to the host's epithelial integrity (De Luca et al., 2010). Excess Th17 cytokines, however, promote inflammation and impair antifungal immune responses when mice are infected gastrointestinally (Zelante et al., 2007).

While it is clear that cell-mediated immunity is essential for resistance to fungal infections, humoral immunity has long been considered to have a secondary role. Studies looking at the passive transfer of immune sera or using B cell-deficient mice, have failed to reliably demonstrate the significance of antibodies in antifungal immunity. Excitingly, however, protective mAbs could be used for the development of novel therapeutics. Furthermore, protective antibody responses generated following the targeting of specific fungal antigens offers the potential for vaccine development. Indeed, antibodies to common antigens, such as β-glucans, could provide cross-species protection (Casadevall and Pirofski, 2012).

While studies with knockout mice and purified β-glucans preparations have implicated Dectin-1 as being central to the Th17 skewing seen in fungal infections, contributions from other receptors have not been excluded (see below). In humans, naïve T cells are thought to commit to the Th17 lineage when exposed to the cytokines TGF-β, IL-1β, and IL-6, IL-21, or IL-23 (Vautier et al., 2010). Terminal differentiation and population expansion requires IL-23 (Lyakh et al., 2008). Interleukin 1β (IL-1β) is an important proinflammatory cytokine that is required for the induction of immune responses involving Th17 cells (Awasthi and Kuchroo, 2009). DCs induce the differentiation of Th cells via the release of IL-1β (Geijtenbeek and Gringhuis, 2009). The expression, maturation and secretion of IL-1β is tightly controlled and is a multi-step process that is an integral part of the innate immune response (Dinarello, 2009). However, to date, little is known about the molecular mechanisms that control IL-1β production in DCs after fungal infection (Gringhuis et al., 2012). In this regard, recognition of β-glucan, *A. fumigatus*, and *C. albicans* by Dectin-1 and TLR2 activates the NLRP3 inflammasome, which leads to the production of bioactive IL-1β that promotes Th17 cell development and antifungal immunity. (Gross et al., 2009; Hise et al., 2009; Said-Sadier et al., 2010; Smeekens et al., 2010). In a pulmonary model of *A. fumigatus* infection, Dectin-1 decreased the production of IL-12 and IFN-γ in innate cells, which favoured Th17 differentiation versus that of Th1 (Rivera et al., 2011). Despite the importance of Dectin-1 and IL-1β to drive antifungal Th17 cells and resistance to *C. albicans*, *A. fumigatus*, and *P. carinii* (Saijo et al., 2007; Taylor et al., 2007; Werner et al., 2009), vaccine-induced Th17 cells and immunity to *B. dermatitidis* are independent of Dectin-1 and IL-1β (Wuthrich et al., 2011a).

Th1 cell subsets also play a key role in antifungal immunity (Romani, 2004). Th1 cells are induced by the action of IL-12, and produce IFN-γ that activates phagocytes. The importance of

both IL-12 and IFN- γ in forming a protective Th1 response has been demonstrated, for example, in a murine study using blocking antibodies against these cytokines in a model of pulmonary *C. neoformans* infection (Hoag et al., 1997). Blocking antibodies resulted in increased lung fungal burdens and a skewing of T cell responses from a protective Th1 to a non-protective IL-4 Th2 response. In a *C. albicans* study, IL-12p40, a subunit of both IL-12 and IL-23, was shown to be important for mucosal, but not systemic, defense (Farah et al., 2006). In contrast, Th2 responses induced in response to IL-4 are thought to be non-protective in fungal infections, via the suppression of protective Th1 responses and the alternative activation of macrophages (Zelante et al., 2011).

The balance between IL-17 producing Th17 and IL-10 producing Treg cells has been demonstrated to be important in control of fungal infection, e.g. in clearance of *Histoplasma capsulatum* (Vautier et al., 2010). In mice, with increased levels of IL-17 there was a decrease in recruitment of Tregs, and *H. capsulatum* infection was more effectively cleared. Conversely, the use of anti-IL-17 antibodies increased Treg numbers and persistence of fungal infection (Puccetti et al., 1995). Genetic studies in humans have revealed a dual role, protective and non-protective, for IL-10 in fungal infection.

Since their discovery, PRRs have been increasingly studied and shown to play a critical role in innate responses to fungal microbes in several experimental *in vitro*, *ex vivo*, and animal models (Figure 17) (Gow and Hube, 2012). However, the exact role of PRRs in the human response to fungal infection in natural conditions has just started to be deciphered, by means of clinical studies of primary immunodeficiencies and epidemiological genetic studies. It is now known that a wide gamut of membrane-bound and soluble/secreted PRRs recognize fungi in mammals and mediate antifungal immune responses (Figure 17) { (Brown, 2011; Wuthrich, 2012). Although TLRs have been described to be involved in fungal recognition, other non-TLR PRRs have been identified for recognition of fungal pathogens and induction of antifungal immunity, the best characterized of which are the CLRs. Currently, it is widely accepted that CLRs expressed on myeloid cells are the key PRRs for fungal recognition (Figure 17) (Brown, 2011; Kerrigan and Brown, 2010, 2011a; Netea et al., 2008; Romani, 2011). The role of TLRs, CLRs, and other non-TLR PRRs involved in antifungal immunity are further discussed in the next section.

1.4.3.2. TLR & non-TLR PRRs in Antifungal Immunity

Cell associated PRRs involved in antifungal immunity include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and cytoplasmic receptors (mainly NOD-like receptors/NLRs). Among them, CLRs are the PRRs that primarily mediate immune responses against fungal infections (e.g., *Candia albicans, Aspergillus fumigatus,* and *Cryptococcus neoformans*) (Figure 17) (Hardison et al., 2012; Willment and Brown, 2008). Much interest had been originally focused on the role of Toll-like receptors (TLR) in antifungal immunity. However, in the past decade there has been growing appreciation that the non-TLRs have more significant roles in the control of infection with these organisms. Non-TLR C-type like lectin receptors (CLRs) and Toll-like receptors (TLRs) are the two main families of pattern recognition receptors (PRRs) in phagocytic cells that recognize fungal components (Figure 17). Both can activate NF-κB and induce the expression of inflammatory cytokines and costimulatory molecules by macrophages and dendritic cells.

Antifungal immunity has come at a time when there has been a resurgence of research into understanding the mechanisms underlying protective immunity to fungal pathogens. This interest has been largely influenced by the worrying increase in fungal infections, especially in an immunocompromised population, over the last few decades. In the last few years, a growing number of PRRs have been implicated in antifungal immunity (Figure 17). Of these, much interest has focused on the Toll-like receptors (TLRs), which are generally considered to be the principal receptors involved in microbial recognition and intracellular signaling (Netea et al., 2006c). Although a number of TLRs, including TLR2, TLR4 and TLR9, have been implicated in the innate response to fungal infections, other 'non-TLR' receptors also play key functions (Figure 17) (Willment and Brown, 2008).

The main TLRs involved in sensing fungal ligands such as zymosan, phospholipomannan, *O*-linked mannans, and fungal DNA are TLR2, TLR4, and TLR9 (Figure 17). Consequently, mice lacking the intracellular TLR signaling adaptor myeloid differentiation primary response protein 88 (MyD88) are highly susceptible to infections by *B. dermatitidis*, *P. brasiliensis*, *A. fumigatus*, *C. neoformans*, and *C. albicans,* emphasizing important roles for TLR signaling in antifungal immunity (Netea et al., 2006a; Netea et al., 2006c; Romani, 2011; Wuthrich et al., 2012). However, conflicting reports on individual contributions of TLRs exist for almost every TLR and

fungal pathogen (Netea et al., 2008; Wuthrich et al., 2012). The precise reason for these discrepancies is unclear, but the contribution of individual TLRs may vary depending on the experimental model and the fungal species.

C. neoformans mannoproteins synergize with TLR1/2, 3, 4, 7/8, and 9 ligands to stimulate enhanced production of proinflammatory cytokines (such as TNF and IL-12) and chemokines from murine DCs (Figure 17){Gow, 2012 #569}. Synergistic stimulation also required the interaction of mannose residues on mannoproteins with the mannose receptor (MR) (Figure 17) (Dan et al., 2008b). The synergy between the MR and TLRs enhanced mannoprotein-specific $CD4^+$ T cell responses. Thus, phagocytes produce augmented proinflammatory cytokines when stimulated with a combination of fungal mannan and TLR ligands *in vitro* (Figure 17).

As described in section **1.2.1.3**, MyD88 is a key adaptor molecule that is central to signaling from most TLR receptors. Because MyD88 is instrumental in priming Th1 cells in response to fungi, it is generally thought that antifungal TLRs induce a Th1 response (Rivera et al., 2006). However, the role of TLRs for the induction of antifungal Th17 cells is less clear (Wuthrich et al., 2012)

Two different NLR inflammasome components, NLRP3 and NLRC4, have been shown to be essential in protective antifungal immunity, particularly for driving Th1 and Th17 responses (Figure 17) (Hardison and Brown, 2012). Both inflammasomes are required for controlling mucosal infections with *Candida* species, but only NLRP3 is involved in preventing dissemination of this pathogen (Figure 17) (Hise et al., 2009; Said-Sadier et al., 2010). Although, CLRs (including Dectin-1) and TLRs can both induce pro-IL-1β, it is still unclear how the NLRs sense pathogens to trigger activation of the inflammasome for production of active IL-1β (Figure 17). It has been proposed that the NLRP3 inflammasome is activated by stimuli such as potassium efflux and ligand-induced intermediates, e.g., reactive oxygen species (ROS) (Franchi et al., 2012; Martinon, 2010). Interestingly, it has been shown that activation of the NLRP3 inflammasome in response to both *A. fumigatus* and *C. albicans* requires SYK kinase, as well as the respiratory burst and potassium efflux, which suggests direct involvement of CLRs such as Dectin-1 (Said-Sadier et al., 2010). In addition, a truncated allele of one of the NLRP3-inflammasome components, which is required for IL-1β processing, was also found to increase susceptibility to fungal infection, with lower IL-1β production and increased incidence of vulvovaginal candidiasis in women with this allele (Lev-Sagie et al., 2009)

1.4.3.3. CLRs Involved in Antifungal Immunity

Besides the TLRs, C-type lectin receptors (CLRs) expressed by myeloid cells are the key PRRs for fungal recognition and for the induction of protective antifungal host defense (Figure 17) (Netea et al., 2008; Romani, 2011; Wuthrich et al., 2012). C-type lectins mostly recognize carbohydrate structures in pathogens. As explained in section **1.4.2**, the cell wall of fungi predominantly consists of carbohydrates, including mannose-based structures, β-glucans, and chitin (Figure 10). Therefore, it is not surprising that CLRs are key PRRs for fungal recognition via binding to these fungal cell wall carbohydrate components. Indeed, these various fungal carbohydrate-based PAMPS are thought to be mainly recognized by CLRs including the macrophage mannose receptor (MR) (CD206), DC-SIGN (CD209) (e.g., SIGNR-1, a homologue of human DC-SIGN), Galectin-3, Mincle, Dectin-1 and Dectin-2 (Figure 17)(Gow and Hube, 2012). Recognition of fungal carbohydrates by these key CLRs activates host innate immune response against fungal infection and modulates adaptive immunity to fungi (Willment and Brown, 2008). Dectin-1, Dectin-2, and Mincle are SYK-coupled CLRs responsible for sensing β-glucans, mannans, and mannose-like structures from fungi, respectively (see section **1.3.4.**) (Figure 10 & Figure 17). Mannans are also sensed by the mannose receptor (MR) and DC-SIGN, both of which are CLRs. Galectin-3 is responsible for the detection of fungal β-mannans (Figure 17) (Gow and Hube, 2012).

The macrophage C-type lectin Mincle senses infection by fungal species such as *Malassezia* and *Candida* (Yamasaki et al., 2009). Soluble Mincle protein binds to *C. albicans* and *S. cerevisiae* extracts and to *Malassezia* sp. by selectively binding α-mannose (Figure 17) (Gow and Hube, 2012). Although in one study Mincle$^{-/-}$ mice demonstrated increased susceptibility to systemic *C. albicans* infection, this receptor is generally believed not to be involved in *C. albicans* eradication (Wells et al., 2008). Similarly, Dectin-2 binds to the cell wall of multiple fungi including *C. albicans*, *P. brasiliensis*, *H. capsulatum*, nonencapsulated *C. neoformans* (Kerscher et al., 2013). DCs activated by Dectin-1 or Dectin-2 are able to instruct T cells to confer protective immunity against *Candida albicans* (Robinson et al., 2009). Terminal mannose residues on the surface of *C. neoformans*, *C. albicans*, and *P. carinii* are notably sensed by the MR (Figure 17) (Brown, 2011). MR-deficient mice do not exhibit impaired resistance to primary

infection with *C. albicans* and *P. carinii*, but are more susceptible to *C. neoformans* infection (Bourgeois et al., 2010). Human DC-SIGN recognizes several *Candida* species, *A. fumigatus*, *Chrysosporium tropicum*, and *C. neoformans* (Cambi et al., 2008; Drummond and Brown, 2013).

The importance of Dectin-1, Dectin-2 and Mincle signaling in fungal infection is especially apparent when these receptors are absent or signaling does not occur properly. Recognition of α-mannans by Dectin-2 induces the differentiation of Th17 cells (Figure 17) (Saijo et al., 2010; Wuthrich et al., 2012). In a model of systemic *C. albicans* infection, blockade of Dectin-2 by monoclonal antibody or infection of Dectin-2$^{-/-}$ mice abrogated the development of *Candida*-specific Th17 cells (Robinson et al., 2009; Saijo et al., 2010). Mincle was initially implicated in host responses to *C. albicans* infection (Wells et al., 2008), later, a glycoconjugate microarray study found that Mincle preferentially recognizes *Malassezia* species via α-mannose structures but not via mannan, and is accordingly suggested to be involved in the host defense against *Malassezia* (Yamasaki et al., 2009). A recent report details the requirement for Mincle signaling in the immune response to *Fonsecaea pedrosoi*, a fungus that causes the chronic skin infection chromoblastosis. It was demonstrated that both Mincle and TLR signaling are required to trigger an immune response against this fungus, thereby highlighting non-redundant functions of different classes of PRR in antifungal immunity (Sousa Mda et al., 2011). Mincle induces the production of TNF-α, CXCL1, CXCL2, and IL-10 upon recognition of *Malassezia*, and *F. pedrosoi*. Further investigations are needed to determine whether Mincle engagement by fungal PAMPs determines antifungal Th subsets (Wells et al., 2008; Yamasaki, 2009). Interestingly, Mincle is an important receptor for macrophage recognition of *M. tuberculosis* via its recognition of the mycobacterial cord factor TDM (trehalose 6,6'-dimycolate). Mincle induces the production of IL-6 following TDM stimulation (Ishikawa et al., 2009), and induces the polarization of Th1 and Th17 cells (Yoneyama et al., 2005). For that reason, TDB are currently being investigated as adjuvants for a subunit vaccine against *Mycobacterium tuberculosis* (Werninghaus et al., 2009).

The MR recognizes the terminal α-(1,2)/(1,3)-mannose of N-linked mannans of fungal cell walls, while SIGNR-1 recognizes branched mannans and Galectin-3 recognizes β-(1, 2)-mannans (Figure 17) (Cambi et al., 2008; Poulain and Jouault, 2004; Saijo and Iwakura, 2011). However, MR-deficient mice show normal host defense during systemic candidiasis and macrophages isolated from MR-deficient mice show normal susceptibility to *Candida albicans* infection (Lee et al., 2003), although this receptor is suggested to be involved in host defense against *C.*

albicans in humans through induction of Th17 cell differentiation (Smeekens et al., 2010; van de Veerdonk et al., 2009a). Moreover, the mannose receptor (MR) has been reported to induce Th17 cell differentiation of human T cells in response to *C. albicans*, but memory—and not naïve—T cells were the major source of the IL-17-producing cells (Levitz, 2009; van de Veerdonk et al., 2009b). Nevertheless, the role of the MR in inducing Th17 cell differentiation remains inconclusive (Wuthrich et al., 2012). Macrophages from Galectin-3-deficient mice show normal binding and endocytosis of *C. albicans* (Jouault et al., 2006). Whether SIGNR-1-deficient mice are sensitive to *Candida* infection has not been reported yet.

Recently, it has been reported that Dectin-1 and Dectin-2 are the specific receptors for β-glucans (Saijo et al., 2007; Taylor et al., 2007) and *C. albicans*-derived mannans (Figure 17) (Saijo et al., 2010), respectively. These two CLRs induce cytokines and reactive oxygen species (ROS) to protect hosts from fungal infection through activation of the spleen tyrosine kinase (SYK)–caspase recruitment domain family member 9 (CARD9)–nuclear factor-κB (NF-κB) pathway in dendritic cells (DCs) and macrophages (Figure 17) (Drummond et al., 2011). Interestingly, Dectin-1, Dectin-2, and Mincle are all SYK-coupled CLRs that signal through the SYK-CARD9 pathway, and given the importance of CARD9 in CLR signaling, it is not surprising that CARD9 deficiency is linked to impaired fungal immune responses (Figure 17) (Rosentul et al., 2011). An early stop codon mutation in the gene encoding CARD9 is associated with an increased incidence of both mucocutaneous and invasive *Candida* infections in human patients (Glocker et al., 2009). Patients homozygous for this mutation have a lower number of Th17 cells than normal controls, again highlighting the importance of this signaling pathway in the development of Th17 responses required for antifungal immunity (Puel et al., 2010). Studies in mice showed that this mutation results in a loss of function of CARD9, and peripheral blood mononuclear cells from patients homozygous for this mutation showed no CARD9 expression (Glocker et al., 2009). While Dectin-1 mutations are associated with a susceptibility to mucocutaneous *Candida* infections (see section **1.5.5**), the mutation of CARD9 results in a more severe phenotype, with a susceptibility to both mucocutaneous and invasive *Candida* infections. This is not surprising, as a defect in CARD9 will have more global effects, impairing signaling from Dectin-1, Dectin-2, Mincle and other PRRs, while Dectin-1 mutations only affect Dectin-1 signaling (Figure 17) (Gow and Hube, 2012). Although Dectin-1 is not a redundant receptor, other PRRs such as Dectin-2, Mincle and some TLRs can contribute to the initiation of an antifungal immune

response (Figure 17) (Gow and Hube, 2012). Therefore, it is clear from the above discussion that CLRs such as Dectin-1, Dectin-2 and Mincle, play central roles in the recognition and shaping of immune responses to fungal pathogens, in part, through the induction and modulation of cytokine responses, which consequently lead to Th17 antifungal responses (Figure 17) (Gow and Hube, 2012; Vautier et al., 2012).

In conclusion, CLRs influence cytokine production and subsequent immune responses in antifungal immunity. The concerted collaboration of TLR and non-TLR pathways in innate immune cells, can mount a specific adaptive immune response tailored to the fungal pathogen (Figure 17) (Geijtenbeek and Gringhuis, 2009). Specifically, cytokine production is controlled by extensive cross-talk between several signaling pathways activated by different PRRs, and this is essential for effective antifungal immunity (Figure 17) (Gow and Hube, 2012). Further understanding of which cytokines induce protective responses to fungal pathogens and how C-type lectins and other receptors direct cytokine production may allow development of novel antifungal therapies (Vautier et al., 2012).

1.5. An Overview of Dectin-1: The β-glucan Receptor

1.5.1. Dectin-1 Discovery, Expression, Structure and Isoforms

Dectin-1 was originally discovered as a receptor on dendritic cells that recognized an unidentified ligand on T cells, and hence it the origin of its name **'Dendritic-cell-associated C-type lectin 1'** (Ariizumi et al., 2000; Brown, 2006b; Brown and Gordon, 2001). Soon thereafter, Dectin-1 was re-identified as a receptor for β-glucan polysaccharides, following a screening approach of a murine macrophage cDNA expression library in order to identify receptors that bind to zymosan, a β-glucan-rich extract of the yeast cell wall (Brown and Gordon, 2001). β-glucans are carbohydrate PAMPs that form a major component of fungal cell walls. These polysaccharides primarily consist of a backbone of glucose residues linked by β-(1,3)-glycosidic bonds with or without β-(1,6)-linked side chains of varying length and distribution (Figure 13) (Adams et al., 2008; Brown, 2006a). Subsequent studies of Dectin-1 established it as a signaling and phagocytic C-type lectin receptor (CLR) of the innate immune system with the ability to detect β-glucans, and mediate phagocytosis and cytokine production in response to fungal pathogens (Brown et al., 2003; Brown et al., 2002b; Goodridge et al., 2012). Since then, Dectin-1 has been one of the most intensively studied and well-characterized CLRs, and has become a model receptor for signaling

CLRs. Indeed, Dectin-1 is the first discovered and the archetype of a signaling 'non-TLR' pattern recognition receptor (PRR) (Brown, 2006a; Tsoni and Brown, 2008a) (see section **1.5.3**). Dectin-1 has also been identified as the major receptor for β-glucans on leukocytes, and thus its name the 'β-glucan receptor' (Adams et al., 2008; Brown et al., 2002b; Marakalala et al., 2011). As mentioned above β-glucans are fungal cell wall PAMPs, and it is now well recognized that Dectin-1 as a C-type lectin PRR and the major β-glucan receptor in mammals, plays a key role in host fungal recognition and antifungal immunity (Brown and Gordon, 2001; Brown et al., 2003; Brown et al., 2002b; Drummond et al., 2011; Tsoni and Brown, 2008a). Moreover, β-glucans are well known for their anti-infective and antitumorogenic activities, and the identification of Dectin-1 as a PRR that recognizes these carbohydrate PAMPs has enabled significant advances in our understanding of the mechanisms underlying these activities (Brown et al., 2003; Martin et al., 2009; Murphy et al., 2010).

Dectin-1 Expression

Dectin-1 mRNA is expressed in a tissue specific manner and is mainly present in immune-rich tissues, such as the thymus, spleen, lung, liver and kidney, in the peripheral blood, spleen and lymph nodes (Leibundgut-Landmann et al., 2008). Also consistent with the role of Dectin-1 as a PRR involved in immune surveillance of fungal microbes, it is prominently expressed at portals of pathogen entry such as the intestinal mucosa and the lung (Reid et al., 2004; Taylor et al., 2002). Although, Dectin was initially identified as a dendritic cell-specific receptor, it is not exclusively present on DCs, and is now known to be expressed by many other cell types. It is predominantly expressed by innate immune cells of myeloid origin, such as monocytes, antigen-presenting cells such as macrophages and dendritic cells, neutrophils and other phagocytes (Figure 11) (Brown et al., 2002b; Drummond et al., 2011; Reis e Sousa, 2004b; Taylor et al., 2002). Several studies demonstrate that Dectin-1 is the primary β-glucan receptor on the surface of these cells (Herre et al., 2004a). Dectin-1 is expressed at its highest levels on blood and splenic monocytes/macrophages, neutrophils and on cells at portals of pathogen entry such as alveolar macrophages, whereas it is expressed at lower levels on all conventional DC subsets in the peripheral blood, spleen and lymph nodes (Ariizumi et al., 2000; Goodridge et al., 2009b; Leibundgut-Landmann et al., 2008; Taylor et al., 2002). Dectin-1 doesn't exist on NK cells, and has been reported to be expressed but at low levels, on other cell types such as epithelial keratinocytes, intestinal enterocytes, subpopulations of human B cells, mast cells, Kupffer cells,

Langerhans cells, endothelial cells, and eosinophils and pneumocytes (Batbayar et al., 2012; Brown et al., 2002b; Goodridge et al., 2009b). Dectin has also been identified on some subsets of T cells including an unidentified subpopulation of T lymphocytes in the spleen, and an IL-17–producing subset of γδ T cells (Martin et al., 2009). Dectin-1 has been demonstrated to induce proliferation of these T cell subsets, however the exact function of Dectin-1 on T cells needs to be further characterized (Martin et al., 2009; Taylor et al., 2002). Moreover, the function of Dectin-1 detected on human B cells has yet to be determined (Taylor et al., 2002).

The levels of Dectin-1 surface expression has been show to be significantly influenced by a variety of factors such as steroids, and immune stimuli including cytokines and microbial components, e.g., β-glucans (Harada and Ohno, 2008). For example, IL-4, IL-13, and GM-CSF (granulocyte–macrophage colony-stimulating factor) cause significant upregulation of Dectin-1 expression. In this regard, *in vitro* differentiation of monocyte-derived as well as, bone-marrow-derived DCs requires IL-4 and GM-CSF, both of which have been shown to increase Dectin-1 surface expression (Willment et al., 2003; Willment et al., 2005). On the other hand, IL-10, LPS, zymosan (a particulate β-glucan) and dexamethasone (DMSO) trigger downregulation of Dectin-1 expression. Also maturation of DCs with TNF-α leads to decreased Dectin-1 activity (Willment and Brown, 2008; Willment et al., 2005). Furthermore, the systemic administration of C. *albicans* resulted in an increase in Dectin-1 expression, whereas on the other hand, Dectin-1 expression was decreased during polymicrobial sepsis (Marakalala et al., 2011; Ozment-Skelton et al., 2009).

β-glucan Receptors

Numerous receptors have been described that recognize β-glucans, and while their relative importance has not been firmly established, Dectin-1 appears to play a predominant role. A number of other receptors present on both immune (mainly macrophages, monocytes, neutrophils, NK cells, DCs, and some T cells) and non-immune (e.g., endothelial and epithelial cells), have also been described to recognize β-glucans, namely, complement receptor 3 (CR3), lactosylceramide, the CLR langerin (on langerhans cells), and the scavenger receptors SCARF1, and CD36 (Brown, 2006a; de Jong et al., 2010; Goodridge et al., 2009b; Means et al., 2009; Zimmerman et al., 1998). While the relative functional relevance of these receptors in mediating β-glucan recognition and cellular responses has not been firmly established, Dectin-1 appears to play a predominant role. Furthermore, Dectin-1 is identified as the primary receptor for β-glucans, at least on leukocytes (mainly macrophages and DCs), and it is the only one of these receptors

with a clearly defined mechanism for intracellular signaling (Brown et al., 2003). Moreover it is unknown how each β-glucan receptor distinguishes its ligands. It should also be noted that the other receptors are particularly important in mediating responses to β-glucans in nonimmune cells. For instance CR3 (expressed mainly on neutrophils, monocytes and NK cells, contains a binding site specific for β-glucans, and is thought to mainly mediate β-glucan recognition by NK cells, which do not express Dectin-1 (Taylor et al., 2002). Additionally, lactosylceramide is a glycosphingolipid that plays a role in β-glucan and fungal recognition in non-immune cells, which could lead to immune responses, yet the underlying signaling mechanisms are unknown.

Dectin-1 Topology and Protein Domains

Dectin-1 is a 28 kDa type II transmembrane protein with a single extracellular CRD (carbohydrate recognition domain) (Figure 12) (Brown and Gordon, 2001; Tsoni and Brown, 2008a). Dectin-1 has the typical amino acid sequence of C-type lectins, and based on its structure, as well as CRD homology, Dectin-1 is located within 'Group V' of the CLR superfamily which consists of the non-classical CLRs known as NKCLs ['NK (natural killer)-like C-type lectin-like receptors'] (see section **1.3.5**) (Aarnoudse et al., 2008; Plato et al., 2013; Zelensky and Gready, 2005). Dectin-1 is also a member of the 'Dectin-1 cluster', a subgroup of the NKCL receptors (see section **1.3.5**).

Human Dectin-1 is a protein of 247 amino acids, 3 amino acids longer than the mouse homologue (consisting of 244 amino acids). Dectin-1, similar to other proteins of the CLR family has six cysteine residues, all of which are highly conserved (Ariizumi et al., 2000) (Figure 6 & Figure 12), and two amino acids, Trp^{221} and His^{223} (in mouse Dectin-1), located after the fourth cysteine residue, have been identified as residues critical for its ligand binding function (Adachi et al., 2004). Human Dectin-1 shares 61% protein sequence identity and 72% DNA sequence homology with the mouse homologue (Figure 12). Dectin-1 is a single-spanning type II transmembrane protein that consists of three major parts: a single extracellular C-terminal **carbohydrate recognition domain** or **CRD**, which is responsible for ligand binding and is connected to a **transmembrane domain** by a short **stalk region** (40 amino acids), followed by an intracellular **cytoplasmic tail** that contains a single tyrosine-based signaling motif known as the hemITAM (immunoreceptor tyrosine-based activation) motif (also called ITAM-like motif) (Figure 12) (Kerrigan and Brown, 2010).

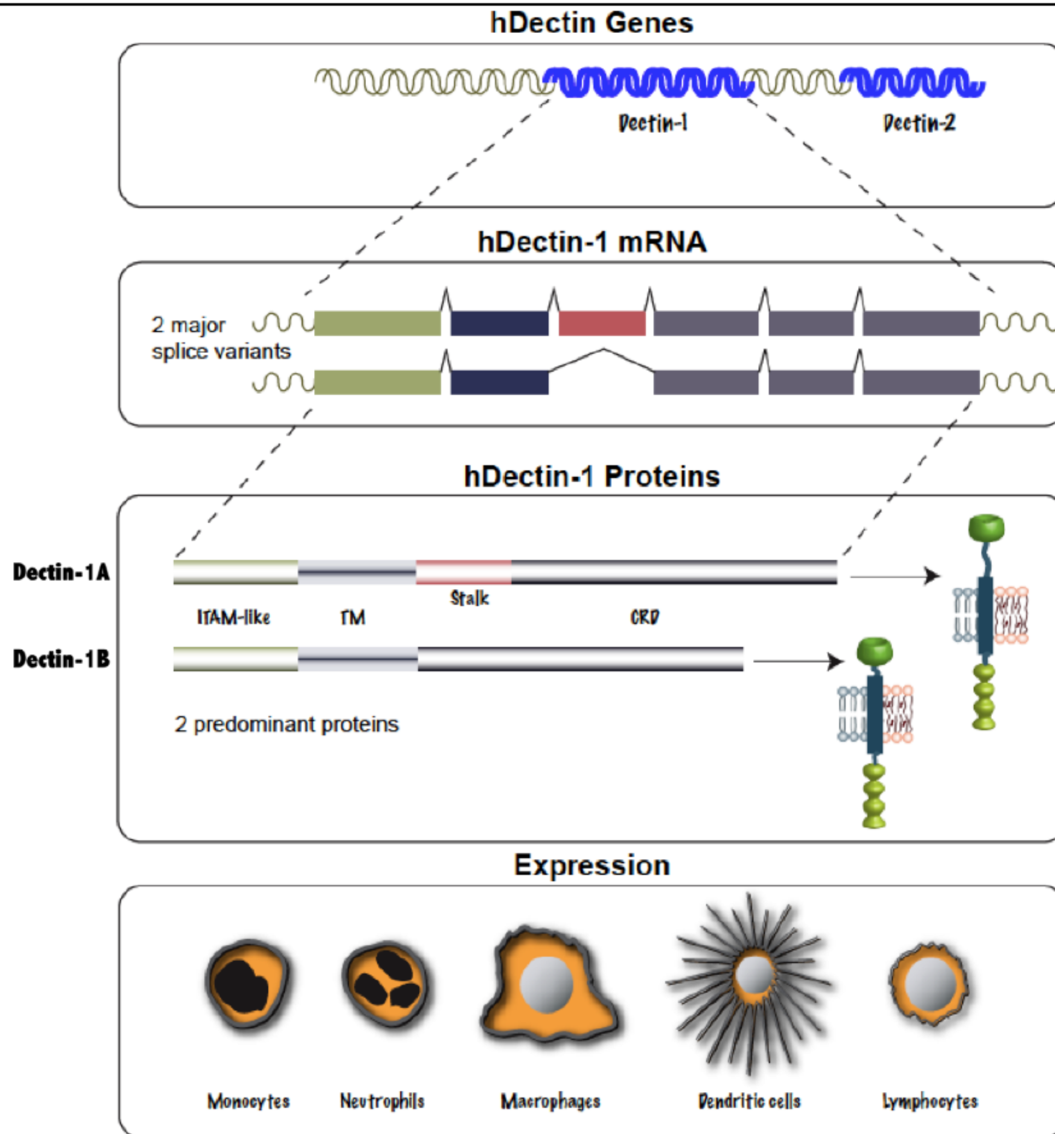


Figure 11: The Dectin Gene, Expression and Isoforms

In humans, two Dectin genes are located within a single genetic locus, the natural killer complex (NKC) on chromosome 12 in humans. Human Dectin-1 (hDectin-1) transcripts can be alternatively spliced into 2 major isoforms, namely Dectin-1A and Dectin-1B. The corresponding proteins are similar except that Dectin-1B is a smaller isoform missing the stalk region. This truncation has no impact on the protein function. Dectin-1A and Dectin-1B are predominantly expressed in cells of the immune system (as depicted in figure), especially on the surface of immune cells of the myeloid lineage. These two Dectin-1 isoforms have also been found on the surface of T lymphocytes.

Dectin-1 Gene and Splice-variants

The approved gene symbol of Dectin-1 is referred to as ***CLEC7A*** (Sattler et al., 2012). The full-length Dectin-1 gene, CLEC7A like all other closely related NKCL receptors in the 'group V' CLRs, is encoded by six exons (Figure 6) (Ariizumi et al., 2000). In most species, Dectin-1 can be alternatively spliced generating two major isoforms (splice variants), namely, Dectin-1A and

Dectin-1B (Figure 11), and a number of minor isoforms (Grunebach et al., 2002; Marakalala et al., 2011; Willment et al., 2005). The Dectin-1B isoform lacks the extracellular stalk domain, yielding a 'stalkless' Dectin-1 isoform (lacking exon 3/E3; see Figure 6) that is shorter than the 'full length' Dectin-1A isoform (Figure 11) (Willment et al., 2001). In humans, eight isoforms of Dectin-1 (A-H) have been described so far, and despite the identification of various Dectin-1 isoforms in humans and mice, isoforms A and B are the most commonly expressed (Willment et al., 2001). Regulation of Dectin-1 mRNA splicing, resulting in translation of the various isoforms, has not been extensively studied. However, it is apparent that transcript levels are differentially controlled within specific cell types, species and mouse genetic backgrounds, and with respect to exogenous and endogenous growth factors (Ferwerda et al., 2009; Goodridge et al., 2009b; Heinsbroek et al., 2006; Kato et al., 2005). In this regard, the major isoforms (A and B) of Dectin-1 show cell-type-specific and species-specific patterns of expression (Heinsbroek et al., 2006). Willment *et al.* (2005) have reported cell-specific isoform expression during monocyte maturation. Monocytes express both Dectin-1A and Dectin-1B, and Dectin-1A expression decreases during maturation to macrophages (Willment et al., 2001). Immature dendritic cells express high levels of both isoforms, and expression of both isoforms is lost when these cells are matured with LPS. So far, only the two most commonly expressed isoforms, Dectin-1A and Dectin-1B can bind to β-glucans and yeast particles. However, although both isoforms are functional for β-glucan binding *in vitro*, they appear to have slightly different functionalities regarding their ability to induce cellular responses (Heinsbroek et al., 2006; Willment et al., 2001; Willment et al., 2005). For instance, one study reported that both Dectin-1A and Dectin-1B murine isoforms are able to induce TNF-α production upon zymosan recognition, but that cells expressing Dectin-1B produce significantly more TNF-α (Heinsbroek et al., 2006). On the other hand, in a coccidioidomycosis model of fungal disease, mice preferentially expressing the stalkless Dectin-1B isoform showed an increased susceptibility to infection as compared with another mouse strain, which predominantly expresses the full length Dectin-1A isoform (del Pilar Jimenez et al., 2008). It has also been suggested that the stalkless Dectin-1B isoform is less efficient in ligand binding, and that the use of differential isoforms might be a mechanism of regulating cellular responses to β-glucan (Ferwerda et al., 2009; Kato et al., 2005). At least six other isoforms (C-H) generated by alternative splicing have been described for Dectin-1, some lacking exons and others with small insertions resulting in premature stop codons. Very little

information exists regarding these other Dectin-1 splice variants, and they are predicted to produce truncated proteins and soluble forms of the receptor (Marakalala et al., 2011). Moreover, none of these minor Dectin-1 isoforms has been shown to bind β-glucans (Willment et al., 2001), and whether or not these isoforms are functional proteins requires further investigation (Goodridge et al., 2009b). One of these minor isoforms of Dectin-1, which has been detected only in humans, is the cytoplasmic splice variant hDectin-1E. This isoform lacks the stalk and transmembrane domains, and is therefore retained in the cytoplasm instead of being expressed at the cell surface and has been shown to interact with other cytoplasmic proteins (Xie et al., 2006). So far hDectin-1E, has a known ligand the cytosolic Ran binding protein (see section **1.5.2.3**) (Xie et al., 2006).

Dectin-1 Post-translational Modifications

Dectin-1 is also N-glycosylated at different positions, a post-translational modification, which regulates the cell surface expression, activity and function of the receptor (Suzuki-Inoue et al., 2006). Indeed, glycosylation of Dectin-1 has been shown to be necessary for maximal surface expression and consequently for the activation of NF-κB, a key transcription factor activated downstream of Dectin-1 signaling (Kato et al., 2005). Mouse Dectin-1 has two N-glycosylations within the CRD (Asn 185 and Asn 233), whereas human Dectin-1 has one N-glycosylation motif located in its stalk region, which is lost in the stalkless Dectin-1B isoform (Willment et al., 2001). In vitro experiments demonstrated that surface levels of human Dectin-1B, can be increased by a glycosylation motif; conversely, removal of N-glycosylation sites from mouse Dectin-1A and Dectin-1B decreases surface expression and β-glucan binding activity (Kato et al., 2005).

The CRD domain is responsible for ligand binding, and has been shown to be involved in the binding of Dectin-1 to zymosan, intact fungal cells and other soluble fungal β-glucans (Ariizumi et al., 2000; Marshall et al., 2004). Dectin-1 is a member of the NKCL 'Group V' of CLRs and is specifically unusual in that unlike NKCL receptors that normally recognize proteinaceous ligands, is able to recognize carbohydrates (Zelensky and Gready, 2005). In fact, Dectin-1 is the first example of an NKCL receptor whose main ligand is a polysaccharide. More intriguing is that although the fold of the Dectin-1 CRD domain is structurally related to that of classical C-type lectins, it lacks all the conserved residues in the CRD typically involved in carbohydrate binding, in addition to those involved in calcium coordination, and is able to recognize β-glucans in a calcium-independent fashion (Brown et al., 2002b; Weis et al., 1998). To date the exact

mechanism of how Dectin-1 recognizes carbohydrates is unclear.

Dectin-1 Oligomerization and Associated proteins

Dectin-1 contains the conserved cysteine residues within the CRD shared by other members of the CLR family (Figure 6), yet unlike other 'group V' CLRs, it lacks the cysteine residues in the stalk region required for dimerization (Ariizumi et al., 2000). Dectin-1 appears to be expressed, and function, as a monomer, and to date there is no physical evidence that Dectin-1 forms either homodimers or heterodimers, like other members of the NKCL family such as Ly49D and CLEC-2 (a member of the 'Dectin-1 cluster' that forms homodimers). Nevertheless, it has been suggested that Dectin-1 might be functional as a dimer (see sections **1.5.4** and **1.5.2.1)** (Adams et al., 2008; Brown, 2006a; Goodridge et al., 2009b). However, Dectin-1 has been shown to form complexes with other receptors and integral membrane proteins and to localize to lipid rafts, which may affect its full function (Kimberg and Brown, 2008). The presence or absence of transmembrane proteins, such as the tetraspanins CD37 and CD63, may modulate the functional activity of Dectin-1 (Mantegazza et al., 2004; Meyer-Wentrup et al., 2007). The presence of CD37 increases Dectin-1 surface expression but reduces Dectin-1–mediated production of IL-6 and TNF-α, although its phagocytic ability is unaffected (Meyer-Wentrup et al., 2007). Dectin-1 mediation of phagocytosis and cytokine production is impaired if during cellular activation, it cannot translocate to lipid rafts to colocalize with SYK and PLC-γ2 (see section **1.5.4**) (Xu et al., 2009a).

Figure 12: Structure of Dectin-1

Dectin-1 is a type II transmembrane protein. The cytosolic N-terminus (N-ter) of the receptor contains an unconventional 'ITAM-like' or 'hemITAM' motif with a phosphorylatable tyrosine residue at position 15. The extracellular portion of Dectin-1 possesses a carbohydrate recognition domain (CRD) and a stalk region (absent in the Dectin-1B isoform) The structure of this carbohydrate recognition domain (CRD) has been resolved by X-ray crystallography (Brown et al., 2007). In absence of ligand, the CRD domain exists as a monomer (top-left panel), whereas upon addition of laminaritriose, the CRD domains dimerizes (top-right panel).

1.5.2. Dectin-1 Ligands

Although many endogenous and exogenous ligands for Dectin-1 have been reported, the receptor mainly recognizes β-glucan-ligands (as explained further below in this section)

1.5.2.1. β-glucans and Structure–Activity Relationships with Dectin-1

β-glucan as PAMP

Dectin-1 is generally recognized as a PRR receptor for β-glucans, which are carbohydrate PAMPs predominantly present in fungal cell walls as major structural components (Herre et al., 2004a). β-glucans are most well known for their immunomodulatory potential, which includes their ability to simulate the immune system, boost resistance to various infectious diseases, as well as promote antitumor activity (Murphy et al., 2010). Although the immunomodulatory potential of β-glucans, as well as their protective effects against infection and cancer have been long known and well described, the molecular basis for these effects are still not fully understood (Murphy et al., 2010).

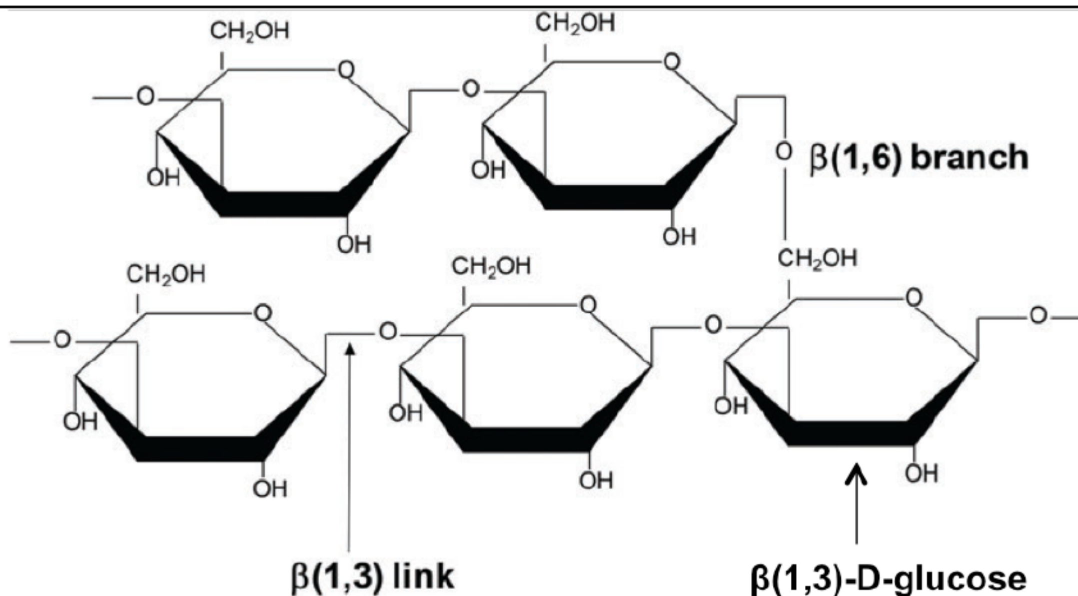


Figure 13: Primary Structure of β-glucan

The primary structure of β-glucans is depicted in above figure. β-glucans consist of a polymer backbone of glucose residues linked by β-(1,3)-glycosidic bonds, often with β-(1,6)-linked side-chains of variable length and distribution, to form side branches. Adapted with permission from (Rahar, S., et.al, 2011)

β-glucans Origin and Structure

β-glucans are naturally occurring carbohydrates that do not exist in mammals, and which are derived from plants, fungi, yeast, some bacteria, as well as from cereals such as oats and barley,

Dectin-1 can recognize a variety of soluble and particulate β-glucans derived from different sources primarily fungi, yeast and mushrooms, as well as from bacteria, and algae (Brown et al., 2002b; LeibundGut-Landmann et al., 2007; Tada et al., 2008; Tsoni and Brown, 2008a). β-glucans consist of a polymer backbone of glucose residues linked by β-(1,3)-glycosidic bonds, often with β-(1,6)-linked side-chains of variable length and distribution, to form side branches (Figure 13).

Dectin-1 Specificity for β-glucans

It has been shown through several ligand binding assays using natural and synthetic water-soluble glucans, as well as non-glucan polymers, that Dectin-1 can recognize yeast particles, in addition to a variety of soluble and particulate glucans (Adams et al., 2008; Palma et al., 2006; Williams et al., 1991). However, Dectin-1 was shown to be highly specific for glucans that have a β-(1,3)-D-glucopyranosyl backbone, and it binds to these glucans in a calcium-independent manner (Adams et al., 2008; Brown and Gordon, 2001; Graham et al., 2006; Palma et al., 2006). Furthermore Dectin-1 can recognize β-(1,3) and β–(1,3)/(1,6) synthetic β-glucans (Adams et al., 2008). These assays also demonstrated that Dectin-1 is unable to bind to glycans containing other linkages including, non-β-(1,3)–linked carbohydrate polymers (such as mannan or the (1,4)/(1,6)-α-glucan pullulan), linear β-(1,6)-linked glucans, plant-derived glucans containing β-(1,4)-linkages (such as barley β-(1,3)/(1,4)-glucan), although some cell-based assays suggest that β-(1,3)/(1,4)-glucans are recognized by Dectin-1 (Brown and Gordon, 2001).

The structure, degree of branching and purity of these glucans can vary considerably (Brown and Gordon, 2005). A variety of natural and synthetic, β-glucan and β-glucan–rich polymers, are used *in vitro* as Dectin-1 ligands—most frequently laminarin, glucan-phosphate, curdlan, β-glucan derived from edible mushrooms (see below section **1.6**), and zymosan, a complex particle derived from the cell wall of *Saccharomyces cerevisiae* that contains multiple ligands for receptors other than Dectin-1 (Figure 14) (Murphy et al., 2010). The size of the β-glucan polymer influences the cellular response triggered through Dectin-1 (Rosas et al., 2008). Moreover, the solubility of the β-glucan also affects its ability to stimulate Dectin-1, and differences in purity can influence stimulation through other PRRs leading to misinterpretation of results from Dectin-1 signaling (Batbayar et al., 2012; Goldman, 1988; Goodridge et al., 2011). Dectin-1, has been demonstrated to bind to particulate β-(1,3)-glucans, such as zymosan (a particulate yeast cell wall extract) (Rogers et al., 2005), as well as soluble (e.g. laminarin), and macroparticulate (e.g curdlan)

glucans (Rosas et al., 2008). *Curdlan* is a high-molecular weight, β-(1,3)-glucan (~molecular weight: 2000 kDa) (Figure 14). It is extracted as a purified macroparticulate β-(1,3)-glucan from the bacterium *Alcaligenes faecalis,* and is largely insoluble in water but soluble in alkaline solutions (see *Chapter 2*) (Lehtovaara and Gu, 2011). Curdlan is well established for its immunomodulatory properties through its induction of Dectin-1-mediated cellular responses (Murphy et al., 2010; Rand et al., 2013). *Laminarin*, is a short (40 glucose residues), branched β-(1,3)-glucan with β-(1,6)-linked branches (consisting of one glucose unit) that occur every 10 residues on the main glucan backbone (Figure 14) (Chen and Seviour, 2007). It is a water-soluble β-glucan of low molecular weight (6–8 kDa) that is extracted from the brown seaweed *Laminaria digitata,* and is is an established ligand for Dectin-1 with a measured affinity of 22nM (Adams et al., 2008; Goodridge et al., 2011). However, laminarin has been shown not to trigger Dectin-1 activation, but instead is commonly used as a specific blocking agent for Dectin-1 function (Brown et al., 2007; Dillon et al., 2006; Goodridge et al., 2007; Goodridge et al., 2009b; Underhill et al., 2005; Yadav and Schorey, 2006). This blocking effect has been proposed to be due to its lower complexity and smaller size (Adams et al., 2008; Goodridge et al., 2011). *Zymosan* is a particulate stimulatory yeast cell-wall extract composed primarily of β-glucans, mannan, mannoprotein, and chitin (Figure 14) (Gow et al., 2012; Netea et al., 2008). It is believed that zymosan recognition triggers the immune response that is primarily designed for fungal pathogens, and accordingly zymosan has been widely used for over 50 years as a model fungal particle for studying recognition of fungi by the innate immune system and the activated immune responses. Several studies have shown that Dectin-1 could interact with zymosan, stimulating cellular responses (explained below) such as phagocytosis, generation of ROS (reactive oxygen species) and synthesis of certain cytokines in a TLR-independent manner (Rogers et al., 2005; Underhill et al., 2005).

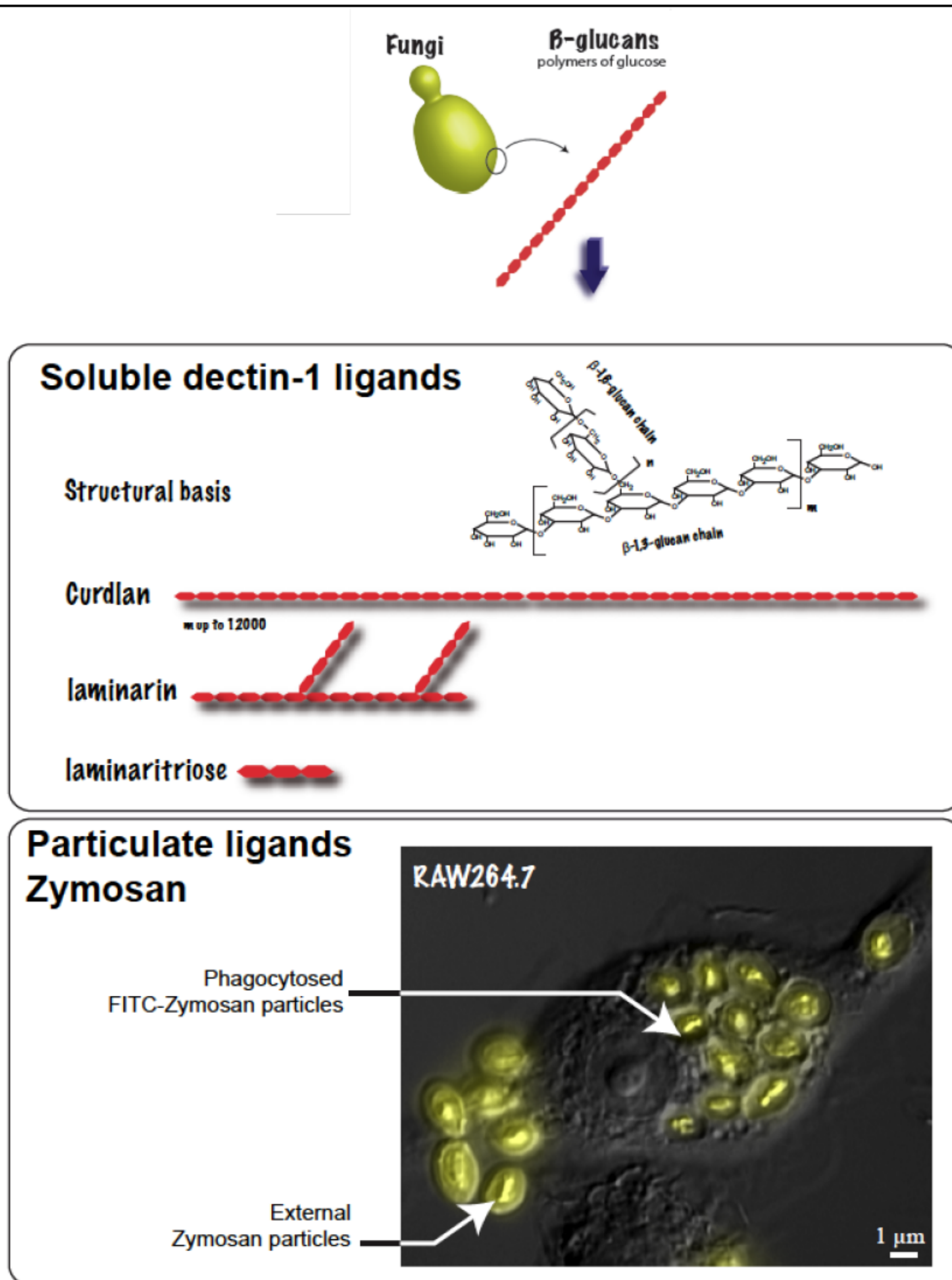


Figure 14: Dectin-1 Ligands: Soluble and Particulate β-glucans

β-glucans are major cell wall components of fungi. Dectin-1 is able to recognize various forms of β-glucans, either soluble (top panel) and in particulate form (bottom panel). Soluble β-glucans consists of a chain of glucose units attached through β-(1,3) linkages with or without β-(1,6) glucose branching. Curdlan is a linear β-glucan that contains up to 12,000 units. Laminarin is smaller but presents β-(1,6)-linked branches .The smaller ligand is laminaritriose and consists of only 3 glucose residues. Because β-glucans are major constituents of the yeast cell wall extract zymosan, these particles are recognized by Dectin-1 on the surface of macrophages, which are then able to internalize them efficiently. Represented on the bottom panel is a DIC picture (acquired by Dr. Nicolas Touret on an epifluorescent microscope) of a macrophage [RAW 264.7 cells (Mouse leukaemic monocyte macrophage cell line]

performing phagocytosis of FITC labeled zymosan. Particles have already been ingested on the right side, whereas some still remained extracellular on the bottom left side of the image.

The smallest Dectin-1 ligand characterized so far is *laminaritriose***,** a β-(1,3)-linked trisaccharide or glucan of only three β-glucosyl residues residues (Figure 14). This ligand was co-crystallized within dimers of the Dectin-1 carbohydrate recognition lectin-like domain (CRD) **(**Figure 12**)** (Brown et al., 2007).

Dectin-1 Structure and β-glucan Binding

As described above in section **1.5.1**, Dectin-1 is a non-classical C-type lectin-like receptor that lacks the residues typically involved in calcium coordination and carbohydrate recognition, and to date, the exact mechanism by which Dectin-1 recognizes β-glucan ligands is still unknown (Brown et al., 2007; Tsoni et al., 2009). However, the crystal structure of the CRD domain that has been determined indicated the existence of a shallow groove on the protein surface of the receptor that could be part of the β-glucan binding site (Brown et al., 2007). Furthermore, mutational analysis identified two highly conserved amino acids in the CRD, Trp221and His 223, which are essentially required for β-glucan binding and consequent activation of Dectin-1 signaling (Adachi et al., 2004). Interestingly, the crystal structure of the Dectin-1 CRD domain revealed that these two residues (Trp221and His 223), which are highly conserved in all identified Dectin-1 homologs, flank the shallow surface groove on the surface of the CRD domain that has been suggested to form the ligand-binding site (Adachi et al., 2004; Brown et al., 2007). Further analysis of the Dectin-1 crystal structure has also revealed that Dectin-1 can dimerize through a novel interface, creating another groove in which the β (1,3)-linked trisaccharide glucan, laminaritriose, was found to bind (Brown et al., 2007). This suggested that Dectin-1 molecules may dimerize *in vivo* through this novel interface, potentially creating another β-glucan binding site, and also proposed that while individual Dectin-1 molecules can bind β-glucans, cooperative binding as dimers may be more efficient Figure 12 (Brown et al., 2007). However, given that the minimum oligosaccharide that is functionally recognized by Dectin-1 is a heptasaccharide of seven glucose units, (explained below) the physiological significance of this potential binding site is unclear (Adams et al., 2008). Moreover, in addition to crystallographic data, other biophysical and binding studies, have also proposed that Dectin-1, although exists as a monomer *in vitro*, could form higher order complexes through oligomerization mediated by binding to β-glucans of higher molecular weight (Adams et al.,

2008; Brown et al., 2007; Palma et al., 2006).

Previous studies have demonstrated that the physicochemical properties of β-glucans such as, primary structure, polymer size, surface charge, solution conformation and side-chain branching, may be important for recognition and interaction with PRRs (Mueller et al., 2000; Muller et al., 1996; Novak and Vetvicka, 2008). However, these studies were performed using cell-based assays, rather than pure receptor, and in many cases the glucan preparations used in the experiments were not properly characterized or were not homogenous, thereby yielding confusing and misleading results. Recent studies have shed new light on how the structure and physicochemical features of β-glucans affect their recognition by Dectin-1 (Adams et al., 2008; Mueller et al., 2000; Palma et al., 2006). These studies demonstrated that Dectin-1 is highly specific for β-(1,3) linked glucans, yet it does not bind to all β-(1,3)-D-glucans equally, and this binding is metal-ion independent. Interestingly, the binding affinity of these interactions has been shown to be extremely high for some of the β-(1,3)-glucans including those such as β-(1,3)-glucan phosphate (2.2 pM) a potent Dectin-1 agonist used as an immune modulator. The binding efficiency was shown to be strongly influenced by the physicochemical and structural features of the glucan, such as backbone chain length and degree of branching of the polymer (Adams et al., 2008; Palma et al., 2006). Dectin-1 differentially interacts with β-glucans over a wide range of binding affinities from very low (2.6 mM) to very high (2.2 pM). This broad range of affinities appears to be due to differences in size and number of branches in β-glucan from various sources. Furthermore, a study by *Adams et al.* (2008) demonstrated that the affinity of Dectin-1 for β-glucans of less than ten glucose units is low, but rises with increasing polymer length (Adams et al., 2008). In the same study the affinity of Dectin-1 was shown to be maximal for synthetic linear β-(1,3) glucans and to increase when the synthetic glucan contained a single glucose β-(1,6)-linked side-chain branch. Depending on the method of analysis, the minimum binding unit of β-glucans recognized by Dectin-1 is a linear 7- or 10-mer β-(1,3) glucan, (Adams et al., 2008; Lowe et al., 2001; Palma et al., 2006). However, the study by Adams *et al.* (2008), in addition to an older report by Brown *et al.* (2001), demonstrate that the minimum recognition motif of β-(1,3)-glucan ligands detected by Dectin-1 is more complex than a linear heptasaccharide and consists of a linear β-(1,3)-linked backbone chain of at least 7 glucose subunits with at least one glucose β-(1,6)-linked side-chain branch (Adams et al., 2008). Despite the above-mentioned studies, which investigated the minimal binding subunit preferentially recognized by Dectin-1,

the mechanism for this binding selectivity is not known to date (Palma et al., 2006). However on the basis of the crystal model of Dectin-1, it was suggested that the binding pocket of Dectin-1 may be a groove that accommodates the helical structure common to most β-glucans, as well as the minimal β-glucan backbone chain length of seven glucose residues (Adams et al., 2008; Brown et al., 2007; Tsoni and Brown, 2008a). It is also worth mentioning that it is important to distinguish between the minimum β-(1,3)–glucan polymer size and structure that is required for Dectin-1 recognition and binding versus the minimum structure that is required for induction of biological activity.

The reports discussed indicated that the minimal glucan recognition subunit for Dectin-1 is a polymer containing between seven and ten (1,3)-β–linked glucose subunits with a single side-chain branch. However, (1,3)-β glucans of this size have not been shown to reliably stimulate intracellular signaling or exert biologic effects when administered parenterally. In vivo studies in mice suggest that β-(1,3)/(1,6)-glucans derived from the yeast species S. cerevisiae and composed of more than 70 glucose subunits are required for induction of intracellular signaling and demonstration of biological activity (Williams et al., 1991). It has been speculated that, in addition to being recognized and bound by membrane-associated receptors, the β-1(,3)-glucan polymer must be of sufficient size to cross-link receptors on the cell surface as a prerequisite for induction of biological activity; however, this has not been proven unequivocally to date. In this regard, several reports have shown that a higher degree of structural complexity and size of β-glucans is associated with more potent immunomodulatory and anti-cancer effects (Batbayar et al., 2012). For instance, according to a study by Cleary *et al.*, β-(1,3)-glucans with a higher molecular weight or a greater degree of β-(1,6)-linkages tend to have stronger stimulatory effects on macrophages in mice (Cleary et al., 1999). Laminarin a low molecular weight, branched β-glucan can bind to Dectin-1 without stimulating downstream signaling and is able to block binding to Dectin-1 of particulate β-(1,3)-glucans, such as zymosan (Brown et al., 2002b). Additionally, a report on TNF-α secretion (a potent proinflammatory cytokine) induced by different β-glucan fractions extracted from *Grifola* (an edible mushroom), demonstrated that higher molecular weight (> 450 kDa) glucan was more potent than lower molecular weight (< 450 kDa) glucan (Okazaki *et al.*, 1995). Ishibashi *et al.* showed that heat-treatment of grifolan (a soluble β-glucan extracted from *Grifola*) reduced the molecular weight and consequently the secretion of TNF-α (Ishibashi et al., 2001). In the same study the precipitated, insoluble fraction

of the heat-treated β-glucan, recovered by centrifugation, but not the soluble fraction, retained the TNF-α secretion inducing ability. These studies suggested that regardless of the solubility of the β-glucan, only the high molecular weight form of grifolan in both insoluble and soluble forms were required for induction of TNF-α secretion by macrophages (Ishibashi et al., 2001).

Conclusion

Despite structural, mutational and biophysical studies it is still not clear how Dectin-1 is actually able to recognize these β-glucans and there is a need to further identify more precisely the structural features of β-glucans that determine the subsequent level of activation of immune responses (Tsoni and Brown, 2008a). Furthermore, additional studies with β-glucans whose chemical structures have been fully characterized are required.

1.5.2.2. Dectin-1 and Fungal Recognition

Generally, β-glucan is a major structural cell wall component of fungal pathogens, and it is therefore a fungal PAMP involved in antifungal immunity (Brown et al., 2003; Herre et al., 2004a). Through its recognition of cell-wall β-glucan, Dectin-1 has been shown to interact with a variety fungal species, including *Candida albicans*, *Aspergillus fumigatus* (Gantner et al., 2005; Luther et al., 2007; Taylor et al., 2007), *Coccidiodes posadasii* (Viriyakosol et al., 2005), *Pneumocysti carinii* (Steele et al., 2005), *Saccharomyces* (Brown and Gordon, 2003) and *Penicillium.* Moreover, through its recognition of cell-wall β-glucan, Dectin-1 has also been implicated in the binding of non-fungal pathogens such as nontypeable *Haemophilus influenza* (Ahren et al., 2003). On the other hand, Dectin-1 doesn't recognize *C. neoformans*, and has been shown to be not essential for the development of host protective responses to this fungal pathogen (Nakamura et al., 2007). It has also been suggested that Dectin-1 plays a role in the recognition non-fungal pathogens. For instance, Dectin-1 recognizes *Mycobacteria*, which do not possess β-glucan, by binding to an unidentified ligand on the surface of these pathogens (Reid et al., 2009; Reid et al., 2004; Rogers et al., 2005; Underhill et al., 2005; Werninghaus et al., 2009), suggesting that there may still be unidentified exogenous ligands of Dectin-1, yet to discovered. Recognition of pathogenic fungi by Dectin-1, on macrophages and DCs, triggers innate immunity and is accompanied by an acute inflammatory response (see section **1.5.5**).

1.5.2.3. Non-β-glucan Dectin-1 ligands

In addition to recognition of β-glucans, Dectin-1 can also bind to other non-β-glucan ligands.

Several studies have demonstrated that Dectin-1 has an unidentified endogenous ligand on T cells (Ariizumi et al., 2000; Brown and Gordon, 2001; Grunebach et al., 2002). Evidence also indicates that Dectin-1 may act as T cell costimulatory molecule on antigen presenting cells (APCs), and induces T cell activation and proliferation through its binding to this unidentified ligand on T cells (Ariizumi et al., 2000; Grunebach et al., 2002). Indeed, expression of Dectin-1 by APCs in the T cell areas of lymphoid tissues supports a role for this receptor in these interactions (Reid et al., 2004). This binding occurs in a non-carbohydrate manner, indicating that this ligand may be a protein (Grunebach et al., 2002). Although this endogenous ligand on T cells has not been identified and the exact nature of its interaction with Dectin-1 is still unknown, evidence suggests that Dectin-1 binds to it in a non-carbohydrate manner, indicating that this ligand may be a protein that interacts with Dectin-1 through a distinct binding site in the CRD domain (Ariizumi et al., 2000; Grunebach et al., 2002; Palma et al., 2006; Weck et al., 2008; Willment et al., 2001). Recent evidence also implicates that Dectin-1, especially the Dectin-1B isoform, may have a role in the recognition and phagocytosis of apoptotic tumour cells through its detection of an unknown ligand (Figure 11) (Ariizumi et al., 2000; Brown et al., 2003; Grunebach et al., 2002; Weck et al., 2008). Dectin-1 has also been shown to bind to an unidentified molecule on mycobacteria, pathogens that do not express β-glucans (Shin et al., 2008). Also the cytoplasmic Dectin-1 E isoform binds to the Ran binding protein (RanBPM), a cytosolic endogenous ligand that can interact with the GTPase Ran, which may act as a scaffolding protein to coordinate signaling from other cell receptors (Xie et al., 2006). From the above discussion, it is clear that there may still be unidentified exogenous and endogenous ligands of Dectin-1, especially ones of proteinaceous nature yet to be discovered.

1.5.3. Dectin-1–mediated Cellular Responses and Functions

Dectin-1 is an example of a multifunctional CLR and the discovery of Dectin-1 and the study of its functions, has helped to resolve many of the previously reported roles for β-glucan receptors in immunity, including the innate recognition and response to fungal pathogens and β-glucan-mediated immunomodulation (Brown, 2006a; Drummond and Brown, 2011). Ligation of β-glucans to Dectin-1, elicits a signaling cascade to activate innate and adaptive immunity. Ligand binding induces a variety of cellular responses, including: ligand uptake by endocytosis and phagocytosis, cellular maturation (mainly DC and macrophage maturation), activation of a

respiratory burst through the production of ROS (Reactive oxygen species), neutrophil degranulation, fungal/microbial uptake and killing, synthesis of transcription factors, the production of numerous cytokines and chemokines, and recently Dectin-1 has been implicated in inflammasome activation, mainly the NLRP3 inflammasome (Figure 15 & Figure 16) (Brown, 2006b; Dennehy et al., 2008; Dennehy et al., 2009; Drummond and Brown, 2011; Gantner et al., 2003; Gantner et al., 2005; Goodridge et al., 2009a; Herre et al., 2004b; LeibundGut-Landmann et al., 2007; Olsson and Sundler, 2007; Saijo et al., 2007; Taylor et al., 2007; Underhill et al., 2005). All of these cellular processes induce an effective innate and adaptive immune response against the pathogen (Plato et al., 2013). The various cellular processes stimulated in response to Dectin-1 triggering, culminate in the generation of a proinflammatory innate immune response and the activation of adaptive immunity through the promotion of T helper immunity. Dectin-1 has been shown to play a key protective role in antifungal immunity through the induction of these diverse cellular responses upon fungal recognition (Brown, 2006a; Goodridge et al., 2009b; Gringhuis et al., 2009b; LeibundGut-Landmann et al., 2007). Indeed several genetic studies in humans and mice have revealed essential functions for Dectin-1 in host protection against several pathogenic fungi (Ferwerda et al., 2009; Taylor et al., 2007).

Dectin-1 is reportedly the first example of a non-TLR PRR that mediates its own intracellular signals after binding of β-glucans, with subsequent synthesis of its own signature profile of cytokines and chemokines including IL-10, and IL-2 (Figure 16) (Brown, 2006a; Drummond and Brown, 2011). Generally, Dectin-1 ligation induces the activation of transcription factors, including NF-κB and NFAT, leading to the production of various inflammatory cytokines, chemokines, and lipid mediators (including prostaglandins and arachidonic acid metabolites) (Figure 16). Cytokines shown to be induced downstream of Dectin-1 activation include TNF-α, IL-2, IL-10, IL-6, IL-1β IL-23, GM-CSF, well as reduced IL-12 production (IL-12 is down-regulated by collaboration of Dectin-1 with TLR2 (see section **1.5.4.4**), and low levels TGF-β (Figure 16) (Gringhuis et al., 2009b; Osorio et al., 2008; Ozinsky et al., 2000a; Sancho and Reis e Sousa, 2012; Slack et al., 2007; Vautier et al., 2012; Vautier et al., 2010; Yadav and Schorey, 2006). Dectin-1, additionally stimulates the production of the chemokines CXCL1, CXCL2, macrophage inflammatory protein (MIP)-1α/ CCL3 and MIP-1β/CCL4, with very low levels of CCL17 and CCL22 (Figure 15 & Figure 16) (Brown, 2006a; Kerrigan and Brown, 2010, 2011b; LeibundGut-Landmann et al., 2007; Reid et al., 2009). Dectin-1 has also been shown to mediate

the production of arachidonic acid (AA), the lipid mediator COX-2 (cyclooxygenase) and PGE2 (prostagalandin E2) expression, which promote acute tissue inflammation (Parti et al., 2010; Suram et al., 2006).

Dectin-1 is a unique PRR that can also function as a phagocytic receptor. After particle recognition, Dectin-1 can mediate phagocytosis in macrophages, neutrophils and DCs and elicit a respiratory burst in these cells, which involves the production of ROS (reactive oxygen species through the multimeric enzyme complexe NADPH oxidase (Figure 15 &Figure 16) (Gantner et al., 2003; Rogers et al., 2005; Underhill et al., 2005). Aside from its involvement in second messenger signaling, ROS is used as a microbicidal agent and is crucial for the degradation of the phagocytosed invader (Kohchi et al., 2009). Products of the ROS-induced degradation pathways can be processed and presented by DCs after phagocytosis. This process links the innate and adaptive immune responses, as antigen-presenting DCs producing varying combinations of cytokines can migrate to the lymph nodes to activate T cells (Plato et al., 2013).

Several studies have demonstrated a pivotal role of Dectin-1 in stimulation of innate immunity, in addition to its ability to drive and modulate adaptive immune responses. A potential role for Dectin-1 in adaptive immunity has been described, particularly for the generation of efficient $CD8^+$ cytotoxic T cell and $CD4^+$ T helper responses, mainly Th17 and Th1 responses (Agrawal et al., 2010; Espinosa and Rivera, 2012; Osorio et al., 2008; Rivera et al., 2011). In this regard, triggering of Dectin-1 induces signaling pathways generally culminate in the induction of a proinflammatory gene program leading to the production of a strong proinflammatory response, mediated primarily via activation of Th17 and Th1 adaptive immune responses (LeibundGut-Landmann et al., 2007; Leibundgut-Landmann et al., 2008).

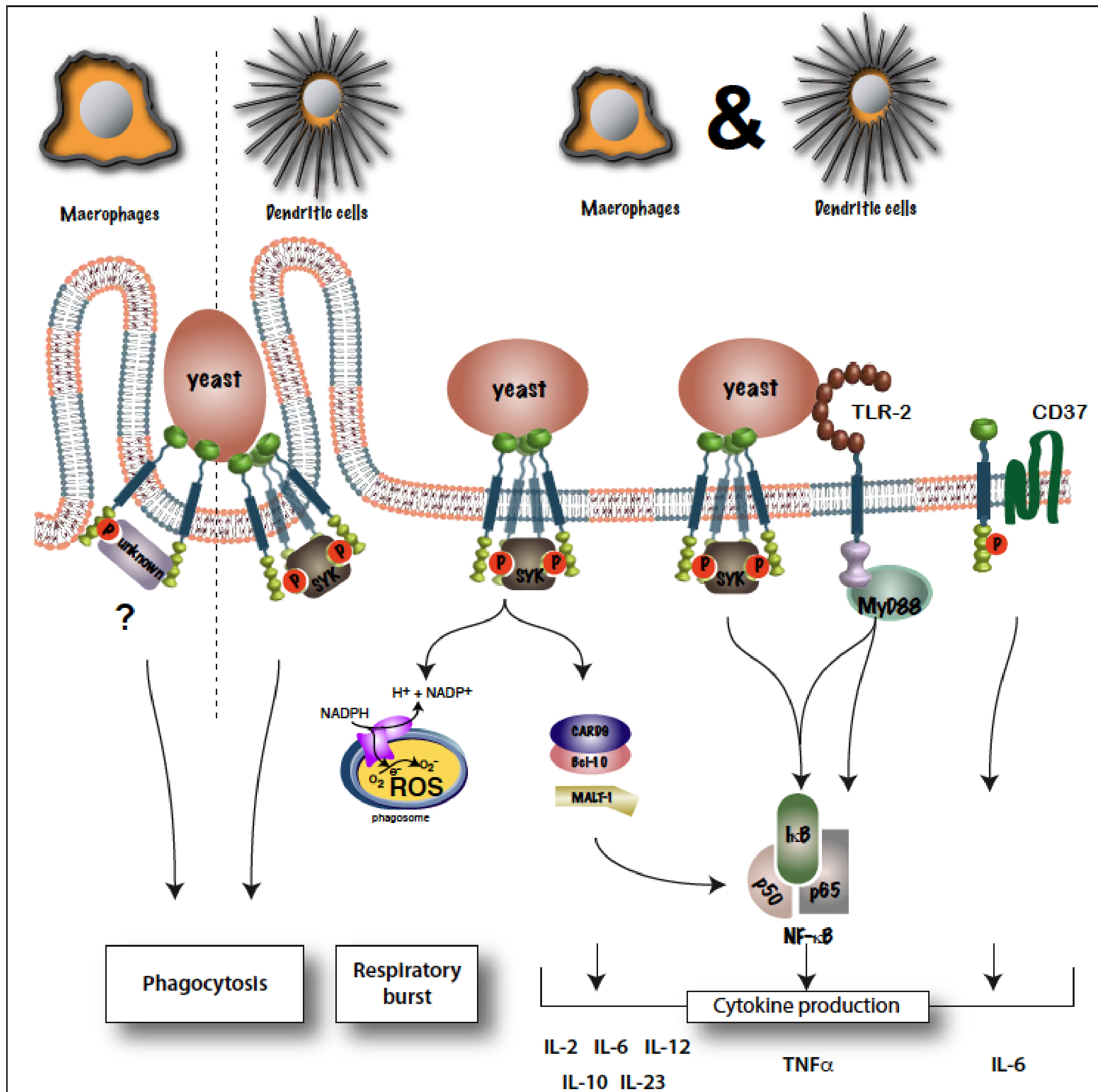


Figure 15: Main Cellular Responses Mediated by Dectin-1 in Innate Immune cells.
Upon ligand binding, Dectin-1 triggers several signaling pathways leading to phagocytosis, activation of the respiratory burst and cytokine production. Dectin-1-mediated phagocytosis has been shown to require the adaptor protein SYK in all innate immune cells except macrophages, however our lab has evidence that phagocytosis in a murine macrophage cell line (RAW 264.7 macrophages) requires SYK (unpublished data). Interestingly, cytokine production follows independent pathway whether or not TLR2 is engaged. SYK-dependent signaling occurs through the CARD9/Bcl-10/MATL1 (CBM) complex, which in turn activate the NF-κB complex and induces secretion of IL-2, -6, -10, -12, -23. The TLR2/MyD88 signaling pathway is also necessary for the induction of TNFα and down-regulation of IL-12. Finally, interaction with the tetraspanin CD37 is important for the retention of Dectin-1 at the cell surface and IL-6 production.

Dectin-1–mediated production of cytokines and upegulation of surface expression of costimulatory molecules, as well as cellular activation of APCs, mainly DCs, promotes Th17, Th1, and cytotoxic T cell responses (Leibundgut-Landmann et al., 2008; Osorio et al., 2008; Vautier et al., 2010) (Figure 18). Dectin-1 can induce several Th17-associated cytokines (e.g. IL-

1β, IL-6 and IL-23, and TGF-β), and downregulate IL-12 (Th1-associated) induced by the TLRs (Figure 16) (Dennehy et al., 2009), influencing the differentiation of activated $CD4^+$ T cells. Indeed, stimulation of Dectin-1 in DCs with the purified agonist curdlan has been shown to induce the production of Th17 responses both *in vitro* (Osorio et al., 2008) and *in vivo* (Huang et al., 2010; LeibundGut-Landmann et al., 2007). Interestingly, Dectin-1 is the first of several pattern recognition CLRs, shown to induce the maturation of DCs capable of instructing the generation of Th1 and Th17 effector cells that are essential to host protection against fungal infection (Agrawal et al., 2010; Dennehy and Brown, 2007; LeibundGut-Landmann et al., 2007). Historically, Th1 were thought to be the main mediators of antifungal immunity, however recently Th17 responses have been shown to play a significant role in fungal clearance and host protective immunity against several pathogenic fungi (see section **1.5.5**). Th17 cells have also been implicated, to play a more prominent role in the immunopathology of autoimmune and inflammatory diseases such as autoimmune forms of rheumatoid arthritis immunity, inflammatory bowel disease (IBD) and diabetes mellitus, and therefore Dectin-1 has been thought to play role in the pathogenesis of these diseases (Cortez-Espinosa et al., 2012; Kerrigan and Brown, 2011b; Reid et al., 2004; Tsoni and Brown, 2008a). Indeed, signaling through Dectin-1 in dendritic cells has been shown to augment autoimmune responses *in vivo* mediated by Th17 cells and Th1 cells (LeibundGut-Landmann et al., 2007; Yoshitomi et al., 2005).

Other branches of the adaptive response influenced by Dectin-1 include cytotoxic T cell responses (Leibundgut-Landmann et al., 2008; Ni et al., 2010). Stimulation of Dectin-1 by agonists can act as adjuvants that prime the production of cytotoxic T cell responses by DCs (Leibundgut-Landmann et al., 2008). Dectin-1 has also been implicated to affect humoral immunity (Kumar et al., 2009b). Furthermore, gamma-delta γδ T cells, which are important early IL-17 producers, were recently shown to express Dectin-1 whose expression was elevated following immunization with curdlan, which also expanded populations of $IL\text{-}17^+$ γδ T cells (Martin et al., 2009).

The discovery of Dectin-1 as the major receptor for β-glucans, as well as a fungal PRR, was followed by subsequent functional studies that facilitated the elucidation of many of the previously reported roles for β-glucan receptors in immunity, including the innate recognition of and response to fungal pathogens and immunomodulatory effects elicited by β-glucan. (Brown et al., 2002b; Goodridge and Underhill, 2008; Herre et al., 2004b; Huysamen and Brown, 2009;

Kimberg and Brown, 2008). Dectin-1 plays a key role in the recognition of several fungal pathogens (See section **1.5.2.2**). Upon recognition of β-glucans in the fungal cell wall of various pathogenic fungi, Dectin-1 initiates a complex signaling network that triggers the above-mentioned cellular responses including fungal uptake and microbial killing, which culminates in host protection against fungal infection mainly via the induction of anti-fungal Th1 and Th-17 responses (Figure 18) (Drummond and Brown, 2011). β-glucans have been long known for their immunomoduatory properties, and have been shown to initiate antimicrobial immune responses mainly through their interaction with a myriad of innate immune cells including DCs, macrophages and neutrophils (Chan et al., 2009). Furthermore, recent results using blocking monoclonal antibodies in vivo indicate that Dectin-1 may also be involved in the anti-cancer activity of β-(1,3)-glucans (Ikeda et al., 2007). Therefore, in addition to the role of Dectin-1 in antifungal immunity, Dectin-1 has importantly been implicated in mediating the biological effects of β-glucans although this has not yet been formally demonstrated *in vivo* (Brown et al., 2003).

1.5.4. Dectin-1–induced Signaling Pathways

Dectin-1 is the archetypical signaling non-TLR pattern recognition receptor (PRR), and to date serves as a paradigm for CLR signaling (Mocsai et al., 2010). Dectin-1 is a prominent example of a "self-sufficient" C-type lectin PRR that can directly (through its intrinsic signaling capacity), and autonomously (independent of other PRRs), couple pathogen recognition to signaling pathways that induce gene transcription programs essential for the activation of host defense. Dectin-1 is considered a unique C-type lectin PRR because of its capacity to induce numerous cellular responses, via its own signaling pathway independent of other PRRs, or in conjunction with other receptors to fine-tune and modulate signaling outcomes (Brown, 2006a). For instance, in contrast to DC-SIGN (a C-type lectin PRR), signaling from Dectin-1 does not require additional TLR signaling for the activation of NF-κB, however, as seen in section **1.5.4.4**. Dectin-1 synergistically collaborates with TLRs to modulate proinflammatory responses (Gantner et al., 2003; Hardison and Brown, 2012) (Figure 15 & Figure 16). The signaling pathways leading to Dectin-1–mediated cellular responses are complex and although extensively studied and characterized, are still not fully understood (Plato et al., 2013). One level of complexity is that Dectin-1–mediated signaling and cell activation is shown to be cell-type specific (Goodridge et

al., 2009a). In this regard, the induction of cellular responses by Dectin-1 has been reported to be controlled by inherent cellular programming, with distinct macrophage and DC populations responding differentially to the engagement of this receptor (Rosas et al., 2008). The differential activity of Dectin-1 is affected by the context within which Dectin-1 is expressed, which depends in part on the presence of growth factors, cytokines or microbial components (e.g., GM-CSF, IL-4 or LPS) as well as the size, type and dose of ligand used to stimulate Dectin-1 (Goodridge et al., 2009a; Rosas et al., 2008). Finally, there is disagreement regarding whether the binding of soluble β-glucans to Dectin-1 induces biological activities, and this is an issue that to date remains controversial (see section **1.6.6**) (Batbayar et al., 2012; Goodridge et al., 2011).

Dectin-1 mediates intracellular signaling via its cytoplasmic hemITAM motif and was the first non-TLR PRR shown to possess this ability (Figure 12) (Brown, 2006a). In fact, Dectin-1 is the archetype for hemITAM signaling. Upon binding of β-glucans to the CRD domain of Dectin-1, the tyrosine residue within the hemITAM (ITAM-like) of the cytoplasmic tail is phosphorylated (Figure 12) (Gantner et al., 2003) by Src family kinases (SFKs) (Olsson and Sundler, 2007). Dectin-1 signaling downstream of ligand binding involves both SYK-dependent and SYK-independent signaling cascades, mainly the Raf-1 (a serine/threonine kinase) signaling pathway (Figure 16), along with pathways from collaborating receptors such as those of TLRs (Batbayar et al., 2012; Plato et al., 2013). The two main signaling pathways induced in response to Dectin-1 ligation, one through SYK and one through Raf-1, have been reported to be independent of each other, yet collaborative (Gringhuis et al., 2009b).

1.5.4.1. The SYK-dependent Dectin-1 Signaling Pathway

SYK (Spleen tyrosine kinase) is a tyrosine kinase at the heart of Dectin-1 signaling (Figure 15 & Figure 16) (Brown, 2006a; Plato et al., 2013). Activation of SYK is one of the hallmarks of the Dectin-1 signaling pathway that distinguishes it from the activation of TLRs, which don't utilize SYK for their signaling. Dectin-1 was the first SYK-coupled CLR shown to be crucial for mammalian host protection. SYK represents a common point in the signaling pathways of several SYK-coupled CLRs including Dectin-1, Dectin-2 and MINCLE (see section **1.3.4**). Dectin-1–induced signaling downstream of SYK leads to the activation of key signaling mediators including PLCγ2 (phospholipase-Cγ2), PKCδ (protein kinase C-δ, PKCD), the novel adaptor

CARD9, MAPKs (MAP) kinases including ERK1/ERK2 (p44/p42), p38 and JNK, as well as the transcription factors NF-κB, AP-1, and NFAT (nuclear factor of activated T cells) (Figure 16) (Dillon et al., 2006; Hara et al., 2007; Kerrigan and Brown, 2011b; LeibundGut-Landmann et al., 2007; Olsson and Sundler, 2007; Plato et al., 2013). Within these networks, pathways leading to the SYK-dependent activation of NF-κB can be categorized into both canonical and noncanonical routes (explained further below). Ligand binding to Dectin-1 can additionally induce SYK-independent signaling through the serine-threonine kinase Raf-1, which converges with the SYK pathway at the level of NF-κB activation for synergistic stimulation of the canonical p65 NF-κB subunit, as well as regulation of NF-κB–induced cytokine responses (see Figure 16) (explained further below) (Gringhuis et al., 2009b). Collectively, intracellular signaling pathways triggered in response to Dectin-1 binding to β-glucans or fungal pathogens, culminate in the stimulation of innate immunity, as well as the induction of Th1 and Th-17 adaptive immune responses, which are essential to host defense against fungi (Geijtenbeek and Gringhuis, 2009; Gringhuis et al., 2009b; Romani, 2011).

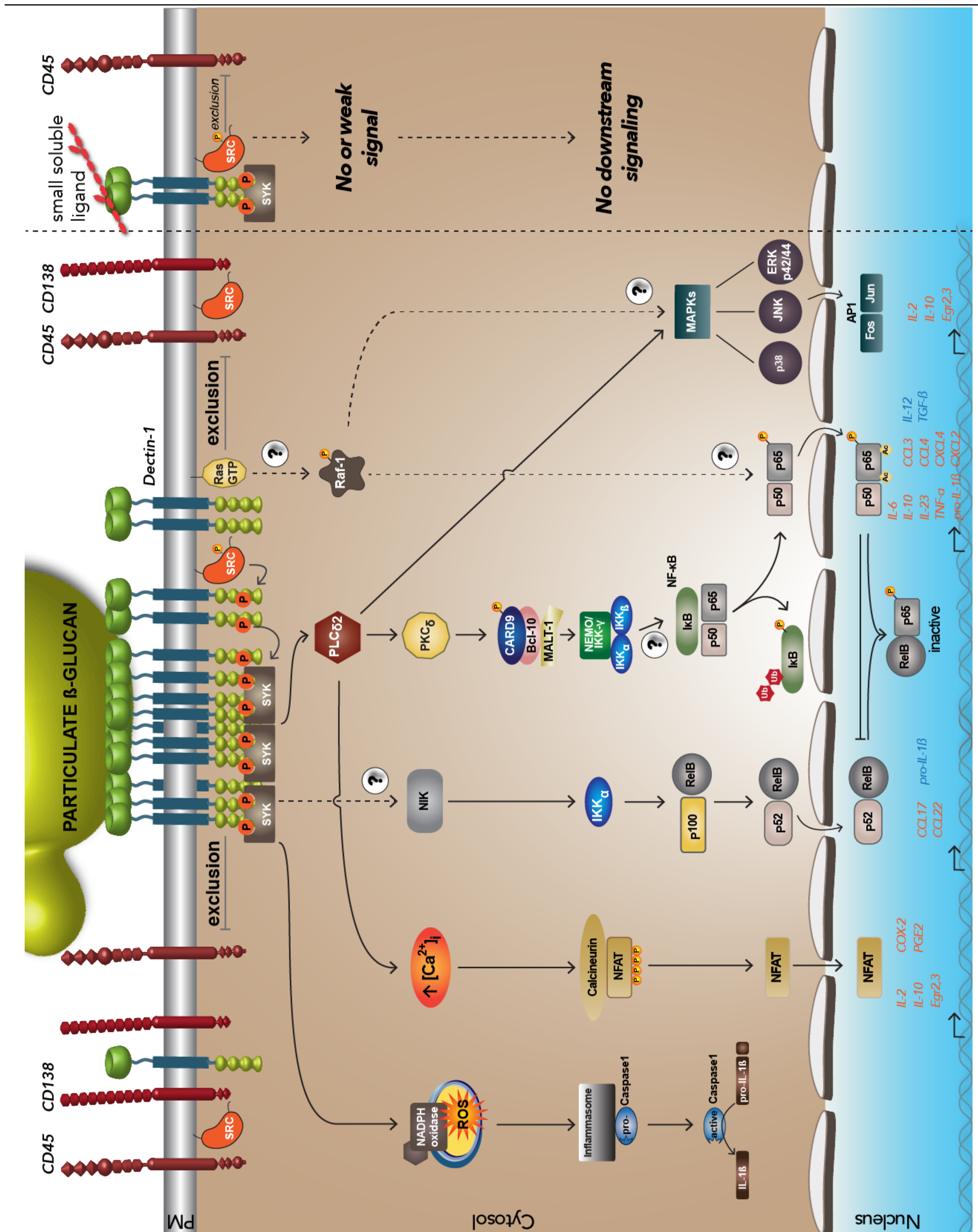


Figure 16: Dectin-1– Signaling Pathways

The above figure demonstrates different pathways involved in Dectin-1 signaling, including the two major pathways: the SYK dependent pathway and the Raf-1 pathway. Figure highlights other side pathways and important signaling players of the pathway such as SYK, Src family kinases (SFKs), CARD, MAPKs. Also illustrated are the canonical

and non-canonical NF-κB pathways activated by Dectin-1. Key cytokines, chemokines and inflammatory mediators activated downstream of Dectin-1 signaling are shown at the end of each signaling cascade. Those labeled in 'red' are upregulated downstream of Dectin-1 signaling, whereas those labeled in 'blue' are either downregulated or produced in low levels. Depicted in the figure are the CD45 and CD148 receptor tyrosine phosphatases that are postulated to be excluded from the Dectin-1 clusters formed upon binding to particulate ligands, thereby allowing activation of SFKs which in turn trigger the signaling cascade. On the other hand it is thought that soluble β-glucans are not large enough to exclude these phosphatases, and are either incapable of activating Dectin-1 signaling or just produce a weak signal that is not sufficient to fulfill Dectin-1 signaling further downstream. P: Phosphorylation; Ac: acetylation. (Figure was conceived and designed by Amira Fitieh, and Graphics were done in Adobe illustrator by Dr. Nicolas Touret).

A) Dectin-1–mediated HemITAM Signaling

Dectin-1 mediates SYK-dependent signaling through its so-called cytoplasmic hemITAM or ITAM-like motif (Figure 8 & Figure 12), which triggers intracellular signaling via engagement of the SH2-containing tyrosine kinase, SYK (Figure 15 & Figure 16). As explained in section **1.3.4**, the hemITAM motif of Dectin-1 is a single tyrosine-based activation motif that is reminiscent of the classical ITAM motifs found in other activation immune receptors, such as the BCR (B cell receptor), TCR (T cell receptor), and the Fc receptors (Figure 8). Classical ITAM motifs are effectively a tandem repeat of the YxxL/I consensus sequence (L is Leu, I is isoleucine, and x designates any amino acid). However, unlike these dual tyrosine-based ITAM-containing receptors, the cytoplasmic tail of Dectin-1 possesses only one YxxL motif and was consequently termed hemITAM. The membrane-proximal tyrosine (Tyr 15) of the Dectin-1 hemITAM motif resides in a YxxL context, whereas the membrane-distal N-terminal tyrosine (Tyr 3) resides in a YxxxL context rather than a YxxL context and has been shown not to be required for signaling (Gantner et al., 2003; Herre et al., 2004a; Rogers et al., 2005) (Figure 8 & Figure 12). On the contrary, the membrane-proximal tyrosine residue (Tyr 15) in the hemITAM of the Dectin-1 cytoplasmic tail is required for signaling, and this single tyrosine is sufficient to mediate signaling via SYK (explained further below) (Brown and Gordon, 2003; Herre et al., 2004a; Rogers et al., 2005; Underhill et al., 2005). Upon ligand binding the single membrane–proximal tyrosine residue of the hemITAM is phosphorylated by Src family kinases (SFKs), thereby creating a docking site for the recruitment of SYK (Spleen tyrosine kinase) via its SH2 domains (Figure 16). This leads to SYK activation, which is a pivotal kinase in Dectin-1 signaling, that further recruits and activates other signaling molecules thereby initiating subsequent downstream signaling cascades (Brown, 2006a). This is a novel mechanism of ITAM signaling that is distinct from classical ITAM signaling which requires dual tyrosine phosphorylation of the ITAM. Interestingly, this hemITAM motif, although first discovered in Dectin-1, has subsequently been

identified in other C-type lectin (CLRs), which appears to serve largely homeostatic functions or act as PRRs (Kerrigan and Brown, 2011b) (see section **1.3.4**). These include CLEC-2 (C-type lectin-like receptor 2; involved in platelet activation), CLEC9A (detects damaged cells) and SIGN-R3 (a mouse homologue of human DC-SIGN, which is a PRR involved in host immunity against *mycobacterium tuberculosis*) (Kerrigan and Brown, 2010). The sequence of the hemITAM differs among receptors possessing this motif, thus, currently a consensus sequence for the hemITAM motif is still missing, and the hemITAM is simply defined by the ability of a given CLR to directly recruit SYK via a single tyrosine (Sancho and Reis e Sousa, 2012).

Signaling from the hemITAM motif is complex and sometimes cell-specific, and involves a number of unique pathways, including a novel interaction with SYK kinase as well as collaborative signaling with the TLRs (Brown, 2006a; Kerrigan and Brown, 2010). SYK possesses two tandem SH2 domains that are both required to bind to phospho-tyrosine residues for full activation (Figure 8) (Osorio and Reis e Sousa, 2011). Accordingly, SYK requires a traditional ITAM motif with dually phosphorylated YxxL sequences for engagement via its tandem SH2 domains in order to be fully activated (Kerrigan and Brown, 2010). However, despite involving both SH2 domains of SYK, only phosphorylation of the membrane proximal tyrosine residue (Tyr 15) of the single YxxL sequence within the Dectin-1 hemITAM motif was found to be unusually sufficient for SYK recruitment, activation and induction of SYK-dependent Dectin-1 signaling (Figure 8) (Rogers et al., 2005). This proposed a model, which involves bridging of SYK between two monophosphorylated Dectin-1 molecules (Figure 8, Figure 16 & Figure 15) (Brown, 2006a; Kerrigan and Brown, 2010). In this model, it is hypothesized that Dectin-1, following ligand binding, may form a dimer structure with SYK (Brown, 2006a). The nature of this interaction is still unknown especially that Dectin-1 lacks the cysteine residues in its stalk region involved in CLR dimerization. However, the current model proposes that dimerization of Dectin-1 occurs via the bridging of two nearby Dectin-1 molecules via the two SH2 domains of SYK (Rogers et al., 2005), where each SH2 domain binds to a single phospho-tyrosine residue within the hemITAM motif of each of the two individual molecules of Dectin-1 (Figure 8, Figure 16 & Figure 15) (Brown, 2006a; Brown et al., 2007; Goodridge et al., 2009b; Osorio and Reis e Sousa, 2011; Rogers et al., 2005). In support for this model, it has been reported that SYK-dependent hemITAM signaling mediated by the hemITAM-containing C-type lectin receptor, CLEC-2, occurs through the formation of “dimers” upon ligand binding, that are

bridged intracellularly by SYK. In one report, SYK activation by CLEC-2, was shown to be mediated by the cross-linking of two CLEC-2 receptors through the tandem SH2 domains of SYK with a stoichiometry of 2:1 (CLEC-2:SYK) (Hughes et al., 2010b). Moreover, another report demonstrated that CLEC-2 exists as an active non-disulfide-linked homodimer, which could allow each SYK molecule to interact with two YxxL motifs, one from each CLEC-2 monomer (Watson et al., 2009). Accordingly, it has been proposed that Dectin-1 hemITAM signaling most likely occurs in a similar fashion to what has been reported for CLEC-2, however, this has not yet been experimentally proved. Moreover, despite this evidence for the existence of CLEC-2 dimers for hemITAM signaling, the exact mechanism of how a single YXXL motif can interact efficiently with SYK, which usually recognizes two tandem YXXL repeats present in a classical ITAM motif, is still unclear and needs to be further elucidated.

As mentioned above, upon ligand binding, the single tyrosine residue within the hemITAM motif becomes phosphorylated by receptor proximal Src family kinases (SFKs), thereby recruiting SYK and mediating its activation, which initiates the subsequent SYK-dependent signaling cascade. However the precise mechanisms by which SFKs are activated at the plasma membrane and in turn phosphorylate the tyrosine residue within the hemITAM motif are yet unclear. It also still remains to be determined which of the SFKs are involved in these membrane-proximal events of Dectin-1 signaling, and whether they have redundant roles or not. There are eight members of the SFK family, and innate immune cells primarily express the isoforms: Fyn, Lyn, Src, and Hck (Lowell, 2011). Although the exact spectrum of the SFKs involved in Dectin-1 upstream signaling events has not been identified, the SFKs, Src, Lyn, and to some extent Hck, have been implicated in such events (Hara et al., 2007; LeibundGut-Landmann et al., 2007; Olsson and Sundler, 2007). In this context, recently, a study by Goodridge *et al.* has demonstrated key roles for the SFKs, Src and Lyn, in activation of Dectin-1 (Goodridge et al., 2011). The authors of this study proposed that particulate β-glucans cluster the Dectin-1 receptor into multimeric complexes, from which the regulatory receptor tyrosine phosphatases, CD45 and CD148, are excluded and dispersed out of the clustering sites, thereby enabling activation and phosphorylation of SFKs (mainly Lyn as shown by their study), which triggers downstream signaling from the hemITAM motif of Dectin-1 (Figure 16) (Goodridge et al., 2011). Furthermore, the authors of this study suggested that Dectin-1 is only activated by particulate β-glucans and not by the soluble forms of the ligand (Figure 16) (see section **1.6.6**) (Goodridge et

al., 2011).

SYK activation in response to Dectin-1 ligation induces the activation of several key signaling intermediates, primarily PLC-γ2, PKCδ and the adaptor protein CARD9 (caspase recruitment domain-9) (Dennehy and Brown, 2007; Gringhuis et al., 2009b; Hara et al., 2007; LeibundGut-Landmann et al., 2007; Tassi et al., 2009; Xu et al., 2009b). Activation of these signaling players further triggers different signaling cascades leading to the stimulation of several downstream mediators and transcription factors (mainly NF-κB, AP-1 and NFAT), which culminate in the transcriptional activation of specific gene programs leading to the production of cytokines, chemokines, co-stimulatory molecules and other key molecules essential for activation of Dectin-1-mediated cellular responses and host immunity. SYK-dependent pathways activated by Dectin-1 include MAPK (p38, JNK and ERK1/2) signaling cascades, activation of the canonical (via CARD9) and non-canonical NF-κB pathway, PLC-γ2–dependent activation of the transcription factor NFAT, and activation of the NADPH oxidase for the production of ROS which in turn activates the inflammasome (Figure 16) [reviewed in (Plato et al., 2013; Sancho and Reis e Sousa, 2012)]. Indeed, SYK deficiency or SYK inhibitors, both in DCs and macrophages, inhibit Dectin-1–dependent cytokine production, as well as MAPK, NFAT and NF-κB activation, further suggesting that SYK is essential for Dectin-1 signaling (Herre et al., 2004a; Rogers et al., 2005; Underhill et al., 2005). Inflammatory mediators and cytokines induced downstream of SYK include TNF-α, IL-2, IL-10, IL-23, IL-1β, IL-6, IL-12, the chemokines MIP1, MIP2, CXCL2 and CXCL1 (Figure 16) (Goodridge et al., 2007; LeibundGut-Landmann et al., 2007; Rogers et al., 2005; Slack et al., 2007; Underhill et al., 2005).

B) SYK-dependent NF-κB Signaling: Canonical and Non-canonical Pathways

The key to the ability of some SYK-coupled CLRs, including Dectin-1, to function as PRRs lies in their capacity to activate NF-κB downstream of SYK (Osorio and Reis e Sousa, 2011). Of particular interest is the ability of Dectin-1 to induce Th17 and Th1 responses via the activation of NF-κB, which in turn provides host protection against fungal infection. NF-κB is a family of five transcription factors: RelA (also known as p65), RelB, c-Rel, p50/p105 and p52/p100. These NF-κB protein subunits form homo- or hetero-dimers via interactions between Rel homology domains, and play major roles in the immune response, as NF-κB controls the transcription of genes involved in cell proliferation, lymphocyte activation, and synthesis of cytokines and chemokines (Oeckinghaus et al., 2011). Because of its pluripotent effects on the immune system,

NF-κB activation is a tightly regulated process. There are two main pathways of NF-κB activation: the **canonical/classical** (mainly induced by TLRs) and **non-canonical/alternative** (mainly induced the TNF receptor family members, e.g.CD40) pathways. In the canonical pathway, the NF-κB dimer is retained in the cytoplasm through its association with an inhibitory protein termed IκB (inhibitor of kappa B), which masks the nuclear localization signal of the NF-κB dimer (Figure 16). Following receptor ligation, the canonical pathway is triggered by activation of the IKK (inhibitor of κB kinase) complex. The IKK complex consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ, also known as NEMO (Figure 16). Activation of the IKK complex occurs following K63-linked ubiquitination of NEMO (Kingeter and Lin, 2012), which triggers phosphorylation of IKKα and IKKβ (Figure 16) [reviewed in (Itano et al., 2011)]. The activated IKK complex then phosphorylates IκB proteins on conserved serine residues, resulting in their K48-linked ubiquitination and subsequent proteasome-mediated degradation, thereby releasing the NF-κB dimers free to translocate into the nucleus and activate gene transcription (Figure 16) (Dodd and Drickamer, 2001). Once in the nucleus, the p65 subunit of the NF-kB dimer is phosphorylated and acetylated, which increases the dimer affinity for DNA and enhances transcriptional activity (Chen and Greene, 2004; de Bakker et al., 2007; Vermeulen et al., 2002). On the other hand, in the non-canonical pathway, receptor ligation triggers the activation of the NF-κB-inducing kinase known as NIK (Figure 16). Activated NIK in turn activates the IKKα subunit of the IKK kinase complex, which phosphorylates p100, triggering the processing of p100 into p52 (Figure 16). The RelB-p52 dimer is then free to enter the nucleus and mediate transcription of target genes (Figure 16) [reviewed in (Sun, 2011)]. Although tremendous progress has been made regarding NF-κB signaling pathways induced by receptors such as TLRs, TNF superfamily receptors and antigen receptors [reviewed in (Oeckinghaus et al., 2011; Vallabhapurapu and Karin, 2009)] the NF-κB signaling pathways induced by CLRs remain to be largely characterized (Kingeter and Lin, 2012).

In Dectin-1 signaling, SYK-mediated activation of NF-κB involves both the canonical and non-canonical routes (Gringhuis et al., 2009b; Gross et al., 2006). SYK-dependent signaling from Dectin-1 leads to activation of the canonical NF-κB subunits including p65 and c-Rel, as well as noncanonical NF-κB subunits, e.g., RelB (Geijtenbeek and Gringhuis, 2009; Gringhuis et al., 2009b). Activation of the canonical pathway has previously been described only for a few members of the TNF receptor superfamily (e.g. CD40), none of which signals through SYK (Sun,

2011). Intriguingly, Dectin-1 is the first, and the only PRR to date, shown to induce the non-canonical NF-κB pathway (Geijtenbeek and Gringhuis, 2009).

Dectin induction of the canonical NF-κB pathway is triggered by SYK activation. CARD9 is a key adaptor protein that couples Dectin-1-mediated SYK activation to canonical NF-κB activation, which is required for inflammatory responses induced by Dectin-1 (Figure 16) (Gross et al., 2006; Gross et al., 2009; LeibundGut-Landmann et al., 2007). CARD9 has been demonstrated to control Dectin-1–mediated myeloid cell activation, and to be essential for cytokine production and stimulation of antifungal immunity (Gross et al., 2006; Robinson et al., 2009). Activated SYK further phosphorylates and activates phospholipase-Cγ2 (PLCγ2), leading to the subsequent activation of the adaptor protein CARD9 and its association with the adaptor proteins, Bcl10 and MALT1, to form the CARD9-Bcl10-MALT1 (CBM) trimeric scaffolding complex required for activation of the canonical NF-κB pathway (Figure 16) (Drummond et al., 2011; Gringhuis et al., 2011; Gross et al., 2009; Rosas et al., 2008). Accordingly, the assembly of the CBM scaffold links SYK to the activation of various canonical NF-κB subunits (Figure 16). In response to Dectin-1 triggering, CARD9 and Bcl10 can activate all canonical NF-κB subunits (p65, p50 and c-Rel) (Figure 16) (Gringhuis et al., 2009b; LeibundGut-Landmann et al., 2007). On the other hand Dectin-1 has been demonstrated to selectively activate the canonical subunit c-Rel via MALT1, thereby preferentially driving the production of the Th17 polarizing cytokines IL-23 and Il-1β (Gringhuis et al., 2011). Overall, the SYK/CARD9 pathway culminates in the production of proinflammatory cytokines including IL−6, TNF-α and IL−23, but little IL−12, which induces Th17 cell responses that are essential to host protection against fungi (Figure 16) (Conti et al., 2009). It has been shown that CARD9-deficient humans and mice, fail to activate NF-κB, and are both immunodeficient and highly susceptible to pathogenic fungal infections, highlighting a key role for the SYK-CARD9 pathway in Dectin-1 signaling and host defense against fungi (Ferwerda et al., 2009; Glocker et al., 2009; Gross et al., 2006; Taylor et al., 2007) (see section **1.5.5**). Furthermore, zymosan failed to activate NF-κB, TNF-α, IL-2 and IL-6 production in CARD9$^{-/-}$ and MALT1$^{-/-}$ mouse dendritic cells indicating the importance of the CARD9-Bcl10-MALT1 complex in this pathway (Gross et al., 2006). However, the exact molecular mechanisms by which CARD9 activates NF-κB in response to Dectin-1 triggering are still not clear. The current models posits that the CBM complex, behaves like the CARMA1-Bcl10-MALT1 complex required for antigen receptor signaling in lymphocytes and transduces

signals that culminate in the activation of the IKK complex which in turn activates the canonical NF-κB signaling pathway (Bouyain et al., 2001; Hara and Saito, 2009; Kingeter and Lin, 2012). Interestingly, SYK activation in response to Dectin-1 ligation was shown to trigger rapid recruitment of CARD9 to the plasma membrane or to phagosomes containing ingested fungal particles (Goodridge et al., 2009a; Rosas et al., 2008). In this study, CARD9, whether at the cell surface or in endosomes, recruited the adaptor proteins Bcl-10 and MALT1 leading to the assembly and activation of the CARD9/Bcl10/Malt-1 (CBM) complex. The association of the CBM complex in turn, led to the phosphorylation of IκB, by yet unclear mechanisms, which in turn promoted its degradation, allowing for canonical NF-κB family members including p65 and c-Rel to translocate to the nucleus (Figure 16) (Gross et al., 2006). Although, it is now well-established that CARD9 controls activation of the IKK complex for the induction of canonical NF-κB signaling, the exact details of how the CBM complex activates NF-κB signaling is still not entirely clear, and requires further investigation (Goodridge et al., 2009a; Gross et al., 2006; Hara et al., 2007; Rosas et al., 2008; Saijo et al., 2010). One proposed idea is that CARD9 regulates ubiquitination of the NEMO (IKKγ) regulatory subunit, which is essential for its activation (Figure 16) (Bi et al., 2010). It has also been suggested that SYK, in addition to induction of CARD9 activation, mediates phosphorylation of the IKK complex (Bi et al., 2010). Another interesting suggestion is that this CBM scaffold, in analogy to the CARMA1-Bcl10-MALT1 complex in lymphocytes, enables the recruitment and oligomerization of downstream signaling effectors, such as adaptor TRAF proteins, the MAP kinase TAK1 and the IKK complex, to activate NF-κB (Rawlings et al., 2006). In fact, it has been recently shown that the assembly of the CBM complex in response to Dectin-1 ligation induces the activation of TAK1, a MAP kinase (MAPK) that is known to activate the IKK (IKBα kinase) complex in canonical NF-κB signaling (Strasser et al., 2012), yet the exact molecular details of these signaling steps are still undefined.

Many studies indicated that SYK functions upstream of the CBM complex, but the molecular mechanisms by which SYK links to this CBM complex have only been recently determined. In Dectin-1 signaling, the association of CARD9 to Bcl10 and MALT1 and subsequent formation of the CBM complex has been shown to be induced downstream of the activation of PLCγ2 (phospholipase-Cγ2) by SYK (Figure 16) (Gross et al., 2006; LeibundGut-Landmann et al., 2007; Marakalala et al., 2010; Mocsai et al., 2010). Indeed, PLCγ2-deficient DCs are impaired in their

activation of NF-κB upon Dectin-1 engagement due to defective assembly of the CARD9-Bcl10-MALT1 complex and impaired IKK α/β activation and IκB degradation. However, until recently the signaling events that link PLCγ2 activation to CARD9 recruitment were unclear. A recent report by Strasser *et al.* (2012), demonstrated that Dectin-1 activation in BMDCs (bone marrow-derived DCs) elicits SYK-dependent phosphorylation and activation of PKCδ (protein kinase C-δ, PKCD) at Tyr31 (Figure 16) (Strasser et al., 2012). Activated PKCδ in turn, mediated the phosphorylation of CARD9 at Thr231, which is required for the proper assembly of the CBM adaptor complex resulting in subsequent activation of the IKK complex essential for the activation of the canonical NF-κB pathway (Figure 16) (LeibundGut-Landmann et al., 2007; Strasser et al., 2012). It is also noteworthy that CARMA1, an adaptor protein closely related to CARD9 is part of the CARMA1-Bcl10-MALT1 complex involved in immune receptor signaling in lymphocytes. CARMA1 is activated downstream of PLCγ2, and which is similar to the CBM complex in innate immune cells (Rawlings et al., 2006). In an analogous manner to PLCγ-dependent activation of CARMA1 in lymphocytes, Dectin-1–induced activation of CARD9 downstream of PLCγ2 is mediated via hydrolysis of the membrane-bound phospholipid PIP_2 (Phosphatidylinositol 4,5-bisphosphate) by PLCγ2, which results in the production of membrane-anchored DAG (diacyl glycerol) and soluble IP3 (inositol-1,4,5-triphosphate), both of which are known to aid in activation of the PKC isoforms (Hara and Saito, 2009; Rawlings et al., 2006). Indeed, it has been recently demonstrated that in the case of Dectin-1 signaling in DCs and human monocytes, PKCδ is activated downstream of SYK-induced PLCγ2 (Figure 16) (Elsori et al., 2011; Strasser et al., 2012). Accordingly, the current model of Dectin-1 stimulation of the IKK pathway for canonical activation of NF-κB, is that induction of this pathway is mediated through SYK association with the hemITAMmotif, where active SYK can phosphorylate PLCγ2, allowing the subsequent activation and appropriate assembly of the trimolecular scaffold (CBM) via activation of PKCδ, which in turn activates the IKK kinase complex and canonical NF-κB signaling (Figure 16) (Plato et al., 2013).

As mentioned above, in addition to activating NF-κB via the canonical pathway downstream of SYK, Dectin-1 is also able to activate the non-canonical pathway through SYK activation (Figure 16) (Geijtenbeek and Gringhuis, 2009). In this regard, SYK activation following Dectin-1 ligation, not only activates the canonical NF-κB subunits p65 (RelA) and c-Rel, but also activates the non-canonical NF-κB subunit RelB, which suppresses Th1 and Th17 differentiation (Figure

16) (Gringhuis et al., 2009b). Intriguingly, Dectin-1 is the first and the only known PRR shown to induce activation of NF-κB via the non-canonical pathway, and in a SYK-dependent manner (Geijtenbeek and Gringhuis, 2009). Previously, non-canonical activation of NF-κB had been reported for only a few members of the TNF receptor superfamily (e.g., CD40), which do so by a SYK-independent mechanism (Kerrigan and Brown, 2010). The intricacies of the Dectin-1–mediated non-canonical NF-κB pathway which relays signals from SYK to the activation of the non-canonical RelB-p52 dimers remain undefined at present. However, it is currently well established that in Dectin-1 signaling, SYK activates the noncanonical NF-κB subunits in a CARD9-independent manner, via activation of the NF-κB-inducing kinase (NIK) (Figure 16). Briefly, stimulation of SYK in response to Dectin-1 ligation activates NIK, which in turn activates IKKα (a subunit of the IKK complex) leading to the production of the non-canonical NF-κB subunit, p52, from its precursor p100 by proteolytic processing. Dimers of the non-canonical subunits RelB and p52 (RelB-p52 dimers) can then translocate into the nucleus and induce transcription of target genes (Figure 16) (Bonizzi and Karin, 2004; Geijtenbeek and Gringhuis, 2009). Activation of the non-canonical pathway by Dectin-1 has important functional consequences for adaptive immunity, since RelB represses the transcription of the cytokines IL-12p40 and IL-1β, which are essential for the induction of Th1- and Th17-dependent immune responses (Figure 16) (Gringhuis et al., 2009b). Strikingly, Dectin-1 induces another SYK-independent signaling pathway through the serine/threonine kinase Raf-1 that partly inactivates RelB, thereby antagonizing the transcriptional outcomes induced by RelB and enhancing IL12p40 and IL-1β transcription (explained further below in **1.5.4.2**) (Figure 16) (Gringhuis et al., 2009b). Nevertheless, the NF-κB non-canonical pathway downstream of SYK induces the production of very low levels of the chemokines CCL17 (CC−chemokine ligand 17) and CCL22, which are involved in the recruitment of other leukocytes to the sites of the infection (Figure 16) (Geijtenbeek and Gringhuis, 2009).

Overall, activation of NF-κB by Dectin-1 stimulation underlies the proinflammatory program of myeloid cell activation (Sancho and Reis e Sousa, 2012). Consistent with this fact, the Dectin-1/SYK/NF-κB signaling route culminates in the induction of DC maturation, secretion of cytokines and chemokines. Downstream of this SYK-dependent NF-κB pathway, the net result of cytokines induced by Dectin-1 is the production of IL-10, pro-IL-1β, IL-6, TNF-α, and IL-23, little TGF-β and IL-12 (Figure 16) (Vautier et al., 2012). This cytokine profile renders DCs fully

competent to direct priming of $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells, and antibody responses (LeibundGut-Landmann et al., 2007; Leibundgut-Landmann et al., 2008), and notably (as described above in section **1.5.3**), the Dectin-1–activated $CD4^+$ T cell response includes both a Th1 and Th17 component essential for host protection against fungi. Chemokines produced in response to SYK-mediated NF-κB activation include: CCL3 (MIP-1α), CCL4 (MIP-1β), CXCL1, CXCL2 and very low levels of CCL17 and CCL22 (Figure 16) (Geijtenbeek and Gringhuis, 2009; Vautier et al., 2012).

C) MAPK and NFAT Pathways in Dectin-1 Signaling

In addition to the activation of NF-κB downstream of SYK, the network of SYK-dependent Dectin-1 signaling pathways also involves the activation of the transcription factor NFAT (as in antigen receptor signaling in lymphocytes), as well as the induction of MAPK cascades including the p38, ERK, and JNK cascades (as in TLR signaling) (Figure 16) (Goodridge et al., 2009a; Goodridge et al., 2007; Gross et al., 2006; LeibundGut-Landmann et al., 2007). Although the MAPK and NFAT pathways are activated downstream of SYK, activation of NFAT and the MAPKs, ERK1 and ERK2 are not dependent on CARD9, and CARD9-deficiency in general was shown not to affect these pathways (Figure 16) (Gross et al., 2006; Hara et al., 2007; Plato et al., 2013; Saijo et al., 2010). This is in contrast to SYK-mediated activation of NF-κB that requires CARD9 activation, as described above. On the other hand, NFAT and MAPK activation by the Dectin-1/SYK route are critically dependent on PLCγ2, which regulates Ca^{2+} signaling and activation of the ERK and JNK pathways in DCs (Figure 16) (Tassi et al., 2009; Xu et al., 2009b). In response to Dectin-1 stimulation, PLCγ2-deficient DCs exhibit drastic impairment of Ca^{2+} signaling, activation of ERK and JNK MAPKs, in addition to transcription factors AP-1 and NFAT. Consequently, these cells are highly defective in cytokine secretion in response to Dectin-1 ligation.

For activation of NFAT, Dectin-1 signals through the classic calcineurin/NFAT pathway through SYK activation of PLCγ2 leading to the generation of DAG (diacyl glycerol) and soluble IP_3 (as described above). IP_3 is able to bind to Ca^{2+} channels of the endoplasmic reticulum, releasing Ca^2 from their intracellular stores, thereby resulting in an influx of Ca^{2+} into the cytoplasm (Kerrigan and Brown, 2010). This increase in calcium concentration induces activation of the phosphatase calcineurin and consequently its functional combination with NFAT, which leads to dephosphorylation of NFAT resulting in its translocation into the nucleus to bind to induce

transcription of target genes (Figure 16) (Goodridge et al., 2007; Greenblatt et al., 2010; Plato et al., 2013; Tassi et al., 2009; Xu et al., 2009b). In response to several Dectin-1 agonists the SYK-NFAT axis imparts a unique pattern of cytokines that includes potent expression of high levels of the cytokines IL-2, IL-10, COX-2 (cyclooxygenase-2) and PGE2 (prostagalandin E2), in addition to regulating the expression of the typical proinflammatory cytokines TNF-α, IL-6, IL-12/23p40 (Fric et al., 2012; Goodridge et al., 2007; LeibundGut-Landmann et al., 2007; Rogers et al., 2005). Intriguingly, nuclear translocation of NFAT is an important difference from TLR signaling (which does not lead to NFAT activation), and underlies a unique signature of Dectin-1 signaling including the induction of high amounts of IL-2 and IL-10 in DCs, and COX-2 and PGE2 in macrophages (Figure 16) (Goodridge et al., 2007). IL-2 regulates the development of lymphocytes, whereas IL-10 drives the development, differentiation and survival of Treg cells. Tregs are a subset of T cells that suppress other T effector cells (Th1, Th2 and Th17 cells), and have been shown to mediate protective effects in certain infections by means of their anti-inflammatory function (Ochoa-Reparaz et al., 2008). Accordingly, Tregs induced by Dectin-1 could potentially induce immunosuppressive and anti-inflammatory responses that are important for controlling and balancing the potent proinflammatory responses induced by Dectin-1 signaling. This also emphasizes a potential role for Dectin-1 in balancing the proinflammatory and anti-inflammatory responses during fungal infection (Drummond and Brown, 2011). Indeed, Tregs were demonstrated as immunosuppressive during disseminated candidiasis (Netea et al., 2004). Furthermore, Dectin-1 activation of NFAT through PLC-γ2, results in the induction of other transcription factors, including early growth response Egr2 and Egr3, but not Egr1. (Goodridge et al., 2007; Tassi et al., 2009).

Dectin-1 also activates MAPKs, which are important for signaling and production of inflammatory cytokines in macrophages and DCs (Figure 16). MAPKs or mitogen-activated protein kinases) are serine/threonine/tyrosine-specific protein kinases. The ERK isoforms, ERK1 (p44) and ERK2 (p42) [also known as ERK1/2], are closely related and ubiquitously expressed MAPKs, and are the main MAPKs activated downstream of Dectin-1 signaling (Figure 16) (Slack et al., 2007). The MAPKs, p38 and JNK, are also activated downstream of Dectin-1 signaling (Figure 16) (Slack et al., 2007). Activation of ERK by Dectin-1 agonists was shown to induce the production of IL-10, IL-2, Egr2 and Egr3 (Plato et al., 2013; Slack et al., 2007). AP-1 (a heterodimeric transcription factor mainly composed of the protein subunits Fos and Jun), is the

primary transcription factor (TF) activated downstream of the Dectin-1–induced MAPK signaling pathway, and is a key TF that participates in the immediate early gene response required for innate immune responses (Figure 16) (Batbayar et al., 2012). Interestingly, ERK, JNK, and p38 can translocate into the nucleus, bind to AP-1 transcriptional elements (e.g., Fos and Jun), and catalyze phosphorylation of these elements to induce their transcriptional activity. Engagement of Dectin-1 by curdlan, a potent Dectin-1 agonist, leads to the induction of AP-1, as demonstrated by the robust binding of AP-1 to consensus DNA sequences by nuclear extracts prepared from BMDCs (Xu et al., 2009b). In contrast, the activation of AP-1 is minimal in curdlan-treated PLC-$\gamma 2^{-/-}$ BMDCs. Moreover, PLC-γ2 defective DCs fail to activate MAPK (Slack et al., 2007; Tassi et al., 2009). These studies demonstrated that the signal transduced by PLCγ2 is required to activate MAPK and subsequently AP-1 during Dectin-1 signaling (Figure 16). However, despite the insight provided by these studies, the MAPK pathway induced downstream of Dectin-1 stimulation is still not fully understood and is one of the least characterized pathways of Dectin-1 signaling. Particularly, the exact mechanisms of how MAPKs, (specifically ERK1/2) are activated downstream of PLCγ2 are still unclear. Nevertheless, a study by Kelly *et al.* (2010) using human macrophages demonstrated that zymosan-induced ERK activation downstream of Dectin-1 is mediated through a calcium-dependent pathway that leads to the activation of Ca^{2+}/calmodulin-dependent kinase II (CAMK) and Pyk2 (a nonreceptor tyrosine kinase of the focal adhesion kinase (FAK) family), causing activation of the ERK-MAPK pathway and production of IL-10 (Kelly et al., 2010; Slack et al., 2007). Accordingly, it has been postulated that PLCγ2 induced by Dectin-1 ligation most likely mediates the activation of the MAPK pathways via the induction of Ca^{2+} signaling (Tassi et al., 2009; Xu et al., 2009b). It has also been further suggested that ERK activation by Dectin-1 could occur via small GTPases, e.g. Ras (Grb2/SOS/Ras route) or through PKC, as in Fc receptor and lymphocyte receptor signaling (Siraganian, 2003; Slack et al., 2007). However, in the study by Strasser *et al.* (2012), PKCδ was to a large extent dispensable for ERK1 and ERK2 MAPK activation, as shown by the fact that ERK1 and ERK2 phosphorylations were only slightly reduced in $PKC\delta^{-/-}$ DCs (Strasser et al., 2012). In BMDMs (bone-marrow–derived macrophages), ERK1 regulation of IL-10 release seems to be promoted by the activation of protein kinases MSK1 (mitogen- and- stress activated protein kinase1) and 2 upon Dectin-1 engagement by Zymosan or β-glucan (Elcombe et al., 2013). Due to the recent novelty of this observation, determining whether this mechanism is valid

requires further investigation.

1.5.4.2. The Raf-1–dependent Dectin-1 Signaling Pathway

In addition to the SYK-dependent pathway, Dectin-1 ligation also induces an important SYK-independent arm of signaling mediated by the serine/threonine kinase Raf-1 (Figure 16) (Gringhuis et al., 2009b). Although this pathway is independent of SYK, it converges with the Dectin-1–induced SYK pathway at the level of NF-κB activation for modulating NF-κB activity to control the production of certain cytokines (Figure 16) (Gringhuis et al., 2009b). Specifically, Raf-1 and SYK fine-tune NF-κB–induced cytokine responses by mediating a cross-regulation between the canonical and non-canonical pathways (Geijtenbeek and Gringhuis, 2009; Gringhuis et al., 2009b; LeibundGut-Landmann et al., 2007). Overall, the signaling pathways downstream of SYK and Raf-1 form a large network that ultimately drives Dectin-1–mediated adaptive responses essential for antifungal immunity, through the production of inflammatory cytokines that activate Th1 and Th17 cells (Gringhuis et al., 2009b).

Raf-1 acts to promote phosphorylation of the canonical p65 subunit, which enhances transcriptional activity of p65 and represses non-canonical RelB activity induced by the SYK-dependent non-canonical NF-κB pathway (Figure 16). Both Raf-1 and SYK pathways collaborate for synergistic activation of the p65 NF-κB subunit. In this regard, Raf-1 independent of SYK induces phosphorylation of the canonical NF-κB subunit p65 which is activated downstream of SYK (Figure 16). Phosphorylation of p65 on Ser276 mediates two distinct effects: influencing both p65 and RelB activity. Firstly, p65 phosphorylation at Ser276 induces acetylation of p65, resulting in increased transcriptional activity of p65 which leads to enhanced and prolonged gene transcription of specific cytokines (IL-10, IL-6, IL-12p35 and IL-23p19) (Gringhuis et al., 2009b). Secondly, Raf-1-induced phosphorylation of p65 on Ser276 induces the formation of p65-RelB dimers that sequester active RelB into transcriptionally inactive RelB-p65 dimers, which prevents RelB binding to DNA, and therefore suppresses production of RelB-dependent cytokines (Figure 16). Active RelB–p52 dimers induced downstream of the SYK-induced non-canonical NF-κB pathway, inhibit transcription from the *Il1b* and *Il12b* promoters, thereby limiting the expression of IL-1β, IL-12 and IL-23, which are cytokines with key functions in Th cell differentiation. Therefore the sequestration of RelB into inactive RelB-p65 dimers has important functional consequences for adaptive immune responses, as this decreases the amount of active RelB-p52

dimers formed downstream of SYK, thereby antagonizing or attenuating the repressive function of RelB, which in turn allows the expression of IL-12p40 and IL-1β essential for Th1 and Th17 responses (Figure 16). Although RelB transcriptional suppression of IL-12p40 and IL-1β is reversed by the "capture" of RelB in inactive RelB-p65 dimers, interestingly p65 activity is not attenuated by RelB-p65 dimerization. Overall, Raf-1 activation enhances the expression of some SYK-dependent cytokines including IL-10, IL-12 p35, IL-12/23 p40, IL-6, and IL-1β, but negatively regulates the RelB-dependent cytokines, including IL-23p19 (Gringhuis et al., 2009b). Collaboration of the Raf-1– and SYK–dependent arms of Dectin-1 signaling mediated by Raf-1 and SYK also induces the limited expression of CC‑chemokine ligand 17 (CCL17) and CCL22, which are involved in the recruitment of other leukocytes, as the expression of these chemokines depends on transcriptional activation by RelB-p52 dimers (Geijtenbeek and Gringhuis, 2009).

Whereas SYK signals result in activation of the canonical and noncanonical NF-κB pathways, Raf-1 activation results in selective phosphorylation and permits subsequent acetylation of the NF-κB p65 subunit (Figure 16) (Gringhuis et al., 2009b). Acetylated p65 can become transcriptionally active in partnership with p50 or can sequester SYK-induced RelB into RelB-p65 inactive dimers that do not bind to DNA. Therefore, the net result of Dectin-1 signaling via the SYK-CARD9, SYK-NIK, and Raf-1 routes is the merging of the canonical and non-canonical NF-κB pathways with Raf-1 activation of NF-κB, which favors the production of the cytokines IL-12 (Th1-polarizing cytokines), as well as IL-1β, IL-23, IL-6, (Th17-polarizing cytokines), thereby skewing T cell differentiation towards Th1 and Th17 subsets that are essential for antifungal immunity (Figure 16) (Brown, 2006a; Geijtenbeek and Gringhuis, 2009; Gringhuis et al., 2009b; Leibundgut-Landmann et al., 2008; Rogers et al., 2005; Sancho and Reis e Sousa, 2012; Underhill et al., 2005).

To date, the membrane-proximal events involved in the Raf-1 pathway are not fully characterized. Dectin-1 stimulation by curdlan in human DCs has been demonstrated to induce the phosphorylation of Raf-1 on Ser338, and on Tyr340 and Tyr341, by Pak and Src kinases, respectively (Gringhuis et al., 2009b). Members of the Ras and Rho protein families of small GTPases are key effectors for activation of the Raf-MEK-ERK signaling cascade, also known as the MAPK cascade (Pylayeva-Gupta et al., 2011). Normally activation of the Raf kinase family members (A-, B-, and C-Raf) occurs by an intricate multistage process that involves homodimer and heterodimer formation, activation of Ras-GTP (a small GTPase) or members of the Rho

family of small GTPases, which then lead to subsequent phosphorylation of Raf kinase (Roskoski, 2010). Rho family GTPases have been implicated in the activation of Raf-1 upon Dectin-1 ligation, and Raf-1 could be activated by the Grb2/SOS/Ras route (Roskoski, 2012). However the exact small GTPase responsible for this upstream step of Raf-1 activation has not yet been identified, and the nature of the molecular steps involved in this activation process is still not elucidated (Gringhuis et al., 2009b).

1.5.4.3. Dectin-1 Signaling Pathways for the Activation of Phagocytosis, ROS and Inflammasome

The consequences of Dectin-SYK activation for host defense extend beyond the activation of transcription. Apart from inducing transcriptional responses, SYK activation in response to Dectin-1 ligation can impact key cellular functions including phagocytosis, microbicidal activity and inflammasome activation (Mocsai et al., 2010; Plato et al., 2013). However, although signaling via SYK appears to mediate the majority of the cellular functions ascribed to Dectin-1, including the respiratory burst and cytokine production, as described below, some responses, such as phagocytosis in macrophages, do not involve signaling through this kinase (Figure 15) (Herre et al., 2004a; LeibundGut-Landmann et al., 2007; Rogers et al., 2005; Underhill et al., 2005).

An obviously unique feature of Dectin-1 is its ability to mediate ligand internalization through endocytosis or phagocytosis, which is a property largely divergent from TLRs that are not considered to be endocytic receptors (Brown, 2006a). Dectin-1, as a phagocytic receptor has been shown to mediate phagocytosis and production of reactive oxygen species (ROS) in response to particulate ligands such as zymosan (Figure 15 & Figure 16) (Drummond et al., 2011; Gantner et al., 2003; Kennedy et al., 2007; Rogers et al., 2005; Underhill et al., 2005). The Dectin-1 cytoplasmic tail has three consecutive acidic amino acids (DED/Asp-Glu-Asp) that might signal for endocytosis and/or phagocytosis (De Rosa et al., 2009; Engering et al., 2002b; Serrano-Gomez et al., 2004). However, its detailed mode of action is still unclear. SYK contributes to phagocytosis of fungal particles by DCs although not macrophages (Herre et al., 2004a; Rogers et al., 2005; Underhill et al., 2005). Upon interaction of zymosan with Dectin-1 in mouse DCs, SYK activates SFKs (Src family kinases) and the Rho GTPases (guanosine nucleotide triphosphatases), Cdc42 and Rac1 and triggers pseudopod extension around the particle, which is suggested to be actin-dependent (Goodridge et al., 2009a; Gross et al., 2006; Hernanz-Falcon et

al., 2009; Herre et al., 2004a; Rogers et al., 2005; Underhill et al., 2005). Interestingly, particle uptake mediated by Dectin-1 is not dependent on the SYK kinase in macrophages, despite requiring both the key tyrosine in the hemITAM motif and the DED motif upstream of signaling (Herre et al., 2004b). On the contrary, reactive oxygen species (ROS) generation within macrophage phagosomes is SYK dependent (Gross et al., 2006; Underhill et al., 2005). Therefore, the requirement for SYK appears to be cell type specific (Figure 15) (Brown, 2006a; Rogers et al., 2005; Underhill et al., 2005). Although the SYK-independent signaling pathways for mediating phagocytosis are unknown and probably novel, it has been proposed that Dectin-1 may also use novel, non-SYK signaling pathways, including the Raf-1 dependent signaling pathway. Interestingly, a recent study by Elsori *et al.* (2011) demonstrated a dependence of Dectin-1 on PKCδ activated downstream of SYK for Dectin-1-mediated phagocytosis of zymosan and regulation of ROS production in primary human monocytes (Elsori et al., 2011).

In addition to internalization, SYK can control events within phagosomes. Activation of Dectin-1 results in SYK-dependent production of reactive oxygen species (ROS) in myeloid cells, which mediates microbial killing (Figure 15 & Figure 16) (Gross et al., 2009; Underhill et al., 2005), Dectin-1 induction of ROS production occurs through SYK-dependent activation of the multimeric enzyme NADPH oxidase (Figure 16) (Gross et al., 2006; Plato et al., 2013; Underhill et al., 2005). NADPH oxidase, with its translocation into the phagosome membrane, reduces O_2 forming reactive oxygen species (ROS) such as H_2O_2 (Brown, 2006a; Elsori et al., 2011; Gantner et al., 2003; Li et al., 2007; Rogers et al., 2005; Underhill et al., 2005). The study by Elsori *et al.* (2011) also showed that in human monocytes, PKCδ controls the production of ROS by modulating NADPH oxidase (Elsori et al., 2011).

Secretion of the cytokine IL-1β, which is an essential cytokine in innate immunity, requires at least two signals: the first signal is cell stimulation that results in the synthesis of pro-IL-1β protein within the cells, and the second signal is the inflammasome-dependent proteolysis and processing of pro-IL-1β into secretory and bioactive IL-1β (Figure 16 & Figure 17) (Franchi et al., 2009; Martinon, 2007, 2010; Martinon et al., 2002; Martinon et al., 2009). Interestingly, the production of IL-1β was recently shown to require collaboration between Dectin-1 and the NLRP3 inflammasome via the Dectin-1–induced SYK-dependent pathway (Figure 16) (Gross et al., 2009; Hise et al., 2009; Said-Sadier et al., 2010). Whereas the SYK-CARD9 pathway in Dectin-1 signaling activates the synthesis of pro-L-1β, SYK can additionally activate the NLRP3

inflammasome in a ROS-dependent manner, resulting in proteolytic processing of IL-1β from inactive pro-Il-1β by the inflammatory protease caspase-1 (Figure 16 & Figure 17) (Gross et al., 2009; Hise et al., 2009; Said-Sadier et al., 2010). Therefore, ROS induced by the activation of NADPH downstream of Dectin-1 signaling, not only contributes to direct microbicidal activity within the phagosome, but can additionally act as a point of regulation for IL-1β production, by inducing the activation of the NLRP3 inflammasome, which in turn mediates the proteolytic processing of pro-IL-1β into IL-1β (Figure 16 & Figure 17) (Gross et al., 2009). Notably, IL-1β has a crucial role in antifungal immunity, where it helps in the polarization of T helper (Th) cells into Th17 (Vonk et al., 2006).

The inflammasome is a cytoplasmic, and proteolytic multiprotein complex that controls caspase-1 activity in the innate immune system, which is required for the processing and activation of the key cytokine IL-1β in response to pathogens (Franchi et al., 2009; Franchi et al., 2012; Martinon et al., 2002; Martinon et al., 2009). Typically, the essential components of the inflammasome are a sensor protein, e.g., NLRs [NOD-like receptor (NLR) family of cytosolic PRRs (see section **1.2.1.3)**, an adapter protein, mainly ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), and the protease caspase-1 (Figure 16 & Figure 17) (Jin and Flavell, 2010). NLRP3, a member of the NLR family of cytosolic PRRs, along with the adaptor protein ASC, and caspase-1 form the 'NLRP3 inflammasome' (Figure 16 & Figure 17) (Gross et al., 2011). Although it is still unclear how the NLRP3 inflammasome on its own responds to diverse stimuli, it has been proposed that the NLRP3 inflammasome is activated by ligand-induced intermediates such as reactive oxygen species (ROS), potassium efflux, and the lysosome destabilization (Franchi et al., 2012; Martinon, 2010), and multiple stimuli including pathogen-derived factors and danger-associated molecules produced by cellular damage (Gross et al., 2009; Hise et al., 2009). In this regard, well-known activators of the NLRP3 inflammasome include PAMPs (DNA and RNA), ATP, self-derived molecules (e.g., β-amyloid), and immune adjuvants (e.g. aluminum salt) (Kumar et al., 2009b; Martinon, 2010; Martinon et al., 2009). In response to these diverse stimuli, NLRP3 assembles together with the adaptor protein (ASC) and casapse-1, into a multimeric proteolytic protein complex to form the NLRP3 inflammasome, which activates caspase-1 and triggers the processing of pro-IL-1β and secretion of IL-1β (Figure 16 & Figure 17) (Franchi et al., 2009; Franchi et al., 2012; Martinon et al., 2002; Martinon et al., 2009). Therefore, the active NLRP3 inflammasome essentially drives proinflammatory innate immune

responses towards invading pathogens and cellular damage, and regulates adaptive immune responses.

IL-1β produced by the NLRP3 inflammasome is essential for antifungal immunity. Consistently, NLRP3-deficient mice are highly susceptible to fungal infection (Gross et al., 2009); however, the exact role of caspase-1, a major component of the inflammasome, as well as the inflammasome itself in human antifungal responses remains controversial (Mencacci et al., 2000; van de Veerdonk et al., 2009a). IL-1β secreted in response to Dectin-1 activation helps in destroying fungal invaders (Gross et al., 2009; Kankkunen et al., 2010; Underhill, 2007). It has been shown that activation of the NLRP3 inflammasome in response to the fungal pathogens, *A. fumigatus* and *C. albicans*, requires SYK kinase, as well as the respiratory burst (producing ROS) and potassium efflux, which suggests direct involvement of SYK-coupled CLRs, such as Dectin-1 and Dectin-2, in inflammasome activation by these fungal pathogens (see section **1.3.4**) (Said-Sadier et al., 2010). The connection between Dectin-1–mediated SYK signaling and IL-1β processing constitutes an example of how SYK-coupled CLRs can affect myeloid cell function independent of a role in regulating gene expression. However ROS produced during Dectin-1 signaling should be tightly controlled, as increased production of ROS may result in uncontrolled activation of the inflammasome leading to excessive tissue damage and chronic inflammation, which can induce autoimmune and inflammatory diseases.

1.5.4.4. Cooperative Cross-talk in Dectin-1 Signaling: Interaction & Collaboration of Dectin-1 with Other PRRs & Membrane Proteins

PAMPs are not individually present in a vacuum, and in fact in a real setting of microbial or viral infection, the host cells encounter different PAMPs that exist on the surface of the same pathogen, which enhances efficient recognition of the pathogen. Accordingly, it is common for several different PRRs to be engaged at the same time following pathogen encounter. When this occurs, extensive cross-talk between various PRRs takes place, integrating signals from these different receptors in order to shape the developing immune response to ensure effective pathogen control and elimination (Figure 17) (Vautier et al., 2012).

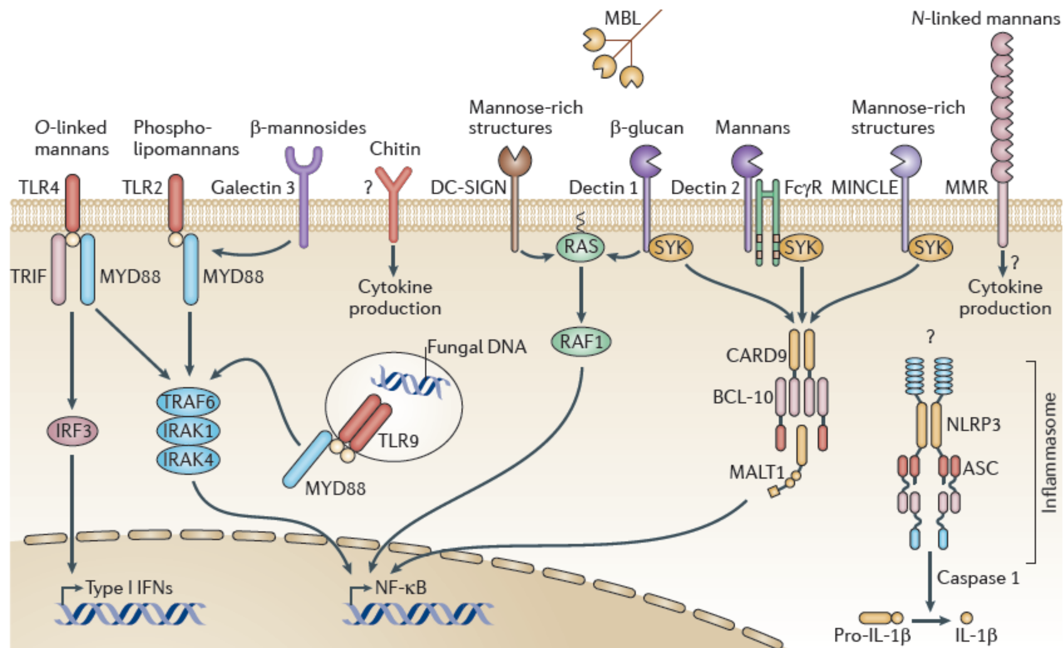


Figure 17: Main PRRs Involved in Fungal Recognition

This figure describes different PRRs involved in fungal recognition, their underlying signaling pathways, and cross-talk between these pathways. The soluble lectin receptor mannose-binding lectin (MBL) can bind mannose-rich structures from *Candida albicans*. In addition, the membrane-bound C-type lectin receptors (CLRs) macrophage mannose receptor 1 (MMR), dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) and macrophage-inducible C-type lectin (MINCLE) also recognize mannose-rich structures. Dectin 1 can bind β-glucans, and Dectin 2, together with the Fcγ receptor (FcγR) [as a signaling adaptor protein], recognizes mannans (mannose polymers). Toll-like receptor 4 (TLR4) recognizes O-linked mannans, whereas TLR2 can recognize phospholipomannans or, together with Galectin 3, recognizes β-mannosides. TLR9 is located in the cytosol and recognizes fungal DNA. Furthermore, the NOD-like receptor NLRP3 (NOD-, LRR- and pyrin domain-containing 3) forms an inflammasome complex with apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and the enzyme caspase 1. The ligand that triggers the NLRP3 inflammasome is currently unknown. CARD9, caspase recruitment domain-containing protein 9; IFNs, Interferons; IL-1β, Interleukin-1β; NF-κB, Nuclear factor-κB. Adapted with permission from (Gow et al., 2012).

Dectin-1 and TLRs

PRR cross-talk can result in the enhancement or abrogation of an immune response. PAMPs other than β-glucans and α-mannans are expressed in fungal cell walls and include TLR2 and TLR6 ligands (Figure 10) (Dennehy et al., 2009; Saijo and Iwakura, 2011). Accordingly, TLR signaling is thought to collaborate with Dectin-1 signaling, resulting in the modification of the Dectin-1 signal (Drummond et al., 2011). Indeed, zymosan, a yeast cell wall extract that has multiple PAMPS (mainly β-glucans, mannan, mannoprotein, and chitin) (Figure 14) (Gow et al., 2012; Netea et al., 2008) induces several PRRs including Dectin-1, TLR1, TLR2, and TLR6 (Figure 17) (Brown, 2006a; Ozinsky et al., 2000a). Signals from both TLR2 (Goodridge and Underhill, 2008) and TLR6 (Ozinsky et al., 2000b) were shown to be required for the induction

of cytokines, such as TNF-α and MIP-2 (Brown et al., 2003 ; Gantner et al., 2003), in response to zymosan. Interestingly, collaboration between Dectin-1 and TLR2, following zymosan binding or interaction with live yeast, enhances NF-κB activation and production of TNF-α, while downregulates the production of IL-12 (Figure 15 & Figure 16 & Figure 17) (Brown, 2006a; Gantner et al., 2003). However, in another study Curdlan, a highly selective and pure Dectin-1 agonist, was able to induce robust cytokine responses from DCs (Geijtenbeek and Gringhuis, 2009; LeibundGut-Landmann et al., 2007; Rosas et al., 2008). In contrast to this study, using highly purified receptor-specific reagents Dectin-1 was shown to synergistically enhance TLR-mediated proinflammatory cytokine and chemokine production (Dennehy et al., 2008). This collaborative signaling required both the Dectin-1/SYK and TLR2/MYD88 pathways, and resulted in enhanced translocation of NF-κB to the nucleus (Dennehy et al., 2008). Furthermore, the SYK-independent Raf-1 pathway is also thought to be important for the crosstalk between TLR and Dectin-1 signaling by inducing p65 acetylation and repressing SYK-induced RelB (Gringhuis et al., 2009b). This suggests the need for simultaneous collaboration between Dectin-1 and TLRs for modulating the immune response.

Numerous studies have shown that Dectin-1 could interact with zymosan, and stimulate cellular responses, such as phagocytosis and generation of ROS (reactive oxygen species) in a TLR-independent manner (Rogers et al., 2005; Underhill et al., 2005). However, on the other hand, as discussed above, evidence suggests that Dectin-1 converges with TLR signaling for the induction of cytokine responses (Dennehy et al., 2008; Ferwerda et al., 2008). Indeed, Dectin-1 acts synergistically with TLR to produce strong inflammatory responses by upregulating cytokines such as TNF-α and IL-2, and downregulating IL-12 in favour of Th-17–polarizing cytokines such as IL-6 (Gantner et al. 2003; Brown 2006). A complex of TLR2 and/or TLR6 with Dectin-1 is required for the collaborative production of proinflammatory cytokines such as TNF-α, in response to zymosan stimulation (Gantner et al., 2003; Herre et al., 2004b). Collaborative signaling between Dectin-1 and TLR2 has been shown to induce Th17 responses via the production of prostaglandin E2, which in turn upregulates the production of the pro-Th17 cytokines, IL-6 and IL-23 (Smeekens et al., 2010). In an earlier study by Dennehy *et al.*, Dectin-1/TLR2 enhanced production of IL-6 and IL-23, IL-10, but decreased IL-12 production (Dennehy et al., 2009). Additionally, Dectin-1/TLR2 collaborative signaling has also been demonstrated, though *in vitro*, to induce the activation of thioglycollate-elicited macrophages with specific

Dectin-1 and TLR2 agonists, where Dectin-1 mediated upregulation of TLR2-induced TNF-α, MIP-1α and MIP-2 (Dennehy et al., 2008). It is worth mentioning, though, that Dectin-1 and TLR2 collaboration can be cell-specific, with macrophages requiring Dectin-1/TLR2 co-stimulation to produce TNF-α, whereas stimulation of Dectin-1 in DCs is sufficient to initiate TNF-α production (although this can be enhanced by TLR signaling) (Kerrigan and Brown, 2010; Taylor et al., 2007). Dectin-1 also collaborates with TLR4, TLR5, TLR7, and TLR9 resulting in synergistic induction of IL-10, TNF-α, IL-2, IL-6, and IL-23 and downregulation of IL-12 (Dennehy et al., 2008; Gerosa et al., 2008). The reciprocal regulation of IL-23 and IL-12 is likely to have consequences on the ensuing adaptive immune response, which is especially important for antifungal immunity. The Dectin-1/TLR2 pathway can further synergize with the CLR Mannose receptor (MR) to augment IL-17 production by human peripheral blood mononuclear cells (PBMCs) (Figure 17) (Levitz and Specht, 2006; van de Veerdonk et al., 2009b).

Dectin-1 and Tetraspanins

Tetraspanins are small four-transmembrane proteins that can assemble immune receptors and signaling molecules into functional membrane microdomains. They are often thought to act as scaffolding proteins, anchoring multiple proteins to one area of the cell membrane (Figdor and van Spriel, 2010). Dectin-1 has been demonstrated to physically, and functionally, interact with tetraspanins such as the leukocyte-specific CD37 and the ubiquitous CD63 (Levitz and Specht, 2006; van de Veerdonk et al., 2009b). Dectin-1–induced cytokine production can be modulated by CD63, which downregulates IL-6 production in response to zymosan (Meyer-Wentrup et al., 2007). CD37 interaction also reduced Dectin-1–induced IL-6 production. As a result of this interaction, Dectin-1 may be organized in a microdomain platform that facilitates receptor mutimerization, clustering with TLRs or other CLRs, cross-talk between PRRs, and integration of signal transduction pathways (Figdor and van Spriel, 2010). However, the exact nature of the interaction of Dectin-1 with tetraspanins, the membrane organization of tetraspanins in relation to Dectin-1, as well as the mechanism of the downregulation of Dectin-1–induced IL-6 production by tetraspanins, are all questions that require further investigation.

Other Dectin-1 Partners

Dectin-1 has also been shown to synergize with Galectin-3, another CLR (Figure 17) (Esteban et al., 2011). These two proteins were shown to physically associate with each other, as evidenced by co-immunoprecipitation, and knockdown of Galectin-3 resulted in a four-fold decrease of

TNF-α production by macrophages stimulated with *C. albicans.* Dectin-1 binds to β-glucans, while Galectin-3 binds to β-(1,2)-oligomannans, which are present in the cell wall of pathogenic fungi, but are absent from the cell walls of non-pathogenic fungi such as *S. cerevisiae* (Figure 17). This suggests that Galectin-3 helps distinguish pathogenic from non-pathogenic fungi, as knockdown of Galectin-3 did not affect macrophage production of TNF-α after stimulation with *S. cerevisiae.* Galectin-3 has also been shown to associate with TLR2, and the data indicates that it plays a similar role in pathogen discrimination (Jouault et al., 2006).

Despite an improving understanding of regulatory mechanisms controlling Dectin-1 signaling, these are still poorly understood; however, lipid rafts (Xu et al., 2009a), cytoskeletal components (Gitik et al., 2010), and tetraspanins (Meyer-Wentrup et al., 2007; van Spriel and Figdor, 2010), have recently been implicated. For example, Dectin-1 signaling is affected by disruption of lipid microdomains, and NF-κB-dependent transcriptional activity is a major outcome (Xu et al., 2009a).

Another well-characterized function of Dectin-1 is the production of lipid mediators (Drummond and Brown, 2013; Suram et al., 2006), and inflammatory chemokines/cytokines (e.g. TNF-α, IL-1β, IL-10, IL-6, IL-23, CCL2, CCL3) (Dennehy et al., 2009; Reid et al., 2009), which demonstrate a cell-specific nature (Saijo et al., 2007; Taylor et al., 2007). Production of these mediators is controlled by several signaling pathways which have extensive cross-talk with those activated by different PRRs, and this is essential for effective antifungal immunity (Netea et al., 2006a). For example, as explained in section **1.5.4.3**, the production of IL-1β was recently shown to require collaboration between Dectin-1 and the NLRP3 inflammasome. NLRP3 is activated in response to β-glucans (such as curdlan), and this is dependent on the Dectin-1 SYK-mediated signaling pathway. Whilst the molecular details of this pathway are still being resolved, it has been shown that ROS production and potassium efflux are required, and there may be a partial dependency on TLR2 and Dectin-2 (Gross et al., 2009; Kankkunen et al., 2010; Kumar et al., 2009b; Said-Sadier et al., 2010).

Conclusion

Antifungal responses require the engagement of several different PRRs for a proper immune response (Robinson et al., 2009). Cross-talk between these multiple pathways and collaboration with various cytoskeletal/membrane proteins (e.g. actin and tetraspanins, respectively) is key to

the building of the most appropriate immune response against the invading pathogen.

1.5.5. Dectin-1: A Key CLR in Antifungal Immunity—Effects of Genetic Polymorphisms & Dectin-1 Deficiency on Host Antifungal Defense

It has been reported that β-(1,3)-glucan administration will increase resistance to fungal infections (Murphy et al., 2010; Rice et al., 2005). These findings have led many investigators to speculate that Dectin-1 plays a central role in the innate immune response to infection, specifically fungal infections (Dennehy and Brown, 2007). These data also implied that β-(1,3)-glucan mediates its anti-infective activity through a Dectin-1–dependent mechanism. Brown *et al.* (2001), (Brown and Gordon, 2001), have identified Dectin-1 as a major receptor for β-glucans that mediates the biological effects of β-glucans (Brown et al., 2003). Aditionally, Dectin-1 functions as a C-type lectin PRR that detects β-glucans in fungal cell walls. Indeed, Dectin-1 is able to recognize a variety of fungal species by means of its β-glucan specificity (see section **1.5.2.2**) (Figure 18), and contributes to generation of the immune response to these organisms (Brown, 2006a; Goodridge and Underhill, 2008). In the past few years, the role of Dectin-1 within antifungal host defense has been highlighted by numerous studies. Although several studies with Dectin-1 agonists have demonstrated the functional capabilities of Dectin-1, how all these functions fit into the antifungal response is still unclear. Several controversies have arisen surrounding the exact role of Dectin-1 in antifungal host immunity *in vivo* and its interactions with live fungi, where Dectin-1 appears to play a different role with different fungal species. Antifungal actions mediated by Dectin-1, as well as its role in antifungal immunity to medically important fungi will be described in this section.

The recognition of fungal β-glucans by Dectin-1 results in a variety of cellular responses essential for antifungal immunity and clearance of the fungal pathogen (Figure 18). These host protective cellular responses include fungal uptake and killing, and the production of inflammatory cytokines and chemokines (Figure 18). Macrophages and neutrophils are essential effector innate immune cells activated in response to Dectin-1 fungal recognition and produce cellular responses such as phagocytosis, oxidative burst, neutrophil degranulation, and the production of cytokines and chemokines that recruit and activate other immune cells (Figure 18) (Brown, 2006a; Plato et al., 2013). Moreover, Dectin-1 recognition of β-glucan speeds up the differentiation of Th17 cells and stimulates the production of IL-1β, IL-23 and IL-17A from these cells (Figure 18). Th17

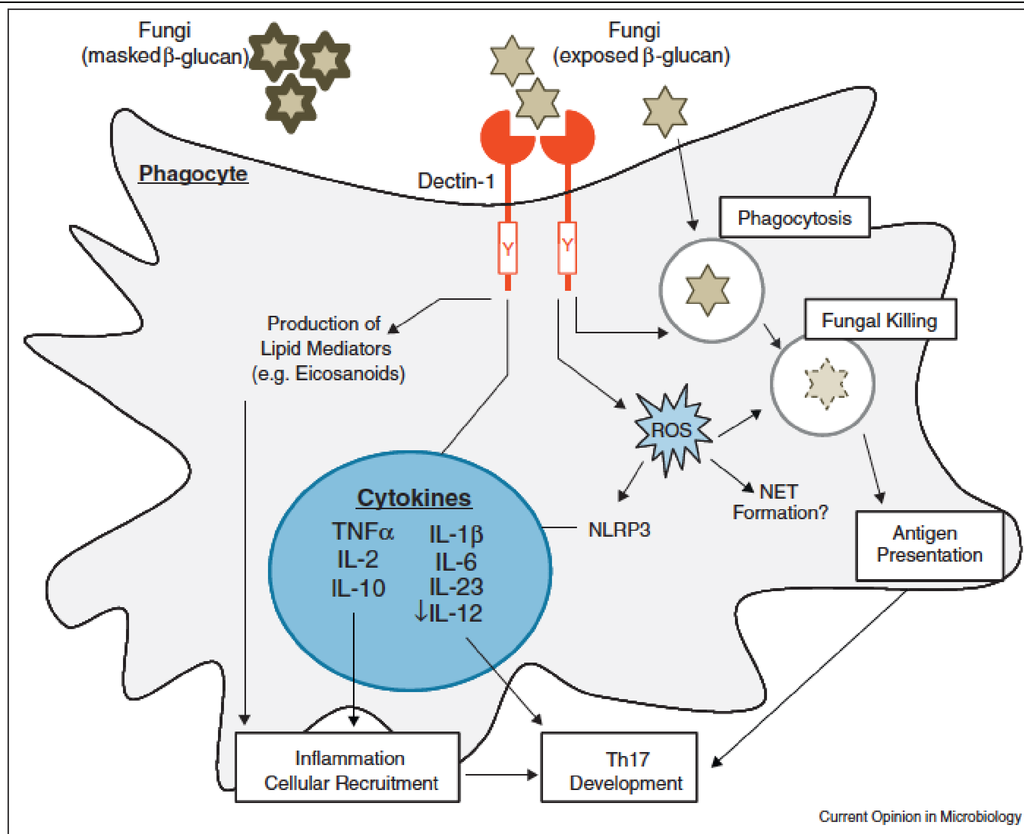


Figure 18: Dectin-1–mediated Antifungal Actions

Dectin-1 mediates several cellular responses following recognition of pathogenic fungi and these are illustrated in a general manner here. Major functions are shown in the white boxes. Note that not all functions shown are relevant to all fungi or fungal morphotypes; for example, some species and morphological forms are not recognized by Dectin-1, as their cell wall β-glucan is masked/shielded. Adapted with permission from (Drummond, R.A., 2011)

cells attract neutrophils to inflammatory sites and activate immune cells to achieve immunological coordination in the host when exposed to fungal infection, especially at the mucosa (see section **1.4.3.1**) (Figure 18) (Korn et al., 2009; Osorio et al., 2008). Interestingly, Dectin-1 also induces the production of IL-10, an anti-inflammatory cytokine whose role during fungal infection is unclear. The presence of IL-10 has traditionally been thought of as disadvantageous to the host during fungal infection. However, recent investigations have led to the suggestion that the inhibitory action of IL-10 on leukocyte activation may be important for limiting host injury during severe inflammation (Figure 18) (Romani and Puccetti, 2006).

Dectin-1 recognizes a number of pathogenic fungal species, including *A. fumigatus*, *C. albicans*, *Coccidiodes sp.*, *spores of C. neoformans*, *H. capsulatum*, *and P. jirovecii,* via β-(1,3)-glucans exposed in the fungal cell wall (section **1.5.2.2**) (Gantner et al., 2005; Rappleye et al., 2007; Saijo

et al., 2007; Steele et al., 2003; Steele et al., 2005; Viriyakosol et al., 2005). Accordingly, Dectin-1 has been demonstrated to be essential for antifungal defense against *C. albicans* (Taylor et al., 2007), and *A. fumigatus* (Werner et al., 2009) and *Pneumocystis jirovecii* (Saijo et al., 2007).

Further support for a central role of the β-glucan receptor, Dectin-1, on the immune function comes from Dectin-1 knockout studies in mice (Herre et al., 2004a; Werner et al., 2009). Dectin-$1^{-/-}$ mice are susceptible to primary pulmonary infection with *Aspergillus fumigatus* and *Pneumocystis marina, P. carinii*, and systemic *Candida albicans* (Saijo et al., 2007; Taylor et al., 2007; Werner et al., 2009). Dectin-1 deficiency has been generally associated with impaired recruitment of inflammatory leukocytes and inflammatory mediator production at the site of fungal infection. Taylor *et al* have generated knockout mice lacking expression of Dectin-1. (Figure 18) (Taylor et al., 2007). These mice are viable but show impaired recognition of zymosan and increased susceptibility to *Candida albicans* infection. Leukocytes from these mice are defective in recognizing fungi, even in the presence of opsonins. Inflammatory cell recruitment after fungal infection is also deficient, resulting in increased fungal burden and dissemination. In all of these knock-out models, regardless of the fungal species used, differences in cytokine production were observed, with, Dectin-1 deficiency resulting in impaired IL-1α, IL-1β, IL-6, IL-23, TNF-α, G-CSF, GM-CSF, CCL3/MIP-1α, CCL4/MIP-1β, and CXCL1/KC production (Figure 18) (Saijo et al., 2007; Taylor et al., 2007; Werner et al., 2009). Impaired cytokine production resulted in insufficient myeloid cell recruitment to sites of infection, allowing uncontrolled fungal growth (Werner et al., 2009).

A recent study by Netea *et al.* clearly demonstrated that Dectin-1 recognized a range of *C. albicans* strains (Netea et al., 2010). However, inconsistent results over the involvement of Dectin-1 in *C. albicans* immunity have been highlighted. The precise reasons for these discrepancies have been attributed to differences in the genetic background of the Dectin-1–deficient mice different. Three different genetic backgrounds were employed to generate the Dectin-1–deficient mice (Saijo et al., 2007; Taylor et al., 2007; Werner et al., 2009). Additionally, different strains and challenge doses of *C. albicans have been* employed in these studies (Saijo et al., 2007; Taylor et al., 2007; Werner et al., 2009). It has been suggested that the variable strains of *C. albicans* used for these Dectin-1 knockout studies may have led to differences in the accessibility of the fungal cell wall β-glucan to Dectin-1 leadng to the observed discrepancies in the results Furthermore, Saijo *et al*. (2007) have reported that Dectin-1 knockout mice are more

susceptible than wild-type mice to *Pneumocystis carinii* (Saijo et al., 2007).

In vivo data using Dectin-1$^{-/-}$ mice also indicate that Dectin-1 is important during murine anti-*Aspergillus* responses. Dectin-1 has been shown to mediate several cellular functions in response to *A. fumigatus* including phagocytosis, induction of killing mechanisms and cytokine/chemokine production, although these are dependent on fungal morphology (Drummond and Brown, 2011). Interstingly, Dectin-1$^{-/-}$ mice exhibited an increased mortality rate and impairment in cytokine production, which resulted in poor neutrophil recruitment and fungal killing (Werner et al., 2009). *Asperigillus fumigates* in Dectin-1 knockout mice; Dectin-1$^{-/-}$ showed an impaired interleukin (IL)-1α, IL-1β, TNF-α, MIP-1α, MIP-1β, and chemokine ligand (CXCL)-1 production that resulted in insufficient lung neutrophil recruitment and uncontrolled *A. fumigates* lung growth (Figure 18) (Werner et al., 2009). Dectin-1 mediated cytokine production has been shown to influence T cell polarization, and in this regard Dectin-1$^{-/-}$ mice infected intratracheally with *A. fumigatus* showed reduced levels of key Th17 cytokines (IL-17, IL-12p40 and IL-23) and a dominant IL-12p40/IFN-γ producing Th1 response, with increased fungal lung fungal burdens relative to wild type infected animals (Figure 18) (Rivera et al., 2011). Depletion of IL-17 with neutralizing antibodies had a similar effect, confirming the protective role of IL-17 in host defense against *A. fumigatus* (Werner et al., 2009).

Nakamura et al. (2007) have reported that Dectin-1$^{-/-}$ mice were not more susceptible to *C. neoformans* infection (Nakamura et al., 2007), whereas in another study a deficiency in the mannose receptor (MR) conferred susceptibility (Dan et al., 2008a). *C. neoformans* yeasts synthesize large polysaccharide capsules within the host lung which are thought to conceal the organism from immune recognition. Early studies showed that a β-glucan receptor could mediate the uptake of an acapsulated mutant of *C. neoformans,* leading to the production of inflammatory cytokines (Cross and Bancroft, 1995). In this regard, Dectin-1 may, however, recognize the spore form of the fungus, which has exposed β-glucans and was recently shown to bind to Dectin-1 on alveolar macrophages *in vitro*. Thus, it is clear that some of the Dectin-1 knockout studies performed in animals for studying the effect of Dectin-1 in antifungal immunity to certain fungal species provide an equivocal picture of the role of Dectin-1 in the *in vivo* response to fungal infection.

The above-described murine and *in vitro* studies support recent genetic studies in humans, which

have revealed Dectin-1 polymorphisms that predispose patients to mucocutaneous fungal infections. The recent discovery of a single nucleotide polymorphism, which introduces an early stop codon in the human Dectin-1 gene, (Y238X), preventing functional Dectin-1 protein from being expressed at the cell surface, showed that this receptor also mediates important antifungal functions in humans (Ferwerda et al., 2009). This polymorphism resulted in recurrent vulvovaginal candidiasis or onychomycosis (Ferwerda et al., 2009), increased oral and gastrointestinal colonization by *Candida* spp. (Plantinga et al., 2009), and increased susceptibility to invasive aspergillosis (Chai et al., 2011; Cunha et al., 2010; Cunha et al., 2011). This increased susceptibility to fungal infection was shown to be due to reduced β-glucan recognition by cells expressing the Dectin-1 polymorphisms resulting in defective production of cytokines, including IL-17, IL- 10, IL-1β, IFN-γ, TNF-α and IL-6 (Figure 18) (Cunha et al., 2010; Gantner et al., 2005; Plantinga et al., 2009). Homozygous patients were more susceptible to mucocutaneous candidiasis, associated with a defect in cytokine production (such as TNFα, IL-17, IL-6) (Ferwerda et al., 2009). In contrast to the Y238X polymorphism, the identification of a second Dectin-1 SNP (Single Nucleotide Polymorphism) identified in an African HIV population, showed a protective effect on oropharyngeal candidiasis, due to reduced production of IFN- γ (Plantinga et al., 2010).

As described in section **1.5.4.1**, Dectin-1 stimulation induces several signaling pathways. Intriguingly, mutations in components of the Dectin-1 signaling pathway and other related CLR signaling pathways also affect antifungal immunity, and are associated with more rare forms of inherited chronic mucocutaneous fungal infections (Figure 16). Dectin-1 is a C-type lectin PRR for fungi, and is part of a novel innate signaling pathway involving the SYK-CARD9 signaling module, which is critical for inducing Th17 responses to fungal infection (Figure 16) (Goodridge et al., 2007; Gross et al., 2006; Rogers et al., 2005; Underhill et al., 2005). Interstingly, a premature stop codon mutation in CARD9 (Q295X) resulted in low expression of CARD9, reduced TNF-α production and greatly reduced numbers of Th17 cells increasing susceptibility to mucocutaneous infection (Glocker et al., 2009). In mouse studies, bone marrow-derived dendritic cells (BMDC) from CARD9$^{-/-}$ mice also exhibited severe cytokine defects in response to *C. albicans*, with delayed NF-κB activation and much reduced TNF-α, IL-6 and IL-2 production (Figure 18) (Gross et al., 2006). These mice are also more susceptible to systemic fungal infection, with reduced survival and higher fungal burdens in the kidneys, lung and liver (Glocker

et al., 2009). In Dectin-1 deficient mice, lung immunity against *A. fumigatus* yields less IL-17, reduced proinflammatory cytokine and chemokine production by alveolar macrophages, and fewer neutrophils, which display defective antifungal defense mechanisms. Furthermore, the (Y238X) Dectin-1 polymorphism in humans, which causes loss of cell surface expression of Dectin-1, rendered individuals susceptible to mucocutaneous infections with *C. albicans,* partly as a consequence of impaired IL-17 production (Ferwerda et al., 2009). Werner *et al.* (2011) have also reported that Dectin-1-dependent IL-17A production in the lungs during invasive fungal infection is mediated in part by neutrophils in a manner that depends on IL-23, a cytokine required for the production of Th17 responses (Figure 18) (Werner et al., 2011; Werner et al., 2009). Gessner et al. (2012) have reported that early innate lung defense against *Aspergillus fumigatus* is mediated by Dectin-1-dependent IL-22 (a key cytokine secreted by Th17 cells production (Gessner et al., 2012). With regard to mycobacteria, *in vitro* studies implied that recognition by Dectin-1 contributed to uptake, respiratory burst induction, cytokine production and the generation of Th1 and Th17 adaptive responses (Rothfuchs et al., 2007; van de Veerdonk et al., 2010; Yadav and Schorey, 2006). Other advances suggest that Dectin-1 signaling mediates the activation of calcineurin, a protein phosphatase required for the activation of the transcription factor NFAT and candidacidal activity of neutrophils, as well as transcriptional responses to *C. albicans* (Figure 16) (Greenblatt et al., 2010). Interestingly, immunosuppressive drugs, such as cyclosporine, have been recently implicated to act on these pathways (at the level of calcineurin activation) and this may confer the observed fungal susceptibility in patients taking these drugs (Greenblatt et al., 2010; Yang et al., 2010).

Recent studies have also demonstrated that Dectin-1 signaling induced by *C. albicans* and *Aspergillus fumigatus* is required for activation of the NLRP3 inflammasome and subsequent IL-1β production, events now known to be crucial for host defense during fungal infection (Figure 18) (Gross et al., 2009; Hise et al., 2009; Said-Sadier et al., 2010). IL-1β production was impaired in cells from homozygous Y238X humans (Plantinga et al., 2009), suggesting that the inflammasome pathway is important in *C. albicans* host defense. Indeed, mice deficient in Dectin-1, caspase-1 or NLRP3 (inflammasome components) were shown to be hyper-susceptible to this pathogen (Figure 18) (Gross et al., 2009; Hise et al., 2009). Moreover, Cheng *et al.* (2011) have recently, reported that the Dectin-1/inflammasome pathway is a mechanism that enables the host immune system to mount a protective Th17 cell response and to distinguish between

colonization and tissue invasion by *Candida albicans* (Figure 18) (Cheng et al., 2011).

Collectively, the studies described above indicate that different components of the Dectin-1 signaling pathway are crucial to mounting effective host antifungal immunity in both mice and humans. In this regard the Dectin-1/SYK/CARD9 signaling cascade, is of particular importance, as it is instrumental in the differentiation of Th17 cells and activation of their downstream functions in response to infection with opportunistic fungi (Hise et al., 2009; Saijo et al., 2007; Taylor et al., 2007). Therefore, recently, the differentiation of Th17 cells in response to fungi has been a subject of intense study.

The generation of ROS is a prerequisite to the formation of neutrophil extracellular traps (NETs) (Brinkmann et al., 2004), recently discovered structures that were shown to be lethal to *C. albicans in vitro* (Figure 18) (Urban et al., 2006). Whilst no direct link between Dectin-1 and NETs has yet been made, there is speculation that Dectin-1 signaling, and the subsequent respiratory burst, may contribute to NET formation (Figure 18). Dectin-1 was shown to mediate the internalization and killing of *P. carinii* cysts by macrophages *in vitro*, and the production of MIP-2 (Steele et al., 2003). Dectin-1$^{-/-}$ mice had higher numbers of cysts in the lung early in infection and this effect was particularly pronounced under immune-compromising conditions (Saijo et al., 2007). However, whilst ROS generation from Dectin-1$^{-/-}$ cells was completely abrogated, cytokine production was shown to be normal and was only affected in MyD88$^{-/-}$ cells, suggesting that TLRs also play an important role in *P. carinii* immunity *in vivo* (Saijo et al., 2007).

Yeast versus Hyphae

Dectin-1 can mediate phagocytosis and ROS production in response to *C. albicans, in vitro*, and this interaction also results in the production of arachidonic acid metabolites (Suram et al., 2006), and cytokines (e.g. TNF, IL-6, CCL2) (Figure 18) (Ferwerda et al., 2009). However, these functions are dependent on fungal morphology as only the yeast form is efficiently recognized (Figure 18) (Gantner et al., 2005). Along that line, Dectin-1 signaling has been implicated in the immune system's discrimination of yeast and hyphal forms of *C. albicans.* Since *C. albicans* commonly colonizes the skin and mucosal surfaces, it is important for the immune system to distinguish between Candida as a commensal organism (yeast form) and an invasive pathogen

(hyphal form) (Gantner et al., 2005; Gow et al., 2012). To that end, *Candida* hyphae were shown to stimulate IL-1β production in macrophages, while *Candida* yeast did not trigger this cytokine production. Both Dectin-1 and the inflammasome were shown to contribute to hyphal-mediated IL-1β production, indicating that Dectin-1 plays a role in the differential recognition of *Candida* (Cheng et al., 2011). The cell wall of *C.albicans* is rich in β-glucans, which are recognized primarily by Dectin-1, yet a recent study, showed that exposure of glucan, by yeast form but not filamentous forms, determines Dectin-1-dependent macrophage recognition and uptake of *Candida albicans* thereby representing a novel immune evasion mechanism by a fungal pathogen (Figure 18) (Gantner et al., 2005). Yeast forms of fungi, as well as inhaled infectious spores (conida) of *Aspergillus* species, are phagocytosed by tissue-resident macrophages and recruited to neutrophils and subsequently destroyed by the release of microbicidal substances to the phagosome. Some conidia and yeast fungal forms escape immune detection and transform into the invasive hyphal forms. Hyphal forms of fungal species such as *Candida* and *Aspergillus* are too large to be phagocytosed and are instead targeted by neutrophils for extracellular killing, whereby they release reactive oxygen species (ROS) and granule contents onto their target (Figure 18) (Romani, 2011). Dectin-1, expressed on alveolar macrophages and neutrophils recruited to the host sites of infection with *A. fumigatus*, has been shown to bind to β-glucans on the surface of these fungi and induce a respiratory burst (Boyle et al., 2011). In this regard, a study demonstrated that Dectin-1 knockout mice are susceptible to infection with *A. fumigatus*, highlighting an essential role for Dectin-1 in host defense against this fungal pathogen (Werner et al., 2009).

<u>Conclusion</u>

The discovery of Dectin-1 as the major β-(1,3)-glucan pattern recognition receptor (PRR) in mammalian systems has dramatically advanced our knowledge of how the innate immune system recognizes and interacts with fungal β-glucans (Brown and Gordon, 2005; Brown et al., 2003; Herre et al., 2004a). Dectin-1 has been reported to be key to the recognition of several clinically important fungal species. Although, the *in vivo* role of Dectin-1 in response to fungal infections still remains controversial, both murine and human studies in the recent years have established a central role for Dectin-1 in antifungal immunity. Indeed, most of the studies described above have pointed towards an important role for Dectin-1 in host antifungal defense, and loss of this PRR in mice has been shown to result in increased susceptibility to fungal infections with

Aspergillus fumigatus and *Pneumocystis marina, P. carinii*, and *Candida albicans* (Saijo et al., 2007; Taylor et al., 2007; Werner et al., 2009).

1.5.6. Dectin-1 in Autoimmunity and Inflammation

Although Dectin-1 may play a protective antifungal and /or beneficial immunomodulatory role as a receptor triggering cellular activation, Dectin-1 may also contribute to the development of inflammatory diseases and autoimmunity. Indeed, recently, a role for Dectin-1 in the induction of autoimmunity and inflammatory disease has been revealed. Through recognition of β-glucan carbohydrates, Dectin-1 can induce autoimmunity and disease in specific genetic backgrounds. Stimulation by purified fungal β-glucan, including curdlan and zymosan, or induction of fungal infection via intact fungal microbes, were shown to induce autoimmune forms of rheumatoid arthritis in genetically susceptible mice (Sakaguchi and Sakaguchi, 2005; Yoshitomi et al., 2005). This β-glucan-induced arthritis could be inhibited by blocking Dectin-1, demonstrating that the inflammatory responses triggered by this receptor were involved in the development of this disease. This study also suggested that the immune responses triggered by Dectin-1 might induce autoimmune diseases in certain genetic backgrounds. Along that line, in SKG mice (which are prone to develop autoimmune arthritis) zymosan and other β-glucan particles stimulate dendritic cells (DCs) to secrete cytokines and to present self-antigens to arthritogenic T cells, leading to chronic arthritis (Yoshitomi et al., 2005). The activation of DCs takes place in a Dectin-1-dependent manner, and the blockage of this receptor prevents the onset of disease. Interestingly, Dectin-1 has been shown to play a role in thymocyte development and/or T cell activation, although the nature of this interaction and the endogenous T cell ligand is unknown.

It is also likely that Dectin-1 is involved in other inflammatory and allergic diseases, including respiratory disorders induced by fungi or their β-glucan components, such as allergic bronchopulmonary aspergillosis, but this has yet to be formally demonstrated (Steele et al., 2005). Although the mechanisms behind these respiratory disorders are not firmly established, Dectin-1 is known to be responsible for pulmonary inflammation following exposure to fungi, such as *Aspergillus fumigatus* (Werner et al., 2009). Recently, Dectin-1 has been shown to be involved in chronic inflammatory conditions such as type 2 Diabetes, (T2D) Cortez-Espinosa et al., 2012). In this study, monocytes from T2D patients with poor glycemic control showed decreased expression and abnormal function of Dectin-1 leading to impaired regulation of inflammatory

responses in these patients (Cortez-Espinosa et al., 2012).

1.6. β-glucans: Bittersweet Ligands of Dectin-1

Over the past four decades, an enormous amount of studies have described the anti-infective, antitumour and immunobiological effects of many glucan preparations in a variety of disease states (Chen and Seviour, 2007; Vetvicka et al., 2008; Zelenay and Reis e Sousa, 2013). Currently, β-glucans are well recognized for their immunomodulatory potential, and have been shown to stimulate immunity and provide a remarkable range of health benefits. β-glucans have been reported to decrease the incidence of infectious complications, such as sepsis, in experimental animals and in surgical and trauma patients (Babineau et al., 1994a; Babineau et al., 1994b; de Felippe Junior et al., 1993). Trials show that β-glucans are especially important against the two most common conventional causes of death in industrialized countries, i.e. cardiovascular diseases (as fibre, β-glucans promote healthy cholesterol and blood glucose levels) (Chen and Raymond, 2008), and cancer (where they enhance immune system functions through induction of β-glucans receptors on immune cells) (Chan et al., 2009). However, the mechanisms associated with β-(1,3)-induced immunomodulation are only emerging. This section covers a summary about β-glucans as ligands of Dectin-1, with focus on their chemistry, physical chemistry, functional role in immunological responses, pharmacokinetics and possible applications as therapeutic tools.

1.6.1. Physico-chemical Properties and Structure-function Relationships of β-glucans

β-glucans are plant- or microorganism-derived polysaccharides made up of a backbone of repeating D-glucose units linked by β-(1,3) glycosidic bonds with or without randomly dispersed β-(1,6)-linked side chains (Figure 13) (Mueller et al., 2000). β-Glucans can vary in terms of physico-chemical parameters, such as backbone structure, frequency and length of branching, molecular weight (from 10^2 to 10^6 Da), the charge of their polymers and structure in aqueous media, degree of polymerization (i.e. the number of repeat units building the polymer), higher-order solution conformation (e.g., random coil, single helix, or triple helix), and solubility (Barsanti et al., 2011; Mueller et al., 2000). Previous studies indicate that the physicochemical properties of glucans (e.g. primary structure, polymer size, surface charge, solution conformation and side-chain branching) may be important for recognition and interaction with PRRs (Novak

and Vetvicka, 2008). Indeed, differences in molecular size, solubility, branching frequency and solution conformation (higher order structure) have been reported to affect the affinity with which Dectin-1 interacts with β-glucans. These structural features of glucans are assumed to be important not only in Dectin-1 mediated recognition of β-glucans, but also for influencing the biological effects of these carbohydrates (Adams et al., 2008; Graham et al., 2006; Murphy et al., 2010).

Curdlan, a high molecular weight (MW) linear β-(1,3)-glucan is extracted as a water-insoluble polysaccharide by the soil bacterium *Alcaligenes Faecalis*. Laminarin, a low MW β-glucan (6-8 kDa) that is readily soluble in water [a small 40'mer β-glucan obtained from the brown seaweed *laminaria digitata,* and is made up of a β-(1,3) D-glucose linear backbone with β(1,6)-linked side-chains (of only one glucose unit every ten residues) (Adams et al., 2008; Barsanti et al., 2011; Chen and Seviour, 2007), and zymosan [β-(1,3)-glucan from Saccharomyces or yeast], and sceleroglucan, a high MW (>1000 kDa) β(1,3)-glucan with one side β-(1,6)-D-glucose side chain every three residues (produced by fermentation of the filamentous fungus Sclerotium rolfsii) (Barsanti et al., 2011; Chen and Seviour, 2007; Huang et al., 2009). Additionally, Grifolan (GRN) [β-(1,3)-linked glucan with a single β-(1,6)-linked branch every third residue], GFPBW2 [with a β-(1,3)- and β-(1,4) backbone and β-(1,6) branches attached to O-6], and Maitake D (MD) fraction [β-(1,6)-glucans with β-(1,3) branched chains), are all highly purified water-soluble β-glucans extracted from the fruit bodies of *Grifola findosa,* an edible mushroom (also known as Maitake in Japan), which exhibit potent antitumor and immunostimulatory activities.

β-glucans adopt one of the three typical conformations in an aqueous environment: a triple helix, a single helix, or a random coil. Following rigorous purification, the structure of glucans such as curdlan, laminaran, lentinan, and scleroglucan have been identified by biophysical/structural methods including x-ray crystallography, which indicate that these glucans have a triple-helix backbone conformation in the solid state (Barsanti et al., 2011). The difference in the β-(1,3)-glucan structures occurs primarily in the side chain. Curdlan, together with paramylon, is structurally the simplest member of the β-(1,3)-glucans with no glycosyl side chain, while laminaran, lentinan, scleroglucan have β-(1/4) or β-(1/6) – glycosyl side chains exposed toward the exterior of the helical structure (Barsanti et al., 2011). Although the triple helical (triplex) structure is stable over a broad range of temperatures in aqueous solution at physiological pH, the hydrogen bonds can be destabilized – i.e. the triplex structure can be forced to undergo a

reversible strand-separation transition (denaturation) into the single helix conformation – by highly alkaline solutions (pH 12), solvents such as DMSO, or by increasing the temperature above the triple-helix melting temperature (135 °C). Furthermore, a triple helix can be converted into a random coil by treatment with NaOH, and the random coil can then be converted into a single helix by neutralization with HCl (Batbayar et al., 2012).

The physicochemical properties of high molecular weight β-glucans are closely correlated to biological activity potency. However, whether single helix or triple helix β-(1,3)-D-glucan are the most biologically active is still an unresolved issue, and the literature appears inconsistent and often contradictory (Barsanti et al., 2011).

1.6.2. Controversies about β-glucan Actions

In some reports, the triple helix conformation of β-glucan has been shown to play an important role in enhancing biological activities, such as cytokine secretion and antitumor activity (Zhang et al., 2005). A recent study of the *in vitro* inhibitory effect of β-glucan against the proliferation of sarcoma-180 tumor cells, revealed that denatured, single-stranded β-glucan lentinan had weaker inhibitory activity than the triple helix conformation (Wang and Zhang, 2009). Contrastingly, in another report, both the single and triple helices demonstrated strong TNF-α and IL-6 cytokine releasing activity in a macrophage cell line (Okazaki et al., 1995). Different immunological effects of β-glucans may be related to the different structures of untreated, denatured, and renatured β-(1,3)-D-glucan molecules, and incorrect conclusions can be easily drawn when comparing results obtained by using such diverse glucans. Despite intensive research efforts, the potency of immunostimulation of the triple helix versus single helix conformation is still controversial. There, is therefore still no consensus on the basic structural requirements for the biological effects of β-glucans, and the exact higher order structure (single or triple helix) of the biologically active β-(1,3)-D-glucan is not well established.

The biological activity of β-glucans is dependent on their conformational complexity including length of polysaccharide chain, extent of branching and length of branches; a higher degree of structural complexity is thought to be associated with more potent immunmodulatory activity. Unfortunately, however, often in the literature, the activity of one β-glucan is inappropriately generalized to all β-glucans. Future research on the comparison of purified β-glucans from different sources is therefore warranted.

1.6.3. Immunomodulatory Effects and Therapeutic Benefits of β-glucans

Medicinal Use of β-glucans

β−Glucans have been long known for their notable physiological effects and immunomodulatory activity with negligible toxicity; this is their most important quality and the reason why so much attention has been devoted to them. Therefore, β-glucans, because of their immunotherapeutic benefits, have been traditionally and currently used as nutritional supplements and immune modulators for boosting the immune system. The literature suggests β-glucans are effective in treating diseases like cancer, a range of microbial infections, hypercholesterolemia, and diabetes.

Proper history of polysaccharides as immunomodulators goes back to the 1940s when Shear and coworkers described a substance, again from *Serratia marcescens* cultures, that caused necrosis of tumors (Novak and Vetvicka, 2008). Subsequently, this substance (so-called Shear's polysaccharide) was identified as a mixture of three polysaccharides with the main chain consisting of D-glucose and D-mannose units connected by (1,3) glycosidic linkages (Srivastava et al., 1962). Moreover, the yeast-derived particle zymosan, composed principally of β-glucan polymers, α-mannan, protein and lipid was first shown in the 1950's to activate immune cells, and has been since used in many physiological and immunological studies (Benacerraf and Sebestyen, 1957; Di Carlo and Fiore, 1958). Zymosan is potent stimulator especially of alveolar macrophages and, among others (Table 3), it induces the release of a series of cytokines from human neutrophils. Although zymosan was able to stimulate immune response, at the onset it was not clear what component of this rather crude yeast cell wall extract is responsible for that activity. When zymosan was examined in detail, yeast cell wall β-glucans were subsequently identified as the active component of zymosan (Di Luzio et al., 1970). Since this discovery, β-glucans have been subsequently isolated and their immunological effects have been thoroughly investigated by an enormous amount of studies. These studies demonstrated the complex role of β-glucans as immunostimulating agents (Murphy et al., 2008; Novak and Vetvicka, 2008). Unlike western countries, which only learned about β-glucans in the 19th century, in Asian countries, mainly Japan, consumption of different medicinal mushrooms (e.g., shiitake, and maitake, etc.) has been a long tradition in oriental medicine. Detailed studies of the biological effects of these mushrooms, especially the anticancer actions, again recognized β-glucan components as the main cause of non-specific immunomodulation (Table 3) (Ren et al., 2012).

Name	Source	Polymer organization
Curdlan	*Alcaligenes faecalis*	linear 1,3- β-glucan
CSBG	Candida albicans	Soluble 1,3- β -glucan with long 1,6-β -glucan segments
OX-CA	Candida albicans	Particle 1,3- β-glucan with long 1,6-β -glucan segments
T-4-N, T-5-N	*Dictyophora indusiata*	branched
Paramylon	*Euglena gracilis*	linear 1,3- β-glucan
Grifolan	*Grifola frondosa*	6-branched 1,3- β-glucan, having one branch chain every third main chain unit
Lentinan	*Lentinus edodes*	6-branched 1,3- β-glucan
Pleuran (HA-glucan)	*Pleurotus ostreatus*	branched
Pachymaran	*Poria cocos*	linear 1,3- β-glucan
Yeast glucan	*Saccharomyces cerevisiae*	branched
Yeast whole β-glucan particule (WGP)	*Saccharomyces cerevisiae*	Crude preparation 6-branched 1,3- β-glucan
Zymosan	*Saccharomyces cerevisiae*	Crude cell wall extract containing 1,3- β--glucan, 1,6- β-glucan, mannan and chitin
Schizophyllan or sonifilan (SPG)	*Schizophyllum commune*	soluble 6-branched 1,3- β-glucans,having one branch chain every third main chain unit
Sclerotinan	*Sclerotinia sclerotiorum*	6-branched 1,3- β-glucan, having one branch chain every second main chain unit
Scleroglucan	*Sclerotium glucanicum, S. rolfsii*	branched
SCG	Sparassis crispa	1,3- β-glucan, having one branch chain every third main chain unit

Table 3: List of Some β-glucans with Immunomodulatory Effects.

The above table lists the β-glucans that have been shown to induce immunstimulatory effects in mammals. The table also illustrates the chemical diversity of these β-glucans, which are derived from different sources. These sources include algae and various microrganisms mainly bacterial and fungal species. The structural features of the polysaccharides are also described. Adapted with permission from (Harada, T., 2008)

β-glucans as Biological Response Modifiers (BRMs)

Currently, β-glucans are biologically classified as potent 'biological response modifiers' (BRMs) or immune modulators with various effects on the immune system, such as the induction of

antimicrobial, fungicidal, radioprotective, antioxidant and anti-tumour effects *in vivo* (Batbayar et al., 2012; Chan et al., 2009; Chen and Seviour, 2007; Herre et al., 2004b; Murphy et al., 2010). β-glucan-mediated anti-infective activities include protection against bacterial, fungal, viral and protozoal infections.

Nevertheless, β-glucan derived as soluble/insoluble fiber from yeast, fungi, grains and seaweed, have shown therapeutic benefits in a variety of animal disease models, (Batbayar et al., 2012; Chen and Seviour, 2007; Murphy et al., 2010). β-glucans have been shown to stimulate innate immunity (Murphy et al., 2010), stimulate antitumour responses (Chan et al., 2009; Hong et al., 2004), increase resistance to infections (Chen and Seviour, 2007) and promote wound repair (Wei et al., 2002).

An example of immunostimulatory glucans is lentinan, a purified β-glucan [β-(1,3)-glucan with a β-(1,6) side branch every 5 residues] extracted from the fruiting bodies of *Lentinus edodes,* a popular edible mushroom in Asian countries. Lentinan has been described as extremely remarkable for its anticancer and immunomodulating activities (Wasser, 2002) and is currently used clinically as an antitumour agent. It has been shown to stimulate natural killer (NK) cell activity, macrophage/monocyte functions (secreting IL-1 and superoxide anion), phagocytosis, and cytotoxicity and to elevate the cytotoxic activity and TNF secretion of macrophages *in vitro* and *in vivo* [reviewed in (Xu et al., 2011)].) Also recently, lentinan a water-soluble β-glucan has been reported to directly stimulate macrophage activation by inducing phosphorylation of the MAPKs (MAP kinases), ERK1/2 and JNK1/2, in addition to nuclear translocation of NF-κB (Xu et al., 2011).

Numerous studies have shown that systemic or oral administration of pharmaceutical grade β-(1,3)/β-(1,6)-glucans stimulates innate immunity (Rice et al., 2005; Vos et al., 2007a; Vos et al., 2007b) increases resistance to infectious challenge, decreases myocardial injury after ischemia/reperfusion injury (Li et al., 2004; Wu et al., 2010), suppresses the growth of transplanted tumors, (Cheung et al., 2002; Hong et al., 2004) and facilitates wound repair (Wei et al., 2002). β-glucans are used as a dietary supplement for enhancing the host immune system and better understanding their mechanism of action would help in the development of future therapeutic products.

β-glucan and Antitumor Treatment

β-Glucan has been used as an immunoadjuvant therapy for cancer since 1980, primarily in Japan

(Chan et al., 2009; Chen and Seviour, 2007; Cheung et al., 2002; Novak and Vetvicka, 2008). Many animal studies have demonstrated remarkable effects of β-glucans (especially those derived from certain mushrooms/yeasts) on a range of tumors, and several human clinical trials have also shown possible treatment benefits (Batbayar et al., 2012; Chan et al., 2009; Chen and Seviour, 2007; Cheung et al., 2002; Rice et al., 2005). β-glucans do not attack tumour cells directly, but exert their antitumour effects by stimulating specific and non-specific immune responses in the host (Chan et al., 2009; Wasser, 2002). Moreover, numerous animal studies have demonstrated the antitumor activities of β-glucans as an immunostimulatory agent, which acts through the activation of macrophage and NK cell cytotoxicity. In this regard, β-glucan can inhibit tumor growth at the promotion stage (Harada and Ohno, 2008; Li et al., 2010). Glucan administered to cancer patients has been shown to enhance the effect of anticancer chemotherapy or radiation therapy and has positive effects on the survival and quality of life of cancer patients. In mice, SPG-mediated antitumor activity was inhibited by blocking of Dectin-1 by using anti-Dectin-1 antibodies (Ikeda et al., 2007). This result indicates that Dectin-1 affects β-glucan-mediated antitumour activity in mice. On the other hand, WPG (whole glucan particle) derived from yeast, potentiated the activity of antitumor monoclonal antibodies, leading to enhanced tumor regression and survival, but not in CR3 (complement receptor 3) knockout mice (Hong et al., 2004). However, there is still not much scientific evidence to explain the precise mechanisms of the actions of β-glucan or the differences of responsiveness to β-glucan in humans. Additionally, the study of animal strain differences of reactivity to β-glucan is important for the clarification of the individual variation in humans. An interesting and important concept from all the basic as well as clinical studies regarding immunomodulatory effects of β-glucans is that the activities of β-glucans are very diverse in each case, thus it has been necessary to develop our molecular understanding of these compounds (Chen and Seviour, 2007; Cheung et al., 2002; Harada and Ohno, 2008).

β-glucan as Hematopoiesis Stimulant

Another beneficial biological activity demonstrated for β-glucans is its ability to stimulate hematopoiesis in an analogous manner as granulocyte monocyte-colony stimulating factor (GM-CSF) (Patchen and MacVittie, 1983). Anticancer drugs impair blood forming functions and have neutropenia as a major side effect, which affects the defense system of patients may accelerate risk of infections (Novak and Vetvicka, 2008). Both particulate and soluble *β*-glucans, all of

which were administered intravenously, caused significantly enhanced recovery of blood cell counts after gamma radiation (Patchen and MacVittie, 1985). Others showed that *β*-glucan could reverse the myelosuppression produced with chemotherapeutic drugs (Wagnerova et al., 1993).

β-glucans as Vaccine Adjuvants

Another intriguing application of the immunomodulatory activity of β-glucans that has recently attracted the attention of researchers is to exploit biologically active β-glucans in the development of glucan-based adjuvant components of vaccine conjugates (Petrovsky and Aguilar, 2004; Petrovsky and Cooper, 2011). It is also of immense potential for immunotherapy and vaccination to target antigens to antigen-presenting cells (APCs), and β-glucans as vaccine components have been used to deliver whole protein antigens to APCs via β-glucan-mediated targeting of Dectin-1 (Further explained in Chapter 4).

Fungal β-glucans as Antifungal Drug Targets

Besides evidence on their antitumour and immunoadjuvant properties, the view has progressively emerged that cell wall β-glucans may also represent an important target for pharmacological or immunotherapeutic interventions in the treatment of fungal infections (Leibundgut-Landmann et al., 2008). β-glucan synthesis is a crucial event for fungal growth and virulence, and its blockade is the common mechanism of action of novel candidacidal and antifungal agents, such as caspofungin and micafungin (Denning, 2003; Sukhithasri et al., 2013). Due to their weak intrinsic immunogenicity, *C. albicans* β-glucans have received very little attention as fungal antigens, however, specific humoral responses directed against these β-glucans can be generated in animal models upon suitable immunization conditions (Bromuro et al., 2002; Uchiyama et al., 2000). Intriguingly, polyclonal or monoclonal antibodies and peptides targeting β-glucans have been shown to cause a marked candidacidal effect in vitro and to confer a significant protection against experimental mucosal or disseminated candidiasis (Magliani et al., 2004; Torosantucci et al., 2005). These responses may confer remarkable anti-*Candida* protection, opening the way to a potential use of β-glucan conjugates as vaccines (See Chapter 4). Indeed, a study by Uchiyama demonstrated that solubilized cell wall β-glucan from *C. albicans*, is an epitope of *Candida* immune mice (Uchiyama et al., 2000). While adding β-glucans to the list of the few potentially protective fungal antigens so far described, the overall body of novel biological findings calls for a well-established characterization of the molecular structure of these compounds and of their organization in the cell wall *C. albicans* and other fungal species (Iorio et al., 2008).

Other Therapeutic Use of β-glucan

Addition of new areas of application of β-glucans as immunomodulatory and therapeutic compounds opens new interesting perspectives and makes the study of β-glucan chemical and biological properties a prospective field of research. For instance, β-glucans have been known to promote wound healing, and β-glucans have been traditionally used as vehicles for fibroblast formulations or as bandage-like coverings for burns and autograft sites (Roy et al., 2011). Recently, in a rat wound model, β-glucans were shown to markedly increase leukocyte recruitment to the wound site, which is essential for an effective wound healing response (LeBlanc et al., 2006). The creation of gels or lattices based on β-glucans has also been proposed for various utilizations for wound healing by stimulating early macrophage recruitment and activation, as well as collagen deposition in the wound area (Ley, 2002). Clinically, β-glucans have been reported to be generally safe and well tolerated, as well as efficient in attenuating the rate of post-operative infection (Babineau et al., 1994b). Collagen matrices with β-glucans have been shown to improve burn wound healing and reduce pain (Delatte et al., 2001), however the molecular mechanisms underlying the effect of β-glucans on wound healing are poorly described. Dermal fibroblasts react to β-glucans by producing interleukin-6 (IL-6) and increased proliferation, which is beneficial for restoring the dermal extracellular matrix and wound healing (Kim et al., 2012; Kougias et al., 2001). In addition, β-glucans induce cytokine production and the release of reactive oxygen species (ROS) by macrophages and dendritic cells (Tsoni and Brown, 2008a; van den Berg et al., 2014), which is thought to be beneficial for neutrophil infiltration, angiogenesis and wound healing (Davis and Perez, 2009). Re-epithelialization of the wounded area is achieved by keratinocyte (KC) migration and proliferation (Martin, 1997). Additionally, β-glucans appear suitable for use in nanomedicine for preparation of natural nanocarriers for drug or biological molecule delivery, and have also been recently used as vaccine adjuvants for enhancing the immune response of vaccines (Huang et al., 2010; Soto et al., 2012).

Non-digestible β-glucans, form a remarkable portion of cereals, mushrooms and yeast and these natural materials facilitate bowel motility and can be used in amelioration of intestinal problems, particularly severe constipation (Battilana et al., 2001; Dongowski et al., 2002). Non-digestible β-glucans present in these substances are also able to modulate mucosal immunity of the intestinal tract (Tsukada et al., 2003). In the central nervous system, β-glucans activate microglial cells that

act as scavengers of the brain cell debris (Muller et al., 1994). The activation of these cells improves the prognosis of neurodegenerative diseases such as Alzheimer's disease and multiple sclerosis (Haga et al., 1989). Furthermore, several β-glucans have been found to be effective in improving glucose metabolism, yet the exact underlying mechanisms of action are still not fully understood. Both oat and fungal β-glucans were demonstrated to reduce blood glucose levels after oral administration in animal experiments and clinical trials, (Barsanti et al., 2011; Lo et al., 2006; Weickert and Pfeiffer, 2008). A study proposed that the β-glucans blood glucose lowering effect is possibly mediated by delaying stomach emptying so that dietary glucose is absorbed more gradually (Hlebowicz et al., 2008). β-glucans reduce the feeling of hunger accompanied by rapid decrease in blood glucose, and thus, maybe used to decrease appetite and reduce food intake (Regand et al., 2009). Moreover, yeast derived β-glucan is able to absorb mycotoxins (such as deoxynivalenol and patulin), probably through hydrogen bonds and van der Waals forces; this β-glucan effect is important particularly for livestock (Novak and Vetvicka, 2008).

In conclusion: possible effects of β-glucans on macroorganisms are thus very diverse and impinge upon not only the immune system, but probably in most of the described activities, are in some form more or less connected with that system.

β-glucan Certifications

Nevertheless, the majority of β-glucans have not been clinically applied and/or not clinically approved (except in China and Japan) with appropriate statistics. Mushrooms, yeasts, fungi, as well as algae have been used as foods products all over the world for thousands of years; thus, these materials may not cause serious problems for human health. Indeed, curdlan, has been applied as an ingredient of various foods for many years, and the safety of curdlan has been approved by the Food and Drug Administration in the US. Moreover, lentinan from *Lentinus edodes* (Taguchi et al., 1983) and Sonifilan (SPG) from *Schizophyllum commune* (Fujimoto et al., 1983) were developed in the 1980s and have been clinically approved and used for cancer therapy in Japan. β-Glucans derived from yeasts, such as *Saccharomyces cerevisiae* and *Candida albicans*, have been prepared and used for basic as well as clinical studies. Double blind clinical trials of yeast β-glucans have been examined extensively for cancer immunotherapy, as immunopotentiators for immunocompromised patients, and for lipid lowering effects since the 1990s (Babineau et al., 1994a; Babineau et al., 1994b; Dellinger et al., 1999). The influence of certain cereals (e.g., barley and oats) and edible mushrooms (e.g., *Grifola frondosa*, *L. edodes*) on

decreasing levels of serum cholesterol and liver low-density lipoproteins, which leads to lowering of arteriosclerosis and heart disease hazards, is also mediated by β-glucan present in these foods. Accordingly, β-glucans are FDA (Food and Drug administration)-approved for cholesterol lowering. The FDA has also approved a health claim that oat and barley β-glucans at a level of 3 gm per day (3g/day) may reduce cholesterol and lower risk of coronary heart diseases (Barsanti et al., 2011).

Conclusion

The glucan field is confusing as each type and source of glucan can be of widely varying quality and purity, and may contain mixtures of different polymer chain structures with variability in the amount of polymer, degree of branching, and polymer chain length. Because these polymer variables are often influenced by the source of the glucan, many glucans are named according to their plant or microbial source. Unfortunately, it is not always clear to what extent such variation in structure, or the presence of contaminants in glucan formulations, may influence their immunomodulatory activity. This variability and lack of characterization is likely to have been a contributing factor holding back the development of glucan-based adjuvants. The proposed mechanisms of the immunomodulatory effects of β-glucans are discussed below.

1.6.4. Proposed Mechanisms for Immunomodulatory Action of β-glucans and a Possible Role for Dectin-1 in Mediating These Effects

β-glucans are known as immune boosters, and are included as food supplements in farmed animals, and have been assessed in a number of clinical trials, investigating protective effects in various cases including HIV and cancer patients, as well as protection against postoperative infections, all with promising results (Chen and Seviour, 2007; Tsoni and Brown, 2008a). Previous studies have shown that β-glucans mediate their anti-infective and anti-tumorigenic properties by possessing the ability to activate leukocytes by stimulating their phagocytic activity and the production of reactive oxygen intermediates and inflammatory mediators, including TNF-α (Czop, 1986; Ross et al., 1999; Williams, 1997; Williams et al., 1999). However, the mechanism of β-glucan-induced activity has not been elucidated precisely because β-glucan preparations are often contaminated with pathogen-derived components.

Binding of β-glucan to receptors, mainly Dectin-1, activates macrophages leading to several interconnected processes including increased chemokinesis, chemotaxis, migration of

macrophages to the site of infection, degranulation leading to increased expression of adhesion molecules on the macrophage surface, adhesion to the endothelium (Novak and Vetvicka, 2008; Schepetkin and Quinn, 2006). In addition, β-glucan binding also triggers intracellular processes, such as the production of respiratory burst after phagocytosis of invading cells formation of reactive oxygen species (ROS) and free radicals [hydrogen peroxide, superoxide radical, etc.], increasing of content and activity of hydrolytic and metabolic enzymes, and signaling processes leading to activation of other phagocytes and secretion of cytokines and other substances initiating inflammation reactions (Schepetkin and Quinn, 2006). It is important for the pharmacological effect of β-glucan that activated macrophages do not act only against the activator but also against any present antigen, microorganism or tumor cell. Due to the fact that mammals lack β-glucanases in their enzyme equipment, macrophages represent what is probably the only tool for liquidation of β-glucan in the body. Within the macrophages, the phagocytized β-glucan is degraded by an oxidative pathway (Nono et al., 1991).

Nitric oxide (NO) is produced in macrophages by inducible nitric oxide synthase (iNOS), and synthesis of this enzyme is triggered by binding of β-glucans to a PRR (possibly Dectin-1) on the macrophage surface (Novak and Vetvicka, 2008). Formed nitric oxide induces a cytotoxic effect upon tumor cells and shows distinct impact on many pathogens (James and Glaven, 1989; Stuehr and Nathan, 1989). On the other hand, it can also damage tissues and DNA; and high concentrations can cause septic shock. The sustained action of the activator induces expression of iNOS, and increased formation of NO results in vasodilatation of veins. The latter, in turn, brings about an intense drop of venous resistance and blood pressure (Vincent et al., 2000). As of now, such an effect of *β*-glucan has not been described, yet it is quite conceivable.

Dectin-1 probably plays a major role in β-(1,3)-glucan–mediated immunomodulation, although this has not yet been formally demonstrated *in vivo*. As we have seen in section **1.5.3**, signaling via Dectin-1 can induce a variety of cellular responses, many of which have long been known to be induced by β-glucans. It is very likely that beneficial effects of these polysaccharides, particularly their anti-infective activities, stem, at least in part, from the ability of Dectin-1 to mediate cytokine production (Dennehy and Brown, 2007). Furthermore, recent results using blocking monoclonal antibodies *in vivo* indicate that Dectin-1 may also be involved in the anti-cancer activity of β-(1,3)-glucans (Ikeda et al., 2007).

The Role of Dectin-1 in β-glucan–induced Immunomodulation

As the principal β-glucan receptor on leukocytes, it is likely that Dectin-1 plays a central role in the immunomodulatory activities of these carbohydrates. Many of the cellular responses triggered by Dectin-1 *in vitro,* correlate with the *in vivo* effects of β-glucans (Brown and Gordon, 2003). Indeed, defects in Dectin-1, and its signaling pathway, influence the inflammatory and adaptive responses induced by purified particulate β-glucans *in vivo* (LeibundGut-Landmann et al., 2007). In addition, studies using blocking monoclonal antibodies have also demonstrated that Dectin-1 contributes to the anti-cancer activity of these carbohydrates (Ikeda et al., 2007). Although not yet examined experimentally, it is likely that Dectin-1 will also be found to contribute to the anti-infective properties of β-glucans (Dennehy and Brown, 2007). In addition to β-glucan-induced immunomodulation, Dectin-1 has been identified as a target for immunization and has been shown to promote antibody and $CD4^+$ and $CD8^+$ T cell responses to model antigens (Carter et al., 2006).

1.6.5. Mucosal/Intestinal Absorption/Uptake and Pharmacokinetics of Orally-administred β-glucans

Due to their immunomodulatory role, β-glucans have been long used as nutritional supplements that boost the immune system and increase resistance against infectious diseases, as well as promote antitumour activity. Most published *in vivo* studies have focused on describing the effects of parenteral administration of β-glucans injected via intraperitoneal [IP], intravenous [IV], or subcutaneous [SC]) routes (Novak and Vetvicka, 2008). However, there is a small, but growing literature, which indicates that β-glucans may be orally effective as well. It is also necessary, for potential clinical practice, to evaluate oral delivery. A few studies have demonstrated the effects of orally ingested β-glucans in experimental animals, and there have been numerous reports of immunomodulatory responses associated with oral ingestion of mushroom and yeast-derived β-glucans, both in water-soluble and particulate forms (Murphy et al., 2010). For instance, Sakurai *et al.* reported that orally inoculated SSG β-glucan from *Sclerotinia sclerotiorum* augmented the phagocytic activity and IL-1 production of alveolar macrophages in mice (Sakurai et al., 1992). Additionally, orally administered, natural β-glucans, such as lentinan (a water-soluble β-glucan, MW: 500 kDa), schizophyllan (a gel-forming β-glucan of MW 450 kDa), and WGP (yeast-derived 'whole glucan particle') have demonstrated immunopotentiating effects, as well as antitumor activity, and the former two glucans have been

long used in tumor immunotherapy (Batbayar et al., 2012; Hong et al., 2004). However these β-glucans are too large to be absorbed in the small intestine, and the mechanisms underlying the uptake of high molecular weight β-glucans into the intestinal lumen are unclear. In contrast to α-glucans, β-glucans are non-digestible due to the absence of the appropriate enzyme; therefore, β-glucans most likely reach the intestinal lumen intact.

A study by Rice *et al.* (2005) compared the fates of water insoluble, particulate, β-(1,3)-glucan, with water-soluble β-(1,3)-glucan after oral administration. The investigators found that unlike soluble β-glucan, levels of particulate β-glucan were undetectable in the blood suggesting that only soluble β-(1,3)-glucans are directly absorbed into the circulation (Rice et al., 2005). However, as discussed further below, a study by Hong *et al.* (2004) reported that particulate β-glucans in the gastrointestinal tract (GIT) are internalized by macrophages, which transport the glucan to various sites throughout the body, and slowly degrade the particulate β-(1,3)- glucans, releasing a bioactive soluble β-glucan product (Hong et al., 2004).

Tsukada *et al.* reported that the number of intraepithelial lymphocytes (IEL) in the intestine was increased by oral administration of β-glucan (Tsukada et al., 2003). Peyer's patches (PP) are aggregations of lymphoid tissue that are usually found in the small intestine in humans, and microfold cells (M cells) are specialized epithelial cells located within the follicle–associated epithelium (FAE) of Peyers patches(Owen, 1999). M cells, being present at the portal sites of intestinal mucosa, continuously sample for pathogens, particulate antigens, and high-molecular weight macromolecules to mediate their transepithelial transport (Owen, 1999). Moreover, Peyer's patches of the intestinal mucosa contain DCs that extend dendrites (projections) across the epithelium to sample and capture antigens, as well as pathogens, present in the intestinal lumen (Donaldson et al., 2012). Possible mechanisms that have been suggested for intestinal absorption of orally administered β-glucans (both particulate and soluble) are either through their uptake and translocation by M cells, or through the projected tips of dendritic cells (DCs) in the follicle-associated epithelium of PP that recognize and capture β-glucans mainly via Dectin-1 on the surface of these DCs (Batbayar et al., 2012; Roy and Klein, 2012). The uptake of high-molecular-weight β-glucans by M cells is followed by their transport to innate immune cells in PP, mainly macrophages and DCs. The β-glucans engulfed by macrophages or DCs are then degraded in lysosomes and are either presented to intraepithelial lymphocytes present in the PP, or secreted as smaller soluble glucans into the PP. The low-molecular weight β-glucans

fragments released, are subsequently detected by PRRs (mainly Dectin-1 and CR3) on innate immune cells that are present in PP as well as in nearby and distant lymph nodes, to further activate appropriate immune responses. It has also been proposed that particulate and high-molecular weight β-glucans translocated via M cells sometimes avoid lysosome fragmentation during intestinal transepithelial passage and therefore arrive to the PP intact. M cells and mucosal DCs in the PP are therefore considered as key initiators of mucosal immunity against immunogens and microorganisms that invade the epithelial barrier. Intriguingly, particulate β-glucan have also been shown to be uptaken by GALT (gut-associated lymphoid tissue)-associated macrophages, which transport glucan to various sites throughout the body (lymph node, spleen, bone marrow) and slowly degrade the particulate β-glucans, most likely via oxidative pathway, and release a bioactive, smaller soluble glucan of about 25 kDa (Chan et al., 2009).

Orally ingested water-soluble β-glucans, similar to particulate glucans, have been shown to mediate immunomodulatory effects depending on their size and other physico-chemical parameters as described above (Chan et al., 2009; Chen and Seviour, 2007; Murphy et al., 2010). Moreover, it has been suggested that dietary administration of β-glucans is as effective as parenteral administration for potentiation of systemic immunity and protection against pathogens (Volman et al., 2008). Data from the oral delivery of fluorescently labeled, water-soluble β-glucans in rats showed that the maximum plasma glucan concentration occurred at approximately 4 hours, and by 24 hours more than 73% was eliminated from the plasma, depending on the β-glucan source (Batbayar et al., 2012). It has been proposed that soluble β-glucans interact with either intestinal epithelial cells and/or intestinal DCs, ultimately resulting in the priming or activation of other immune cells. Indeed, a study by Rice *et al.* demonstrated that orally administered water-soluble glucans have been shown to translocate from the gastrointestinal (GI) tract into the systemic circulation by binding to both the GI epithelium and gut-associated lymphoid tissue (GALT) cells, and this uptake process is not necessarily Dectin-1-dependent (Rice et al., 2005). This oral delivery and GIT absorption of soluble β-glucans also stimulated increased protection against infection, through the modulation of the expression of PRRs in the GALT cells, and increased production of cytokines such as IL-12 (Rice et al., 2005). Although the movement of soluble glucan from the GI tract into the systemic circulation mainly depends on the presence of Dectin-1 positive cells in the GALT, intestinal epithelial cells not expressing the receptor were also observed to uptake and internalize labeled glucan.

Sandvik *et al.* reported successful detection of plasma β-glucans in rats after oral administration of soluble *Saccharomyces cerevisiae*-derived β-(1,3) (1,6)-glucan for 14 days. However, only a minute fraction of a single oral dose was translocated to the plasma. They proposed that the mucosal DCs sample or interact with soluble β-glucans locally via projections across the mucosal epithelium, and then migrate via afferent lymphatics to the mesenteric lymph nodes, where immune modulation is initiated (Sandvik et al., 2007).

An interesting study by Rice *et al.* (2004) (compared the pharmacokinetics of three well-characterized water-soluble β-(1,3)-glucans (namely, laminarin, sceleroglucan and glucan-phosphate) with varying physicochemical characteristics (molecular size, branching frequency, and solution conformations) following oral administration (Rice et al., 2005). It is interesting to note that the largest glucan studied, sceleroglucan (MW >1000 kDa), despite its high molecular weight was completely eliminated from the serum in 24 hours, while smaller glucans remained. Interestingly the physicochemical properties of these 3 glucans vary considerably, and these three glucans have been demonstrated by previous studies to display different bioactivity. Despite that these three glucans have been shown to bind to Dectin-1, laminarin doesn't stimulate innate immunity, whereas sceleroglucan and glucan-phosphate enhance immune function (Mueller et al., 2000; Williams et al., 1999). This data suggests that the *in vivo* pharmacokinetics could affect the bioactivity of β-(1,3)-glucans.

However, the exact mechanisms of the antitumor and anti-infective activities of orally ingested soluble β-glucans through mucosal immunity appear to be complex, and additionally individual β-glucans differ in their effectiveness as immunomodulators. These aspects of β-glucan require extensive investigations on the mechanisms of the antitumor, anti-infective and generally immunomodulatory activities of β-glucan. Nevertheless as highlighted above, many studies imply Dectin-1 as a key factor for the immunodulatory response to β-glucans (Batbayar et al., 2012). In fact, it has been suggested that controlling the level of expression of Dectin-1 might enhance protective effects and improve therapeutic efficacies in the case of infection and cancer respectively (Harada and Ohno, 2008). Therefore, understanding the mechanisms of β-glucan recognition by Dectin-1, as well as further characterization of β-glucan-mediated Dectin-1 signaling pathways, could provide valuable mechanistic insights into the immunomodulatory and biological effects of β-glucans.

1.6.6. Biological Activities of Particulate Versus Soluble β-glucans

β-glucans have been reported to function as a potent adjuvant to stimulate innate and adaptive immune responses. However, as described above, β-glucans from different sources have different physicochemical properties (e.g. structure, and conformation), and thus biological activity. Different preparations of β-glucans, soluble versus particulate, further complicate their mechanism of action. Structure has an impact on the water solubility of β-glucans.

Although both particulate and soluble forms of β-glucans have demonstrated macrophage-activating ability, the immunomodulatory potential and biological activities of water-soluble and particulate β-glucans are still controversial (Batbayar et al., 2012; Cleary et al., 1999; Gallin et al., 1992; Williams et al., 1992; Williams et al., 1991). As described above in section 1.5.3, triggering of Dectin-1 by β-glucans activates phagocytosis, respiratory burst, and the production of cytokines in both macrophages and DCs (Reid et al., 2009). In general, large particulate β-glucans, such as insoluble macroparticulate curdlan and zymosan, are the most potent β-glucans in activating Dectin-1–mediated cellular responses, and demonstrate robust anti-infective and immunomodulatory properties both *in vitro* and an *in vivo* (Tsoni and Brown, 2008a). It was recently noted that particulate β-glucans have stronger immunostimulating activities than soluble ones (Goodridge et al., 2011; Qi et al., 2011). Qi *et al.* (2011) reported that orally administered, yeast-derived, particulate β-glucan activated DCs and macrophages via the Dectin-1 pathway in mice. They also reported that although water-soluble β-glucan binds to DCs and macrophages, it does not activate these cells (Qi et al., 2011). In a recent report by Goodridge *et al.* (2011) that compared particulate and soluble β-glucans, only particulate β-glucans, such as whole glucan particles (WGP) and zymosan, induced Dectin-1-dependent activation of innate immune cells, including phagocytosis and induction of TNF-α, IL-6, and reactive oxygen species (ROS) from bone marrow-derived macrophage and DCs (Goodridge et al., 2011). Accordingly, the authors of this study proposed that Dectin-1 signaling is activated only by particulate β-glucans, not by soluble β-glucans. They suggested a model mechanism, which includes the formation of a "phagocytic synapse" between Dectin-1 on bone marrow-derived macrophages and β-glucans. In this model, CD45 and CD148 tyrosine phosphatases are excluded from the clustered sites of Dectin-1 induced by particulate β-glucan, thereby enabling downstream signaling from Dectin-1 (Figure 16). In contrast they proposed that soluble β-glucan, despite binding to Dectin-1, cannot induce clustering of Dectin-1 nor exclusion CD45 and CD148 around Dectin-1, and thus signal

propagation fails (Goodridge et al., 2011; Goodridge et al., 2012). This model could explain how innate immune receptors distinguish direct fungal contact with β-glucans on fungal microbes from soluble β-glucans shed by fungi in remote sites of the body (Goodridge et al., 2011; Goodridge et al., 2012). In addition to these studies, β-(1,3)-glucan particles from *S. cerevisiae* have been reported to elicit strong humoral and cellular responses to antigens entrapped in glucan particles in mice, demonstrating their possible utility as a vaccine delivery tool (Huang et al., 2010). Particulate β-glucan isolated from *S. cerevisiae* also induced TNF-α in macrophages isolated from murine wounds (Roy et al., 2011).

In contrast to particulate β-glucans, there have also been many reports on immunomodulation by water-soluble β-glucans (Fang et al., 2012) showed that water-soluble β-glucan from *Grifola frondosa* (an edible mushroom), with a molecular weight of 300 kDa, strongly induced TNF-α and IL-6 production, activation of SYK and NF-κB signaling in peritoneal macrophages, and inhibition of sarcoma-180 growth in mice (Fang et al., 2012). Masuda *et al.* (2012) showed that highly purified, soluble β-(1,3)/(1,6)-glucan obtained from *Grifola frondosa* rapidly induced GM-CSF production through Dectin-1-independent ERK and p38 MAPK activation. Subsequently, β-glucan induced GM-CSF-enhanced proliferation of resident macrophages and Dectin-1 expression, which permitted Dectin-1-mediated TNF-α induction through the SYK pathway (Masuda et al., 2012). In addition, soluble yeast β-(1,3)-glucan itself was reported to induce the production of significant amounts of IL-8. This glucan had a strong synergistic effect on LPS-induced secretion of IL-8 and IL-10 (Engstad et al., 2002). A recent study by Hino *et al.* (2012) reported that macrophages released soluble glucans into the medium after phagocytizing insoluble β-glucan particles (Hino et al., 2012). Interestingly, these particles were fragmented by ROS produced by macrophages, and that the released soluble β-glucan was reactive to Dectin-1 and biologically active in terms of macrophage activation (Hino et al., 2012).

1.7. Thesis Overview

1.7.1. Rationale and Hypothesis

In the past two decades, invasive fungal infections have emerged as a major threat to human health especially in immunocompromised patients, whose numbers are increasing due to the advent of HIV, organ transplantations, and the use of immuno-suppressive therapies for autoimmune diseases and cancer. Fungal infections are ranked the seventh most common cause

of infection-related death in North America. Furthermore, current drug regimens are inefficient in curing patients with fungal infections. While fungal infections have been currently recognized as a global and public health problem, little is known about our anti-fungal immune responses. **It is thus a healthcare priority and an exciting challenge to understand at the molecular level the mechanisms that our immune system utilizes to fight fungal infections. Therefore, the main goal of my Ph.D. study was to elucidate such molecular mechanisms.**

To achieve this goal, I have been particularly interested in studying the first and crucial step of this process, which is the recognition of specific components of the fungal pathogen by pattern recognition receptors (PRRs) expressed on the surface of innate immune cells. *This thesis focuses on studying the molecular mechanisms of Dectin-1 activation, the pattern recognition receptor (PRR) that plays a central role in antifungal immunity*, *and which detects β-glucans that are fungal PAMPS present in the fungal cell wall.* Although a lot has been known about the Dectin-1 signaling pathway in the recent years, *the exact molecular mechanism of how Dectin-1 is activated by β-glucans to initiate intracellular signaling events is not* yet known *and needs to be elucidated.* Accordingly, **the main objective** *of this study was to determine such molecular mechanisms by which Dectin-1, following its first encounter with the fungal pathogen, is able to activate the underlying signaling events to trigger an appropriate immune response against the pathogen.*

Previous studies have shown that depending on the size of the β-glucan recognized, Dectin-1 could activate, block or modify receptor signaling (Adams et al., 2008; Goodridge et al., 2011; Xie et al., 2010). While it is known that β-glucans of varying molecular weight have different efficacies, the reasons why are unclear. I therefore, hypothesize that upon binding of fungal particles, Dectin-1 receptors cluster and assemble into multimeric complexes, where Dectin-1 becomes tyrosine phosphorylated intracellularly. These phosphotyrosines act as docking sites for recruitment and activation of SYK (Spleen tyrosine kinase), and other signaling molecules, which in turn trigger the subsequent signaling events. Therefore, I propose a **"Clustering Model"** for Dectin-1 activation by its ligands.

1.7.2. Thesis Outline and Specific Study Objectives

The main aim of this thesis was to dissect and deepen our understanding of the molecular machinery underlying Dectin-1 activation and signaling in response to ligands of varying size. *To*

achieve this goal the specific objectives of this Ph.D. study were:

Aim 1: To examine the effect of Dectin-1 clustering and its activation (covered in *Chapter 3*).

Aim 2: To investigate the nature of the immune response produced by Dectin-1 clustering via targeting of Dectin-1 by a tricomponent vaccine conjugate containing oligovalent β-glucan (Covered in *Chapter 4*).

Detailed in *Chapter 1* of this thesis is a general introduction to innate immunity, fungal infections, and the mechanisms governing antifungal immunity including related Pattern Recognition Receptors (PRRs), as well as the rising threat of fungal infections to global and public, human health. This is then followed by an overview of the β-glucan receptor Dectin-1, with emphasis on its key role in antifungal immunity. *Chapter 1* finally closes with an overview of the physicochemical properties and beneficial effects of β-glucans as immunomodulatory agents. This is followed by *Chapter 2* (Materials and Methods) documenting experimental techniques and approaches, as well as developed protocols. Next, two result chapters (*Chapters 3 & 4*) follow, in which I illustrate, summarize and discuss major findings from my Ph.D. research project. Lastly, at the end of this thesis manuscript, I conclude with a general discussion of the biological relevance of results presented in the thesis, limitations of the study, as well as a final model of my results and future perspectives (*Chapter 5*).

Specifically in this thesis I have made several steps towards the objectives of my study listed above. I have exploited various experimental methods, including biochemical approaches, advanced microscopic techniques, together with cell biological and immunological techniques. These methods are covered in detail in *Chapter 2.* The effect of a panel of different-sized ligands on Dectin-1 activation and the underlying signaling pathway is described in *Chapter 3*. In this chapter, I sought to determine if differences in β-glucan size would affect Dectin-1 clustering, and in turn Dectin-1 signaling at the molecular level. *Chapter 4* examines the effect of laminarin-mediated targeting to Dectin-1 in dendritic cells on inducing Dectin-1 clustering to promote adjuvanticity and enhance immune responses to an anti-*candida* vaccine conjugate. This chapter provides an excellent example and application of how Dectin-1 clustering can induce, as well as augment a desirable immune response against the fungal pathogen. *Chapter 5* summarizes the general ideas, limitations of these studies, in addition to providing directions for future discoveries. I was able to analyze and extract valuable insight into the molecular machinery

underlying Dectin-1 activation and how the activation of Dectin-1 by clustering could be translated into appropriate cellular and immune responses. Finally, findings from my work, as well as from other published data in the literature, are combined in a general model for Dectin-1 activation and signaling.

Chapter 2. MATERIALS AND METHODS

2.1. Antibodies and Reagents

2.1.1. Antibodies

Rabbit anti-phospho SYK (Tyr525/526), rabbit anti-phospho SYK (Tyr352) and rabbit anti-phospho Src (Tyr352) were purchased from Cell Signaling Technology. Monoclonal anti-p65 NF-κB was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and mouse anti-actin was from Sigma-Aldrich (St. Louis, MO). Goat anti-rabbit IgG-horseradish peroxidase, goat anti-mouse IgG-horseradish peroxidase, and fluorescently conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Fluorescently labeled antibodies used for flow cytometry were from eBioscience (San Diego, CA) and included those specific for CD11c, CD11b, CD80, CD86 and F4/80. Anti-human Dectin-1 was from R&D systems (Minneapolis, MN) and anti-mouse Dectin-1 was from AbD Serotec (AbD Serotec). Ultra-pure LPS (Lipopolysaccharide from Escherichia coli 0111:B4, γ- irradiated) was purchased from Sigma Aldrich. Recombinant mouse IL-4 was from eBioScience. Laminarin (soluble β-glucan from Laminaria digitata) and Curdlan (from Alcaligenes faecalis) were both from Sigma-Aldrich.

Antibodies against the following epitopes were used for immunostaining: rat IgG2a isotype control from AbD Serotec (Raleigh, NC, USA); P-SYK at Y352 (#2701) from Cell Signaling Technology (Danvers, MA, USA); HA epitope (HA.11 clone 16B12, #MMS-101R) from Covance (Princeton, NJ, USA); mouse anti-human Dectin-1 (#MAB1859) and goat anti-human Dectin-1 (#AF1859) from R&D Systems (Minneapolis, MN, USA); (NFκB p65 (#sc-8008) from Santa Cruz Biotechnology (Dallas, TX, USA). Antibody against global tyrosine phosphorylation anti-P-Y (clone 4G10) was obtained from EMD Millipore, Canada (a division of Merck KGaA, Darmstadt, Germany).

Antibodies against the following epitopes were used for immunoblotting: HA epitope (HA.11 clone 16B12, #MMS-101R) from Covance (Princeton, NJ, USA); anti-P-Src at residue Y416 and other P-Src Family Kinases at the equivalent residue (#2101), P-SYK at residues Y525/526

(#2711), P-SYK at Y352 (#2701), P-PLCγ2 at Y579 (#3874), P-PKCδ at Y311 (#2055), P-c-Raf at S338 (#9427), P-p44/42, MAPK at T202/Y204 or equivalent (#4370), P-IKKα/β at S176/180 or equivalent (#2697), IκBα (#4814), P-IκBα at S32 (#2859), P-NF-κB p105 at S933 (#4806), P-NF-κB p65 at S536 (#3033), and P-RelB at S552 (#5025) from Cell Signaling Technology (Danvers, MA, USA); P-SYK at Y525/526 (#PK1010), P-SYK at Y323 (#07-915) and Actin from EMD Millipore (Billerica, MA, USA); rat anti-mouse Dectin-1 (#MAB17561) and goat anti-human Dectin-1 (#AF1859) from R&D Systems (Minneapolis, MN, USA); Actin (#sc-1616-R) from Santa Cruz Biotechnology (Dallas, TX, USA); β-Tubulin (#T5201) and Caveolin-1 (#C4490) from Sigma-Aldrich (St. Louis, MO, USA). HRP-, Cy3- and DyLight- coupled secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA), AF488- coupled secondary antibodies were from Life Technologies (Carlsbad, CA, USA), and IRDye-coupled antibodies were from LI-COR Biosciences (Lincoln, NE, USA).

2.1.2. Miscellaneous Reagents and Inhibitors

Unless stated otherwise, general laboratory reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Inhibitors were purchased from Abcam (Cambridge, MA, USA). PP2, Piceatannol, and BAY 61-3606 were purchased from EMD Millipore (Billerica, MA, USA). Alexa Fluor (AF) 546 and Alexa Fluor (AF) 488 were obtained from Invitrogen. Pharmacological Inhibitors were purchased from EMD Millipore (Billerica, MA, USA) (previously known as calbiochem), unless otherwise stated.

2.2. Stable Cell lines and Primary Cell Culture

2.2.1. Exogenous Expression of Dectin-1 Constructs in RAW 264.7 Macrophages and HeLa Cells

The mouse macrophage cell line RAW 264.7 cells (ref. TIB-71, American Type Culture Collection, Manassas, VA) were maintained in Alpha MEM medium (Gibco) with 10 % heat-inactivated serum (Wisent Bioproducts, St Bruno, Quebec, Canada). To generate RAW 264.7 cells stably expressing human Dectin-1, the open reading frame of human Dectin-1 (GenBank NM_197947) was subcloned into the pFB-Neo retroviral vector (Stratagene, Agilent Technologies, Santa Clara, CA) containing the neomycin-resistance gene (neor) for selection by G418. The pFB-neo Dectin-1 vector was transfected together with the pVPack-GP and pVPack-

VSV-G vectors in HEK 293T cells (ATCC CRL-11268) allowing the formation and secretion of non-reproductive Moloney murine leukemia virus (MMLV) that are next incubated with RAW 264.7 cells for 24 h before addition of growing media containing 1 mg/mL of G418. After 2 weeks of selection, Dectin-1 expression was confirmed by immunofluorescence and Western-blot analysis and the generated cell line of murine macrophages stably expressing Dectin-1 was further designated as 'RAW Dectin-1 Cells'. On occasion, the human cervical adenocarcinoma cell line HeLa (no. CCL-2, ATCC, Manassas, VA, USA) was cultured similarly to RAW 264.7 cells, except in DMEM (Life Technologies) containing 10% fetal bovine serum (Wisent Bioproducts, St-‑Bruno, QC, Canada). HeLa cells retrovirally transduced with the pFB-Neo-Dectin-1 construct (as above) containing an additional hemagglutinin epitope (HA) tag were termed "HeLa Dectin-1-HA". Experiments on live cells were performed in HEPES-buffered RPMI media (HRPMI or hRPMI, Wisent Bioproducts) or serum-free MEMα (Life Technologies). Short washes were performed with PBS solution. Similarly, a cell line of HeLa cells stably expressing Dectin-1 (HeLa Dectin-1) was developed.

The murine leukemic monocyte/macrophage cell line RAW 264.7 (ATCC no. TIB-71, American Type Culture Collection, Manassas, VA, USA) was maintained in MEMα (Life Technologies) supplemented with 10% heat inactivated fetal bovine serum (Wisent Bioproducts, St.Bruno, QC, Canada) under a humidified 5% (v/v) CO2 atmosphere at 37°C. The growth medium was replaced every 2-4 days to maintain optimal viability. Confluent cells were detached with 0.25% trypsin-1 mM EDTA (Life Technologies) and subcultured at a ratio between 1:20 to 1:5. Cells were not cultured beyond 20 passages. To prepare for fluorescence microscopy experiments, cells were plated on top of sterilized 18 mm #1½ coverslips (Electron Microscopy Sciences, Hatfield, PA, USA) the day before. For RNA interference and electroporation protocols, cell density was determined with a hemocytometer under the light microscope.

The stable expression of full-length human Dectin-1 in RAW 264.7 cells via the pVPack vector retroviral transduction system (Stratagene, Agilent Technologies, La Jolla, CA, USA) was previously described (Lipinski et al., 2013). Briefly, the open reading frame encoding human Dectin-1/CLEC7A isoform A (Genbank accession no. NM_197947.2) was subcloned into the pFB- Neo vector, which also encodes a gene product permitting resistance to the antibiotic G418 (neoR). Next, replication-incompetent Maloney murine leukemia viral particles containing the construct were generated and allowed to infect RAW 264.7 cells. Transduced cells were selected

by the addition of 1 mg/mL G418 (Life Technologies) to the growth medium. The resulting cells were termed **"RAW Dectin-1"** to distinguish them from the original RAW 264.7 cells, known as "RAW Wild-Type or **"RAW WT".**

2.2.2. Primary Cell Culture: BMDCs and BMDMs

2.2.2.1. Preparation of Primary Bone Marrow-Derived DCs (BMDCs)

C57BL/6J mice were kindly provided by the laboratory of Dr. Hanne Ostergaard (Department of Medical Microbiology and Immunology, U of A, Alberta), who purchased the animals from the Jackson Laboratory (Bar Harbor, ME). Mice were bred, housed in a viral-antigen-free animal at the Faculty of Medicine and Dentistry animal facility, University of Alberta (Heath Sciences Lab Animal Services, U of A, Edmonton, AB, Canada). All animal experimental procedures were approved by the Health Sciences Animal Care and Use Committee at the University of Alberta (Protocol Number 055) and conform to guidelines put forward by the Canadian Council on Animal Care (see preface of this thesis). Bone marrow–derived DCs (BMDCs) were prepared from bone marrow collected from wild-type (WT) C57BL/6 mice maintained at the Faculty of Medicine and Dentistry animal facility (U of A) as described above. BMDCs were prepared as previously described (Lutz et al., 1999), with minor modifications. Briefly, bone marrow cells (BMCs) were harvested from femurs and tibias of normal C57BL/6 mice and washed with PBS. To generate DCs, BMCs were resuspended in complete medium: RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FCS (Wisent Bioproducts), 100 U/ml penicillin (Life Technologies), 100 mg/ml streptomycin, 10 mM HEPES (Life Technologies), 0.1 mM nonessential amino acids (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 50 mM 2-ME (BioShop Canada), and 20% v/v supernatant from Chinese hamster ovary cells transfected with the plasmid pCDNA3 containing the mouse rGM-CSF gene (transduced Chinese hamster ovary cells secreting mouse rGM-CSF were generously provided by Dr. Kevin Kane, Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB, Canada). Cell suspension (10 ml) containing 2 x 10^6 BMCs was cultured in 100-mm–diameter dishes (day 0) and incubated for 10 d at 37°C in a humidified 5% (v/v) CO2 atmosphere. During the incubation period, cells were fed with fresh medium containing 20% GM-CSF supernatant on days 3 and 5. A total of 10 ng/ml mouse rIL-4 (eBioSciences) was added to the medium on day 6. On day 10 of culture, suspension and loosely adherent cells (immature DCs) were harvested and

resuspended in complete medium containing 10% (v/v) mouse rGM-CSF supernatant. Cell suspension was used for in vitro phenotypic analysis of immature DCs and for coculture for an additional 24 h with vaccine conjugates, or LPS.

2.2.2.2. Preparation of Primary Bone Marrow-Derived Macrophages (BMDMs)

BMDMs were generated upon extracting bone marrow cells of wild-type C57BL/6 mice (a generous gift from Dr. Shairaz Baksh of the Department of Pediatrics, University of Alberta, Edmonton, AB, Canada) by flushing femurs with 1 mL of growth medium (DMEM containing 10 % FCS and 1% Penicilin-Streptomycin). The cell suspension was recovered in a 15 mL tube and centrifuge for 5 min at 15000 rpm. The cell pellet was next resuspended in growth medium and directely plated onto plastic dishes. After 2 days, immature macrophages (non-adherent) were recovered from the supernatant and plated in another plastic dish and grown until confluency. L cell Conditioned Medium (LCM) was added on day 5 and then every second day. LCM medium was obtained by combining growth medium with 20 % of the supernatant of L929 cells grown to confluency. L929 cells produce M-CSF which promote the differentiation of murine macrophages. Cells were split by trypzination and then replated into petridishes as required for the experimental procedures.

2.3. Flow cytometry Analysis of BMDC Marker Expression

On day 10 of culture, analysis of surface marker expression (surface phenotype analysis) on immature DCs was performed before stimulation with ligands. Briefly, cells were pelleted by centrifugation and resuspended at a concentration of 2 x 10^7 cells/mL in cold FACS buffer (PBS containing 2 % FCS). Cell suspensions were then incubated on ice for 10 min with anti-FcRcII/III antibody (anti-mouse CD16/32, eBioscience) for Fc receptor blocking. Aliquots of 1 x 10^6 cells in 50 µl were then stained with a master mix of fluorochrome-conjugated antibodies to stain for the various DC markers described in Table 4 (*Chapter 4*), or with a mix of corresponding IgG isotype controls. Briefly, antibodies used were from eBiosciences (Table 4) and are as follows: PE-Cy7 conjugated anti-CD11c; FITC-conjugated anti- CD86 (B7-1); FITC-conjugated anti-F4/80; PE-conjugated anti-CD80 (B7-2), eFluor 450 conjugated anti-MHC II (I-A/I-E); allophycocyanin (APC)-conjugated anti-mouse Dectin-1. Isotype controls were done with similarly conjugated IgG. One aliquot of cells was left unstained for use as a control. After incubation with the antibody mix for 30-40 minutes at 4°C in the dark, cells were washed twice

and resuspended in cold FACS buffer, and samples were acquired on an LSR II cytometer (BD Biosciences, San Jose, CA). Compensation was performed before each data collection using BD Bioscience anti- Rat/Hamster Ig κ/Negative control compensation particles set according to manufacturer's recommendations. Gating was performed for elimination of dead cells. Software analysis of flow cytometric data was performed on FSC3 Express from De Novo software (Los Angeles, CA).

2.4. DNA Constructs

2.4.1. Sources

DNA constructs encoding fusion protein of mEmerald, mEOS or PAmCherry to a ten amino acid linker, and human Dectin-1 (Genbank no. NM_197947.2) at the C-terminus, were a generous gift from Michael Davidson (Optical Microscopy Division, National High Magnetic Field Laboratory, Florida State University) and termed mEmerald-Dectin-1-C10, mEOS2-Dectin-1-C10 and PAmCherry-Dectin-1-C10, respectively. Plasmids encoding GFP- or HA-tagged cDNAs (DNA construct for HA tag was purchased from Biomatik, Ontario) for proteins of interest were used for fluorescent microscopy experiments. SYK-GFP, CARD9-myc, BCL-10-FLAG DNA constructs were purchased from Addgene. PLC-gamma-PH was a kind gift from Dr. Sergio Grinstein (The Hospital for Sick Children, Toronto, ON, Canada).

2.4.2. Amplification of DNA Constructs

Library Efficiency DH5α Competent E. coli cells (Life Technologies) were transformed with the plasmid of interest by heat shock as per the manufacturer's instructions. The cells were spread onto Miller's Luria-Bertani media (LB)-agar (BD Diagnostic Systems) plates supplemented with either [100 μg/mL] ampicillin or [50 μg/mL] kanamycin (Life Technologies), for selection of transformants, and grown overnight at 37°C. Isolated colonies were picked and grown for 8 hours in 2 mL of sterilized LB media (Difco, BD Diagnostic Systems, Quebec, QC, Canada) containing the appropriate antibiotic. Culture conditions were maintained at 37°C and continuous [250] rpm agitation. The entire culture was then inoculated into 250 mL of the identical media and grown for 16 hours under the same conditions. After pelleting the cells by ultracentrifugation, the cDNAs inside were purified with the Plasmid Maxi Kit (Qiagen) and resuspended in sterilized TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The concentrations of DNA in solution were

determined by measuring absorbance of 260 nm light with a Nanodrop 2000c UV-Vis Spectrophotometer (Thermo Scientific) and adjusted to between 1-2 µg/mL.

2.4.3. Transient Expression of DNA Constructs by Lipofection

RAW 264.7 and HeLa cells were seeded onto 18 mm glass coverslips and grown for 16-24 hours to attain 40-60% confluency. Prior to transfection, the cells were rinsed three times in sterilized PBS and replaced with Opti-MEM I Reduced Serum Media (Life Technologies) + 3% heat-inactivated fetal bovine serum. For each coverslip, a mixture was prepared in a sterile microfuge tube, with 1 µg of plasmidic DNA being added to 3 µL of Fugene HD (Promega) in 0.2 mL of Opti- MEM I. The mixture was resuspended and left for 30 minutes, after which the entire volume was added to the cells. Experiments were performed 16-24 hours later.

2.5. Preparation of Dectin-1 ligands (β-Glucans) and Vaccine Conjugates

Most of the Dectin-1 ligands described in this section have been kindly prepared by the laboratory of Dr. David Bundle (Department of Chemistry, University of Alberta, Edmonton, AB, Canada).

2.5.1. Soluble β-Glucans and BSA-laminarin Conjugates

2.5.1.1. Soluble β-glucan preparation

Laminarin (soluble β-(1,6) branched (β-(1,3)-glucan from *Laminaria digitata*) and Curdlan (linear (β-(1,3)-glucan from *Alcaligenes faecalis*) were both purchased from Sigma-Aldrich (Figure 19). Scleroglucan (produced by fermentation of the filamentous fungus *Sclerotium rolfsii*) was bought from invivoGen (Figure 19). Commercially available curdlan obtained from Sigma is largely insoluble. Accordingly, the chemists in the Bundle Lab, Dept. of Chemistry, U of A (mainly by Dr. Lipinski), phosphorylated curdlan to render highly soluble curdlan, which was later named “phospho-curdlan” or ‘P-curdlan’ for our study (Figure 19) (Further described below). Solubilized P-curdlan was then prepared in sterile eppindorff tubes as 10 mg/ml stocks in PBS or hRPMI, sterile filtered and stored frozen until use. Scleroglucan, a branched β-glucan, although considered partially soluble, was also aminated by the Bundle to enhance its solubility. (Figure 19). Wellmune WGP Soluble (WGPsol) manufactured by Biothera (Eagan, MN, USA) was obtained from Invivogen (San Diego, CA, USA) and from Quadra Ingredients (Vaudreuil-

Dorion, QC, Canada).

As mentioned above, Curdlan is highly water-insoluble. Therefore, to produce water-soluble curdlan that is largely soluble in working solutions (PBS and culture medium) that we use for Dectin-1 ligand stimulation experiments (described below), curdlan was phosphorylated by the Group of Dr. Bundle (U of A). To modify curdlan to a water-soluble form, Bundle and coworkers phosphorylated curdlan by reacting it with phosphoric acid, based on a procedure for preparing the soluble β-glucan phosphate (Williams et al., 1991). The resultant compound was termed phospho-curdlan (P-curdlan) (Figure 19). All β-glucans were solubilized in sterile PBS (Phosphate-buffered saline) or hRPMI medium (Gibco, Life Technologies, Grand Island, NY), sterile filtered, and stored as 10 mg/mL stock solutions until use. Briefly, the soluble β-Glucans P-curdlan, scleroglucan, WGPsol, and laminarin were dissolved in sterile, endotoxin-free PBS to 10 mg/mL and sterilized by passage through a 0.22 μm filter. The stock solutions were stored at 4°C (WGPsol) or -20°C (P-curdlan, laminarin, and scleroglucan) and their sterility was actively maintained. All β-glucan preparations were endotoxin-free and were used at a working concentration of 100μg/mL unless otherwise stated.

Soluble multivalent protein-β-glucan conjugates, where multiple (e.g. 17) laminarin molecules were covalently coupled to bovine serum albumin (BSA) as protein carriers, were also prepared by Bundle and coworkers and termed BSA-n-laminarin (e.g. BSA-17-laminarin). Dr. Bundle’s group also assisted in the generation of fluorescently labeled laminarin and BSA-17-laminarin (Figure 19). This was accomplished by reacting succinimidyl ester-Alexa Fluor (AF) 546 (Life Technologies) with amine groups present in the ligands, as previously described (Lipinski et al., 2013). As β-glucans do not contain amino groups, laminarin was chemically derivitized to place an amino group at its reducing end as described in (Lipinski et al., 2011). For the BSA-17-laminarin conjugate, primary amines were present in the protein component BSA (Figure 19).

2.5.1.2. Phosphorylation of Curdlan.

Phosphorylation of Curdlan in this section was performed by Dr.Lipinski and is adapted from our published paper that is reproduced for Chapter 4 (Lipinski et al., 2011). Curdlan (from *Alcaligenes faecalis*, Sigma-Aldrich) (1 mg), urea (18 mg) and formamide (50 mL) were placed

in a 500 mL round bottom flask with magnetic stirring bar mounted in a temperature controlled oil bath. The mixture was first heated to 130 °C to aid dissolution of curdlan. After complete dissolution the temperature was reduced to 100 °C. Phosphoric acid (85 %, 4 mL) was added in small portions over a 2 h period to avoid excessive foaming while maintaining rapid mixing. The reaction mixture was heated for 6 h after addition of the last portion of phosphoric acid. To the cooled mixture an equal volume of water was added and the solution was transferred to a dialysis bag. Dialysis was first performed against running tap water and then against milliQ water. A small amount of insoluble material was removed by centrifugation (20 min x 10,000 g) and purified phospho-curdlan was lyophilized.

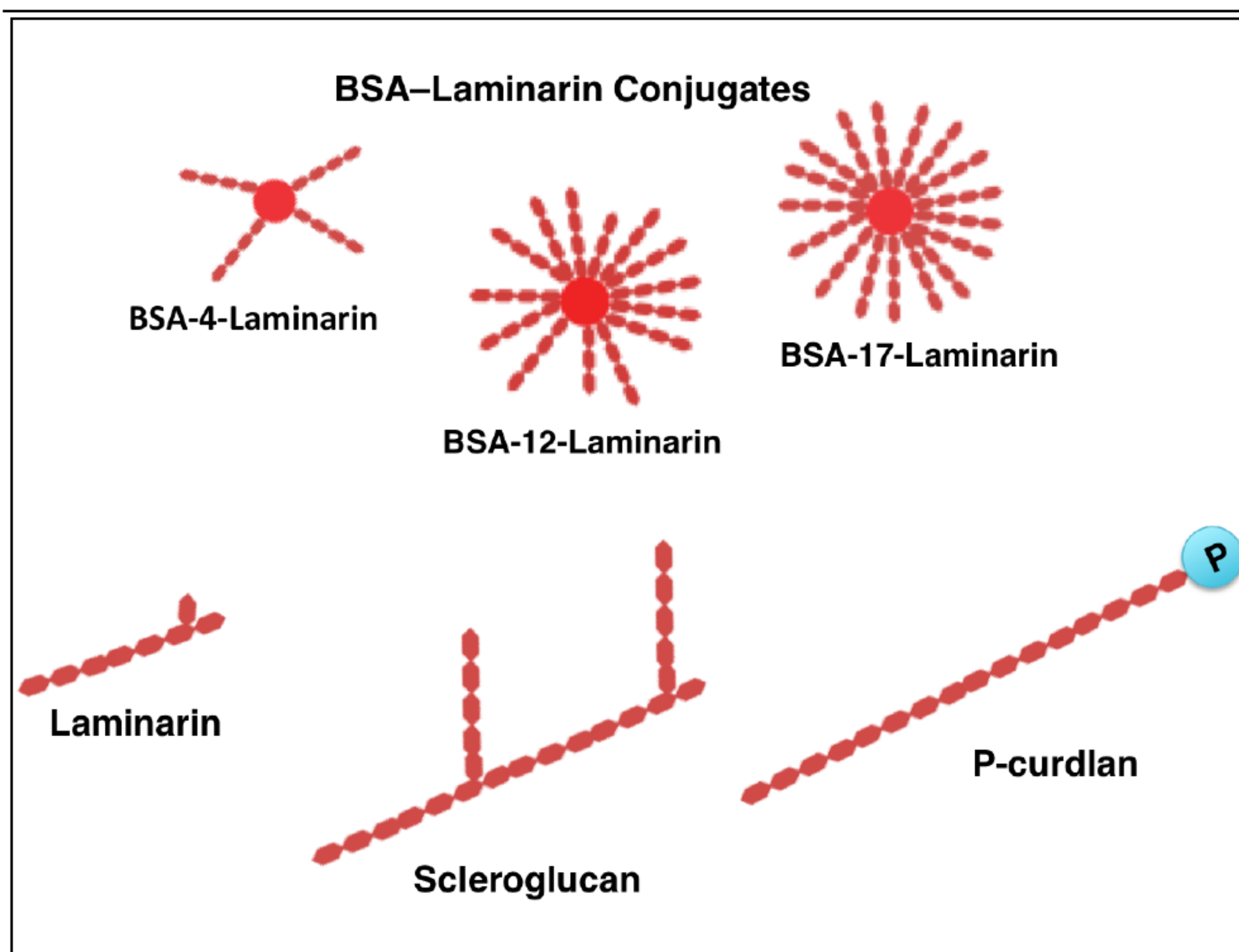


Figure 19: Dectin-1 Ligands Most Relevant to this Study

Above figure shows the various soluble β-glucan ligands that were used for this study. These ligands were mostly prepared, and chemically modified for solubility, by the laboratory of Dr. David Bundle (Department of Chemistry, University of Alberta, Edmonton, AB, Canada). Multivalent BSA-laminarin conjugates with different numbers (n) of covalently linked laminarin were prepared including ones with 4, 12 and 17 laminarin molecules. Laminarin and the BSA-n-laminarin molecules were also fluorescently labeled by the Alexa Fluor546/AF546) red dye for visualization by fluorescent microscopy (not shown in figure) (Figure drawn by Amira Fitieh).

2.5.1.3. Ligand Stimulation Experiments and Preparation of Soluble Ligands

Immediately prior to use, frozen stocks of the soluble β-glucan ligands, laminarin, scleroglucan and Phosphorylated–curdlan (P-curdlan) stored at 10 mg/ml were thawed in a waterbath or heat block at 37°C for 5-10 minutes, mixed on the vortex for 1 minute, and centrifuged at 18000x g for 2 minutes at 37°C to promote maximum dissolution of the compounds and to spin down any aggregates or insoluble components. They were then diluted to a working concentration of 100 µg/mL in warmed media for stimulation of the cells. For ligand stimulation RAW Dectin-1 cells were incubated at 37°C in MEMα lacking or containing soluble β-glucan ligands at 100 µg/mL for various timepoints. The cells were incubated at 37°C in hRPMI media lacking or containing soluble β-glucan ligands at 100 µg/mL for various timepoints. Next, cells were chilled on ice and washed three times in cold PBS and then further processed for either immunofluorescence or immunoblotting experiments.

2.5.1.4. Assessment of Ligand Solubility: Phenol Sulphuric Acid Method

As mentioned above in section **2.5.1.3**, β-glucans including laminarin, P-curdlan, and scleroglucan were suspended in PBS at a concentration of 10 mg/ml and centrifuged at 8000 x rpm for 10 min, and the supernatant was used assumed to soluble β-glucan. The final precipitate was resuspended in PBS washed three times and centrifuged as above, and the final precipitate was considered insoluble β-glucan. We wanted to to ensure that the β-glucans present in the supernatant fraction of the resuspended ligand solution were largely soluble. Neutral carbohydrate content/concentration in both fractions, soluble (supernatant) and particulate (precipitate), was determined by the *Phenol-Sulfuric Acid Method* (Dubois et al., 1951). *Phenol-Sulfuric Acid Method* is a simple and rapid colorimetric method to determine total carbohydrates in a sample (Masuko et al., 2005). It is a highly reliable and fast method amongst the quantitative assays for carbohydrate estimation, and it is mostly used in measuring neutral sugar content in oligosaccharides (Masuko et al., 2005). Briefly, in hot acidic medium (concentrated sulphuric acid) glucose is dehydrated to hydroxymethyl furfural. This forms a yellow-brown coloured product with phenol and has absorption maximum at 490 nm.

The *Phenol-Sulfuric Acid Method* was carried out by preparing a set of solutions with known glucose concentrations and mixing them with the phenol-sulphuric acid reagent. The absorbance

(OD/optical density) of each solution was then measured by a spectrophotometer at a wavelength of 490 nm and a standard curve was constructed by plotting the OD at 490 nm versus the concentrations of the standard glucose samples. Unknown sugar samples, in this case soluble and insoluble β-glucan fractions, were prepared in the same way as standard glucose samples, and their corresponding concentrations were derived from the standard curve. The efficiency of solubilization of the β-glucan sample was assessed by comparing the sugar/glucan content in the supernatant versus precipitate fractions. If the sugar content in the centrifugation supernatant is predominantly higher than that in the precipitate fraction, then the β-glucan suspended in the supernatant fraction is regarded as apparently highly soluble β-glucan. For phospho-curdlan (P-curdlan) and laminarin the carbohydrate content in the supernatant fraction was more than 90% higher than that in the precipitate portion indicating effective solubilization of these glucans in PBS. Finally, size distribution of each β-glucan species present in different samples was performed by chemists in the Bundle Group, Chemistry Dept., U of A.

2.5.2. Trisaccharide–Tetanus Toxoid (TS-TT) Vaccine Conjugates

Trisaccharide-Tetatanus toxoid (TS-TT) vaccine conjugates (used for *Chapter 4*) were entirely prepared by Dr. David Bundle and co-workers mainly, Dr Lipinski (Department of Chemistry, Faculty of Science, U of A) as described below. These vaccine conjugates were maintained at 4°C in complete HRPMI medium at a working concentration of 100 μg/ml (Figure 78). Techniques covered in this section were performed by Dr.Lipinski and are adapted from our published paper that is reproduced for Chapter 4 (Lipinski et al., 2011).

2.5.2.1. Propargylated Laminarin Preparation.

Laminarin (100 mg, soluble β-glucan from *Laminaria digitata*, Sigma-Aldrich, St. Louis, MO) was dissolved in 0.2 M phosphate buffer pH 6.0 (1 mL) in a 6 mL Kimball glass vial with magnetic stirring bar. Propargylamine hydrochloride (120 mg) was added followed by sodium cyanoborohydride (5 mg) and stirred in incubator at 37°C for 7 days. Additional portions of sodium cyanoborohydride (5mg each) were added on days 2 and 4. The reaction mixture was then diluted with water to 10 mL and precipitated with 4 volumes of ethanol. After centrifugation (7000 g x 20 min) the precipitate was dissolved in a minimal amount of water and passed through a Sephadex G-25 column (2.5 x 25cm). The column was eluted with water, fractions containing sugar were collected and lyophilized. Analysis led to the conclusion that 15 % of laminarin

molecules were substituted with a propargyl group. According to previously published results, only about 20 % of laminarin molecules in commercial preparations are available for derivatization via the reducing end since the majority of chains are capped by mannitol. Here, we found that 15 % of laminarin molecules were substituted with a propargyl group (data not shown).

2.5.2.2. Preparation of Laminarin-containing Tetanus-toxoid Vaccine Conjugates

Tetanus toxoid (36 mg) (State Serum Institute, Copenhagen) was azidinated (its amino groups were converted to azide groups) in 0.5 M carbonate/bicarbonate buffer pH 9.8 at a protein concentration ~20 mg/mL with 1.2 M excess of the azo transfer reagent, imidazole-1-sulfonyl azide hydrochloride (NOTE: This reagent must be treated with extreme care. There has been one report of a serious explosion during its preparation alternates and 2 mM CuSO4 as catalyst for 9 h with stirring on magnetic stirrer. The solution was diluted with 50 mL water containing 0.1 % Tween 20 (to prevent protein aggregation) and washed by ultradialysis using a TF membrane (Pellicon XL 50, 10kDa cutoff) with EDTA to remove copper, then with 0.15 M NaCl100 mM 4-methylmorpholine and concentrated to 18 mg/mL. The protein solution was transferred to a 4 mL Kimball vial, and 5.9 mg of trisaccharide (26 molar equivalents) was added followed by 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (16 mg). The progress of conjugation was monitored by TLC for consumption of trisaccharide. After ~5 h another portion of DMTMM (8 mg) was added and the pH adjusted to >8.0 by addition of 4-methylmorpholine. The reaction was complete when only trace amounts of unconjugated trisaccharide were detectable by TLC. The conjugate was dialyzed against 20 mM TrisHCl pH 8.5 and loaded on a DEAE Sepharose CL column equilibrated with the same buffer. Conjugate was eluted with 0.1 M TrisHCl pH 7.2, 1M NaCl. This step was performed to remove Tween present in the conjugate. The conjugate was fractionated on Superdex S-200 column (2x100 cm, PBS), material corresponding to a monomeric fraction was collected, concentrated and divided into two equal portions. The first portion constituted the reference vaccine–trisaccharide-tetanus toxoid (TS-TT).

The second portion of TS-TT (10 mg of protein) was conjugated with propargylated laminarin. Briefly, propargylated laminarin (12.5 mg) was dissolved in 0.2M TrisHCl buffer pH 8.0 containing conjugate (10 mg by protein content) in a 4 mL Kimball vial, copper powder (~ 20 mg) and isobutanol (50 µl) were added. The vial was closed with a septa and purged with argon.

Reaction was started by addition of bathophenantroline/Cu+1 catalyst (25 µl per 1 mL of reaction mixture). After conjugation the reaction mixture was filtered, dialyzed against PBS and purified on a Superdex S-200 column. This tri-component conjugate vaccine, trisaccharide-tetanus toxoid with covalently attached laminarin was designated TS-TT-Lam vaccine. The azide groups of both conjugates (TS-TT-Lam and TS-TT) were reduced back to amines by reaction with trimethylphosphine. Conjugates (10 mg/mL) in 0.5 M sodium carbonate were reacted with trimethylphosphine (50 µL of a 1M solution in THF) in a 4mL Kimball glass vials closed with a septa at room temperature, 18h. Conjugates were then dialyzed against PBS, concentrated and sterile filtered through 0.22 µm filters to yield solutions of TS-TT-Lam (2.7 mg/mL) and TS-TT (4.1 mg/mL). Labeling conjugates with Alexa Fluor 546. To the solutions of the tetanus toxoid conjugate vaccines in PBS (TS-TT and TS-TT-Lam) containing 400 µg of protein, 1/10 volume of 1 M sodium bicarbonate solution was added followed by Alexa Fluor 546 NHS ester (Molecular Probes, Life Technologies, Grand Island, NY) (20 µg) dissolved in DMSO (~ 30 µl). The tubes were wrapped in aluminum foil and left on an inverting mixer for 18 h. Purification was performed on PD-10 desalting columns (GE Healthcare) equilibrated with PBS. Fractions containing labeled conjugates were collected and concentrated on Amicon Ultra-4 Centrifugal Filter Units (10 kDa) to a final concentration of 2.5 mg/mL.

2.6. Cytokine Quantification in Culture Supernatants using ELISAs

For analysis of cytokine production, immature BMDCs were harvested on day 10 of culture and resuspended in complete RPMI 1640 medium containing 10 % (v/v) recombinant mouse GM-CSF supernatant. Cells were plated at 1x106 cells per well in a 12-well plate and stimulated with 100 µg/mL of vaccine conjugates (TS-TT or TS-TT-Lam conjugates), or with 100 µg/mL of Dectin-1 ligands (Phospho-curdlan or laminarin), or with 1 µg/mL of LPS. Cell culture supernatants were collected after 24 h incubation. Concentration of the cytokines IL-4, IL-6, IL-12p70, TNF-α and TGF-β1 in supernatant was assayed by pre-coated sandwich ELISA kits (R&D Systems, Minneapolis, MN). All of the samples were measured in triplicate according to the manufacturer's instructions. Statistical analysis was performed with Prism software using Student t-test (GraphPad Software, Inc.).

2.7. NF-κB Nuclear Localization/ Translocation

RAW-Dectin-1 cells were plated overnight onto glass coverslips in a 12 well plate. The following

day, cells were stimulated at 37 °C for 10 minutes with vaccine conjugates or soluble β-glucan ligands at 100 μg/mL in serum-free HEPES containing RPMI medium (hRPMI culture medium) (Wisent Bioproducts). After stimulation, cells were washed 3 times with warm PBS and fresh hRPMI media (without the ligands) was added for another 20 min to allow for NF-κB nuclear translocation. Cells were then fixed with 4 % paraformaldehyde (PFA), blocked and permeabilized with PBS containing 0.5 % Triton X-100 for 5 min. This was followed by immunostaining the cells with antibodies against the p65 subunit of NF-κB (anti-p65 Ab from Santa Cruz Biotechnology) for 30 min at room temperature (RT). Subsequent incubation with AF488- or Cy3-conjugated secondary antibodies (goat anti-mouse IgG conjugated to AF488 or Cy3) (Molecular Probes) was then performed for an additional 30 minutes at (RT). DAPI staining was simultaneously performed to label the cell nucleii by adding DAPI [2 μg/mL] to the secondary Ab solution. Following staining, coverslips were washed, mounted with DAKO and p65 nuclear localization was examined by confocal microscopy The localization of p65 relative to nuclei (stained with DAPI in blue) was examined under the confocal microscope at Z-sections passing through nuclei.

2.8. SDS-PAGE and Immunoblotting of Cell Lysates

For analysis of SYK phosphorylation by immunoblotting, cells were cultured overnight in 6-well plates. The following day, cells at 80 % confluency were serum-starved for 5 h prior to stimulation. After incubation at 37 °C with 100 μg/mL of Dectin-1 ligands or vaccine conjugates for 10 min, cells were chilled on ice, washed with cold PBS and lysed by addition of phosphorylation lysis buffer (20 mM MOPS, 1 % Triton X-100, 2mM EGTA, 5mM EDTA, 1 mM sodium orthovanadate, 2mM EGTA, 5mM EDTA, pH 7.0) containing phosphatase inhibitor cocktail (PhosSTOP, Roche Applied Science, Laval, QC, Canada) and protease inhibitor cocktail (Sigma). Cells were scraped and cell debris were removed by centrifugation at 14,000 rpm for 20 min at 4 °C. The protein content of lysates was quantified using the Bio-Rad BCA protein assay. For Western blot analysis, cleared lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and were immunoblotted with the following antibodies: rabbit anti-phospho SYK (Tyr525/526), or rabbit anti- phospho SYK (Tyr352), or rabbit anti- phospho Src (Tyr352), or with mouse anti-actin for loading control. This was followed by incubation with appropriate HRP-conjugated secondary antibodies and detection by an enhanced chemiluminescence kit from Roche.

2.8.1. Preparation of Cell Lysates

A monolayer of cells was seeded on 6-well plates and grown for 16-24 hours to attain 70-100% confluency. Experiments were performed as described. After three washes in cold PBS, lysates were prepared by replacing each well with ice cold TNT Buffer (50 mM Tris, 150 mM sodium chloride, 1% Triton X-100, 0.2% sodium dodecyl sulfate, pH 7.2) containing 1/100 protease inhibitor cocktail (Sigma-Aldrich) and incubating for 20 minutes on ice with gentle rocking. The wells were scraped on ice and extracts were collected in pre-chilled microfuge tubes, after which they were centrifuged for 20 minutes (18000x g, 4°C) to pellet cellular debris. The supernatant was collected and frozen until use.

2.8.2. Protein Denaturation before SDS-PAGE

Prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 2X or 4X concentrated Sample Buffer was added to ice-thawed lysates to result in the following composition at 1X: 62.5 mM Tris (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 0.01% bromophenol blue, and 5% 2-mercaptoethanol. The resulting mixtures were heated with agitation at 99°C for 5 minutes to promote protein denaturation, after which they were immediately loaded onto SDS-PAGE gels.

2.8.3. SDS-PAGE and Wet Transfer

Discontinuous SDS-PAGE gels were prepared with a top stacking layer of 5% acrylamide (pH 6.8) and a separating layer of 7.5 – 10% acrylamide (pH 8.8). Lysates were loaded onto the gels and resolved on Mini-PROTEAN Tetra Cell units (Bio-Rad Laboratories, Inc.) with constant current at 20-30 mA per gel and a running buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS (pH 8.3). Empty wells in the gels were filled with 1X sample buffer. Precision Plus Dual Color Protein Standards (Bio-Rad Laboratories, Inc.) were electrophoresed simultaneously and served as the molecular weight ladder. Following SDS-PAGE, proteins were electrotransferred to Trans-Blot nitrocellulose membranes in Mini-PROTEAN Tetra Cell units containing the Mini Trans-Blot module (all from Bio-Rad Laboratories, Inc.) at a constant voltage of 110 V. The transfer buffer consisted of 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.3). To verify appropriate transfer of proteins, the membranes were washed in ultrapure water and stained with 0.1% Ponceau S solution (Sigma-Aldrich). The stain was

removed upon several washes in transfer buffer.

2.8.4. Immunoblotting

Nitrocellulose membranes were blocked at room temperature for 1 hour in 3% gelatin from cold water fish skin (FSG) (from Sigma-Aldrich) dissolved in TBST buffer (Tris-Buffered Saline and Tween 20) (together, referred to as **"IB Blocking Buffer"**). Primary antibodies against the antigens of interest were diluted in IB Blocking Buffer and then bound to the membranes overnight at 4°C. To remove non-specific binding, the membranes were washed in 0.1% Tween-20 buffer (TBST) three times, 5 minutes each, with gentle shaking. Primary antibodies were detected either by fluorescence or chemiluminescence, the choice of which was dependent on the antibody. When membranes were processed for detection by chemiluminescence, the appropriate horseradish peroxidase (HRP)-coupled secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) was diluted in IB Blocking Buffer and bound to the membranes for 1 hour at room temperature. The membranes were washed again in PBSTw three times, 5 minutes each, prior to detection. Detection was achieved by incubating the membranes in BM Chemiluminescence Western Blotting Substrate (POD) (Roche Diagnostics) for 2 minutes, and exposing them to autoradiography film (Super RX Fuji Medical X-Ray Film, Fujifilm) in the dark room. Films were developed with an SRX-101A Medical Film Processor (Konika Minolta Medical & Graphic, Inc.).

2.8.5. Stripping and Reprobing of Western blot Membranes

When necessary, especially for the purpose of examining protein loading control, re-probing of blots was performed after stripping them with Stripping Buffer (7 M guanidine hydrochloride, 50 mM glycine, 100 mM potassium chloride, 0.05 mM EDTA, 20 mM 2-mercaptoethanol) for 10-30 minutes with vigorous agitation, followed by several washes with ultrapure water and three 5-minute washes in TBST.

2.8.6. Quantification of Western Blots

Where chemiluminescence was used for detection, the films were scanned to obtain a digital image. Care was taken to ensure the immunoreactive bands quantified were not above saturation. The intensities of the bands were then quantified by densitometry using ImageJ software (National Institutes of Health). Quantification Data was then plotted using prism software. Error bars represented S.E.M. (standard error of mean) of at least 3 independent experiments.

2.9. Statistical Analysis for the Flow cytometry and ELISA experiments

Data values were entered into Graphpad Prism Version 6.0c and are represented as mean ± standard error (S.E.). Paired, two-tailed parametric t tests were performed with the software to determine the statistical significance of the differences between the means of 2 experimental conditions.

2.10. Pharmacological Inhibition

The inhibitors were dissolved in DMSO as the vehicle and frozen. These stock solutions were diluted to their desired working concentrations in warm serum-free MEMα immediately before use, with final vehicle concentrations not exceeding 0.2%. The cells were then pre-treated with the resulting solutions for 30 minutes (or as indicated) at 37°C. To stimulate Dectin-1, the cells were replaced with the same solutions supplemented with β-glucan ligands. Care was taken to preserve the stability and solubility of the inhibitors during use.

2.11. Deglycosylation Assay

Lysates of RAW Dectin-1 cells and HeLa Dectin-1-HA cells were denatured in Glycoprotein Denaturing Buffer (New England Biolabs, Ipswich, MA, USA) at 65°C for 10 minutes and treated with either water or the enzymes Peptide-*N*Glycosidase F (PNGase F) or Endoglycosidase H (Endo H) for 1–2 hours at 37°C, according to instructions provided by the products' manufacturer (New England Biolabs). The deglycosylated lysates were prepared for SDS-PAGE, which was then performed, followed by immunoblotting for Dectin-1. Electrophoretic mobility shifts in enzyme-treated Dectin-1 led to insights into its glycosylation modifications necessary for appropriate Dectin-1 surface targeting.

2.12. General Procedures for Immunofluorescence

2.12.1. Cell Preparation

For all immunofluorescent staining experiments, cells were seeded on 18 mm glass coverslips and grown for 16-24 hours.

2.12.2. Fixation

Prior to fixation, cells were washed three times in PBS. Fixation was performed in cold 4% paraformaldehyde (PFA) [Sigma] for 20 minutes, then quenched in 0.1 M ammonium chloride. Both reagents were diluted in cold PBS, and both steps were performed on ice. (On occasion, a 10 minute treatment in 3% PFA + 0.1% glutaraldehyde at room temperature, followed by 7 minute reduction of unreacted aldehyde moieties with 0.1% sodium borohydride, replaced the fixation procedure). 3 post-fixation PBS washes ensured removal of unreacted fixative.

2.12.3. Blocking and Permeabilization

After fixation/quenching, blocking of non-specific antigens was achieved by incubating the cells in 5% donkey serum (Jackson Immunoresearch Laboratories) for at least 30 minutes. All subsequent antibody-binding steps were performed in the presence of 5% donkey serum or Goat serum (depending on the species of the secondary antibody), or 3% Fish skin gelatin (FSG), or 3%BSA. Membrane permeabilization was accomplished by incubating the cells in 0.1% Triton X-100 for 20-30 minutes, or in 0.2% Triton X-100 for 10 minutes for permeabilization of the nucleus. Blocking was done at either room temperature or on ice, and permeabilization was done at room temperature in a dark chamber.

2.12.4. Antibody Binding

Primary antibodies were bound to the cells for at least 30 minutes at room temperature, followed by six washes in PBS to remove non-specific binding. This was followed by the binding of appropriate fluorescent dye-conjugated secondary antibodies for 30 minutes at room temperature. Cy3, Cy5, or DyLight -coupled secondary antibodies were from Jackson Immunoresearch Laboratories; AF488-coupled antibodies were from Life Technologies. Unbound secondary

antibodies were removed by six washes in PBS.

2.12.5. Mounting of Slides

When immunostaining was complete, cells were rinsed twice in ultrapure water, drained for excess liquid, and mounted on microscope slides (Fisher Scientific) in 20 µL of DAKO Fluorescent Mounting Medium (DAKO Canada, Inc., Burlington, ON, Canada). After hardening of the media, the slides were visualized by fluorescence microscopy.

2.13. Visualization of Exogenously Expressed Surface Dectin-1 versus Total Dectin-1

Cells were fixed in 4% PFA and blocked in 5% donkey serum. To visualize the surface fraction of human Dectin-1, the cells were labeled with goat anti-hDectin-1 antibody followed by Cy3-conjugated anti-goat secondary antibodies. To visualize the total Dectin-1 population, the cells were permeabilized with 0.1% Triton X-100, blocked, and labeled again with goat anti-hDectin-1 antibody. This time, AF488-conjugated anti-goat IgGs were used as the secondary antibody. To label total Dectin-1, the cells were permeabilized with 0.1% Triton X-00 then incubated again with goat anti-human Dectin-1 antibody in the presence of 0.1% Triton X-100. Confocal microscopy was used to visualize the samples.

2.14. Co-Immunoflorescence of Dectin-1 and Other Proteins in Response to Ligand Stimulation

RAW Dectin-1 cells were incubated at 37°C in MEMα lacking or containing soluble β-glucan ligands at 100 µg/mL for various timepoints. Next, they were chilled on ice and washed three times in cold PBS (to inhibit internalization of Dectin-1 by endocytosis) then fixed in 4% PFA. To label surface Dectin-1, cells were blocked and incubated with goat anti-hDectin-1 antibody, followed by fluorochrome-conjugated anti-goat secondary antibodies. For the purpose of simultaneous visualization of other proteins along with Dectin-1, immunostaining of protein of interest was employed in addition to Dectin-1 staining. Dectin-1 and other membrane proteins were immunostained as described above, followed by permeabilization of cells with 0.1% Triton X-100 at RT for 30 minutes. Cells were then incubated again with primary antibodies against required proteins followed by secondary antibodies conjugated to fluorochrome dyes that are different from the one conjugated to Dectin-1. Samples were analyzed on a spinning disc Olympus confocal microscope with the 60X/1.40 oil objective (Cell Imaging Centre, Faculty of

Medicine Core Imaging Facility). Confocal microscopy in different channels was employed to visualize the samples. Z-stacks and multiple sections of each cell were taken to confirm proper co-localization of proteins observed but in most cases only single sections are shown in results. Puncta that overlapped in dual or triple fluorescent channels revealed localization of Dectin-1with other proteins of interest.

2.14.1. Pearson's Correlation Analysis of Immunofluorescent Colocalization

To determine Dectin-1 colocalization with other proteins of interest at the cell membrane, Pearson's correlation coefficient (R) values were calculated using the "Coloc2" plugin in Image J/Fiji software. Data values from three independent experiments were calculated for multiple cells and expressed as histograms with error bars representing SEM (standard error of the mean)

2.15. Immunoflorescent Staining of β-glucans

To detect β-glucans, mouse anti-β-glucan antibody was bound to the cells, followed by incubation with fluorescently labeled anti-goat secondary antibodies.

2.16. Analysis of Signaling Readouts by Immunofluorescence and Western blotting

Phosphorylated proteins were analyzed by immunofluorescence or by immunoblotting after 5 or 10 min stimulation at 37°C with 100 μg/mL of vaccine conjugates or Dectin-1 ligands.

For analysis of phosphorylated proteins by immunoblotting, cells were seeded on 6-well plates and grown for 16-24 hours to achieve 70-90% confluency. After three washes in PBS, cells were replaced with serum-free MEMα medium (Life Technologies) and incubated for 4-6 hours. Cells were then treated at 37°C with β-glucan ligands prepared in pre-warmed serum-free MEMα (as described above) for the indicated times at 37°C: 5 mins (Src phosphorylation) or 10 minutes (SYK phosphorylation), or as indicated for other phospho-proteins (see *Chapter 3*). After three washes in cold PBS, the cells were lysed and prepared for SDS-PAGE as in sections (Preparation of Cell lysates…) and (Protein Denaturation before SDS-PAGE….) as described above, except with the replacement of TNT Buffer with Phosphorylation Lysis Buffer (140 mM sodium chloride, 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 1% Triton X-100, 1 mM sodium

orthovanadate, 1/100 protease inhibitor cocktail (Sigma-Aldrich), PhosSTOP phosphatase inhibitor cocktail (Roche Diagonistics) prepared according to the manufacturer's instructions, pH 7.0). SDS-PAGE of cell lysates was then performed followed by immunoblotting to probe for phosphorylated proteins of interest.

For immunofluorescence, cells were grown overnight on coverslips at 40 % confluency, and the following day cells were serum starved for 4-5 h in serum-free alpha-MEM medium prior to stimulation. After stimulation, cells were washed 3 times with ice- cold PBS, fixed on ice in 4 % paraformaldehyde, and blocked in PBS containing 3 % BSA + 3 % fish skin gelatin (Sigma). To stain for surface Dectin-1, cells were incubated before permeabilization with mouse anti-human Dectin-1 followed by a dye-coupled anti-mouse secondary antibody. Next, cells were permeabilized with 0.1 % (v/v) Triton X-100 in PBS, and stained for 30 min with either rabbit anti- phospho-SYK (Tyr352) or rabbit anti-phospho Src (Tyr416) or for other phosph-proteins. Cells were then washed and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) and DAPI (4',6-diamidino-2-phenylindole) was used to highlight cell nuclei. Following staining, coverslips were washed, mounted with DAKO mounting medium (DAKO, Agilent Technologies, Santa Clara, CA) and viewed on a spinning-disk confocal microscope (WaveFx from Quorum Technologies, Guelph, ON, Canada).

2.17. Confocal Microscopy and Digital Analysis/Processing

Confocal microscopy was performed on the WaveFX Spinning Disk Confocal System configuration (installed by Quorum Technologies, Guelph, ON, Canada). A CSU 10 or CSU X1 spinning disk confocal scan-head (Yokagawa Electric Corporation, Japan) was set up on an Olympus IX-81 motorized inverted microscope base (Olympus Canada, Richmond Hill, ON, Canada). Samples were illuminated by an LMM5 Laser Merge Module with pumped diode lasers of 405, 491, 561, and 642 nm (Spectral Applied Research, Richmond Hill, ON, Canada). Unless stated otherwise, images were captured through a 60X objective lens (1.42 numerical aperture) with a C9100-13 electron multiplying charge-coupled device camera (Hamamatsu Photonics K.K., Japan). Z-axis slices of 0.2 µm thickness were acquired through the cells using Volocity software (Improvision, Perkin- Elmer, Waltham, MA, USA) and an MS-2000 motorized XY stage with a piezo Z-axis insert of 100 µm travel (Applied Scientific Instrumentation, Eugene, OR, USA). In certain experiments, acquisition parameters (exposure time, camera sensitivity,

laser intensity) were maintained between experimental conditions to allow direct comparison between the images. Images were contrast-enhanced in Volocity prior to channel-splitting in Photoshop CS4 (Adobe Systems Inc., San Jose, CA, USA) for presentation.

2.18. Dectin-1 Ligand Binding Assay

RAW 264.7 murine macrophages referred to as RAW WT and RAW Dectin-1 cells were plated overnight on glass coverslips in a 12-well plate. The following day cells at 60 % confluency were incubated on ice for 5-10 min (to minimize internalization of Dectin-1 by endocytosis during the binding experiment) with Alexa Fluor 546-labeled vaccine conjugates dissolved at 100 μg/ml in ice-cold serum-free hRPMI culture medium or PBS. Cells were then washed four times with ice-cold PBS to remove unbound ligand, fixed with 4 % paraformaldehyde, blocked with PBS containing 3 % BSA + 3 % FSG. For detection of surface Dectin-1 on the plasma membrane, non-permeabilized cells were incubated with mouse anti-human Dectin-1 antibodies followed by incubation with Alexa Fluor 488 conjugated anti-mouse IgG and DAPI. Coverslips were mounted with DAKO and examined using confocal microscopy.

2.19. Mice Vaccination (Performed by the Bundle Group)

The effect of vaccination with the TS-TT-Lam conjugate was studied in three mouse strains: Balb/c, C57BL/6 and CD1 (outbred strain). Groups of 10 mice from each strain were immunized with β- mannan trisaccharide-TT conjugate. Control groups (10 mice each) were given the same conjugate that lacks the laminarin substituent. Both antigens were given as emulsion in mineral oil (incomplete Freund`s adjuvant). Each dose contained 20 μg of a conjugate (equivalent of 1.25 μg of trisaccharide) in 300 μl of the formulation administered intraperitoneally (200 μl) and subcutaneously (100 μl). C57BL/6 and CD1 mice were immunized 4 times at 3 weeks intervals and Balb/c mice received 4 injections at 2 weeks intervals. Sera were collected 10 days after each immunization. Ten days after the 4th immunization mice were bled and exsanguinated.

2.20. Ligand Stimulation for BMDMs and BMDCs

Adherent BMDMs from day 7 cultures were washed, incubated with ligands of interest at 37°C for desired time. After incubation, cells were then placed on ice to inhibit further signaling, the supernatant was aspirated, and cells then lysed directly in the plate with P-lysis buffer. Cell lysates were further prepared as described above. For western blot analysis lysates were loaded

onto 8 % SDS-PAGE gel and subsequently probed for phosphorylation of proteins of interest using phospho-specific antibodies Ligand stimulation experiments were done at least twice for each treatment. For BMDC stimulation cells were harvested and cells were replated in 12-well plates (10^6 cells/well) overnight in normal growth serum without GM-CSF. Media was replaced with 37°C RPMI containing 25ng/ml of recombinant GM-CSF (Peprotech, Rocky Hill, NJ) for the indicated amount of time, at 37°C. After incubation, the supernatant was aspirated, cells were lysed directly in the wells with TNT buffer and lysates were boiled for 3 minutes.

2.21. Chemical Cross-Linking of Dectin-1

Cells were seeded on 10 cm dishes and grown for 18 hours to achieve 100% confluency. After 10 minutes (only 10 minutes to avoid internalization of the receptor) of β-glucan stimulation at 37°C, surface-exposed proteins were cross-linked at 4°C for 2 hours with the amine-reactive, membrane impermeant, cross-linker Sulfo-EGS [ethylene glycolbis (sulfosuccinimidylsuccinate), Thermo Scientific], at 1.5 mM in cold PBS. The reaction was quenched at 4°C for 15 minutes in a buffer of 20 mM Tris and 150 mM sodium chloride (pH 7.5). Lysates were promptly prepared in ice cold TNT Buffer, 4X Sample Buffer without 2-mercaptoethanol was added to a final concentration of 1X and the mixture was heated at 65°C for 5 minutes. The lysates were then run for SDSPAGE on a 7.5% polyacrylamide gel and immunoblotting for Dectin-1 was further performed.

2.22. Antibody Cross-linking

An alternative way to induce Dectin-1 clustering is by antibody crosslinking of Dectin-1 on live cells (ice cold) using bivalent mouse anti-human Dectin-1 antibodies (R&D Systems). This will have a mild cross-linking effect as it only brings two receptors together (bivalent). Subsequent incubation with a secondary antibody will induce formation of larger Dectin-1 clusters. We have also prepared monovalent Fab fragments from the mouse anti-Dectin-1 antibody that provide a non–clustering control for these various Dectin-1 probes. Antibody cross-linking of Dectin-1 was either examined by Immunofluorescence (IF) or by western blotting experiments. Briefly, cells were seeded 24 hours before the experiment either on coverslips (for IF experiments) or in a 6-well plate (for western blot experiments). The following day, cells were washed 2x with ice cold PBS and then treated with primary antibody (prepared in cold PBS) against Dectin-1 or the corresponding isotype control for 10 minutes on ice. This was followed by washing 5x with cold

PBS, and then the addition of fluorescently-labeled secondary Ab (prepared in pre-warmed PBS and at double the concentration/half the dilution factor used for primary antibody) against the species used for the anti-Dectin-1 Ab or isotype control. Cells were incubated with the secondary Ab for 5-10 minutes at 37°C, and then cells are put back on ice and washed 5x with cold PBS. In IF experiments incubation with secondary Abs was followed by fixation with 4% PFA and permeabilization and blocking if followed by Immunostaining if other cytoplasmic proteins of interest were desired to visualize in relation to with Dectin-1 (e.g. Tyrosine phosphorylation, and SYK or Src phosphorylation). For examining the effects of Dectin-1 cross-linking by Abs through western blotting, standard preparation of lysates followed by western blotting was performed as described in section 2.8.

2.23. Dectin-1–mediated Phagocytosis Assay

Laminarin molecules were conjugated to 3µm polystyrene beads (from Spherotech Inc., Lake Forest) in the Bundle lab (Department Chemistry U of A, Edmonton, AB) and then coupled to FITC (Fluorescein isothiocyanate) a green coloured fluorescent dye (Molecular probes, Life Technologies Inc., ON) to form FITC-labeled laminarin beads (FITC-laminarin beads) (Figure 29). Cells were then seeded on glass coverslips in a 12-well plate 24 hours prior to the experiment. The following day the FITC-labeled polystyrene beads were washed 3x with PBS, centrifuged at 8000 rpm, and then resuspended in PBS at an appropriately low concentration to avoid aggregation of beads. Cells were washed 2x with warm PBS and then treated with FITC-labeled beads for 30 minutes at 37°C or for the indicated times. Cells were synchronized by centrifugation at 8000 rpm to allow the beads to evenly distribute on the cells. The 12-well plate was then placed on ice and washed 3X with cold PBS, followed by fixation for 10 min with 4% PFA (paraformaldehyde). Cells were then blocked in blocking buffer consisting of 5 % DS (Donkey Serum) in PBS. Blocking was then followed by labeling non-permeabilized cells with a Cy5–labeled anti-FITC antibody (anti-FITC-CY5), at a dilution of 1:1000 in blocking buffer for 30 min. This will enable us detect non-phagocytosed beads in the CY5 channel (blue). Coverslips were then mounted with DAKO and visualized by confocal microscopy.

2.24. Single Molecule Fluorescence Microscopy Using PALM Superresolution Imaging

In vivo, biomolecules work in dynamic and complicated heterogeneous systems, in which a variety of molecules such as signaling proteins and receptors assemble and interact in a unique

and timely manner. These reactions are generally stochastic; therefore the dynamic behaviors of individual molecules are averaged and hidden in ensemble-averaged measurements such as classical biochemical techniques. It is therefore difficult to quantitatively detect in a spatio-temporal manner the behaviors of single molecules in such systems. To overcome these difficulties, imaging techniques with higher resolution and sensitivity have been developed.

Single molecule fluorescence microscopy has recently attracted a great deal of attention in the field. It is a high-resolution optical method that allows the detection of individual molecules in the cell without being obscured by the ensemble averaging inherent in conventional biochemical methods (Owen et al., 2010). SMI techniques directly reveal the dynamic behavior of individual molecules with high spatio-temporal resolution and sensitivity, which for the purpose of this study would provide us with valuable insight into the molecular events leading to Dectin-1 activation. Single molecule imaging is being performed in our laboratory using a microscope equipped with an EM-CCD camera combined with TIRF (Total internal reflection fluorescence) excitation set-up. Acquiisition of image sequences are done using the software Volocity (Perkin-Elmer). Quantitative image analysis was performed by Dr. Nicolas Touret and John Maringa in collaboration with Dr. Khuloud Jaqaman, UT Southwestern Medical Center (Dallas, Texas).

To obtain the localization of a majority of Dectin-1 receptors, we use an approaches based on PALM *(Photo-activated localization microscop*y) using RAW cells stably transfected with Dectin-1-PAmCherry/PhotoActivatable mCherry, Figure 20, provided by the lab of Michael Davidson from Florida State University) (Owen et al., 2010). With most microscopic approaches, fluorescent probes are too densely distributed to be individually resolvable, as the optical resolution of light microscopy is diffraction-limited. The principle of PALM is based on the sequential and stochastic switching “on” and “off” of fluorophores between a non-fluorescent (dark) state and a fluorescent (bright) state (Lippincott-Schwartz and Manley, 2009; Lippincott-Schwartz and Patterson, 2009) (Figure 21). The activation of fluorophores (here PAmCherry) is done using weak 405 nm laser light intensity. The fluorescence signal is recorded on the EM-CCD camera at a frame rate of 10 fps (frame per second), for a total of up to 5000 frames, following excitation with a 561 nm laser and selection of the emission signal through a 665 – 585 bandpass emission filter. During one imaging cycle (equivalent to one frame = 100 ms) most molecules remain dark and only a small number of molecules are randomly switched on, imaged and recorded. This ensures that the images of individual fluorophores do not typically overlap,

thereby allowing each fluorophore to be localized with high precision. The process is then repeated for numerous cycles until the majority of molecules have been accurately detected and precisely localized into single molecule centers. This produces a series of a few thousand images that can then be processed by the computer software and reconstructed into a high-resolution image.

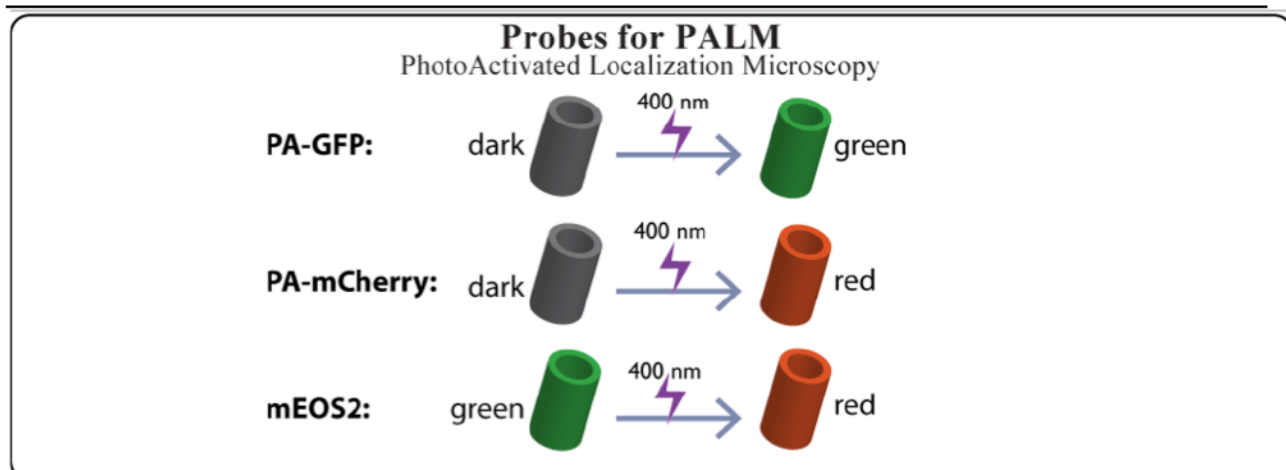


Figure 20: Fluorescent Probes Used for PALM Superresolution Imaging

PALM (Photo-Activated Localization Microscopy) superresolution microscopy uses genetically encoded photoconvertible (photactivatable/ photoswitchable) organic probes, e.g., PA-GFP, PA m-Cherry and mEoS2. PA: photoactivatable

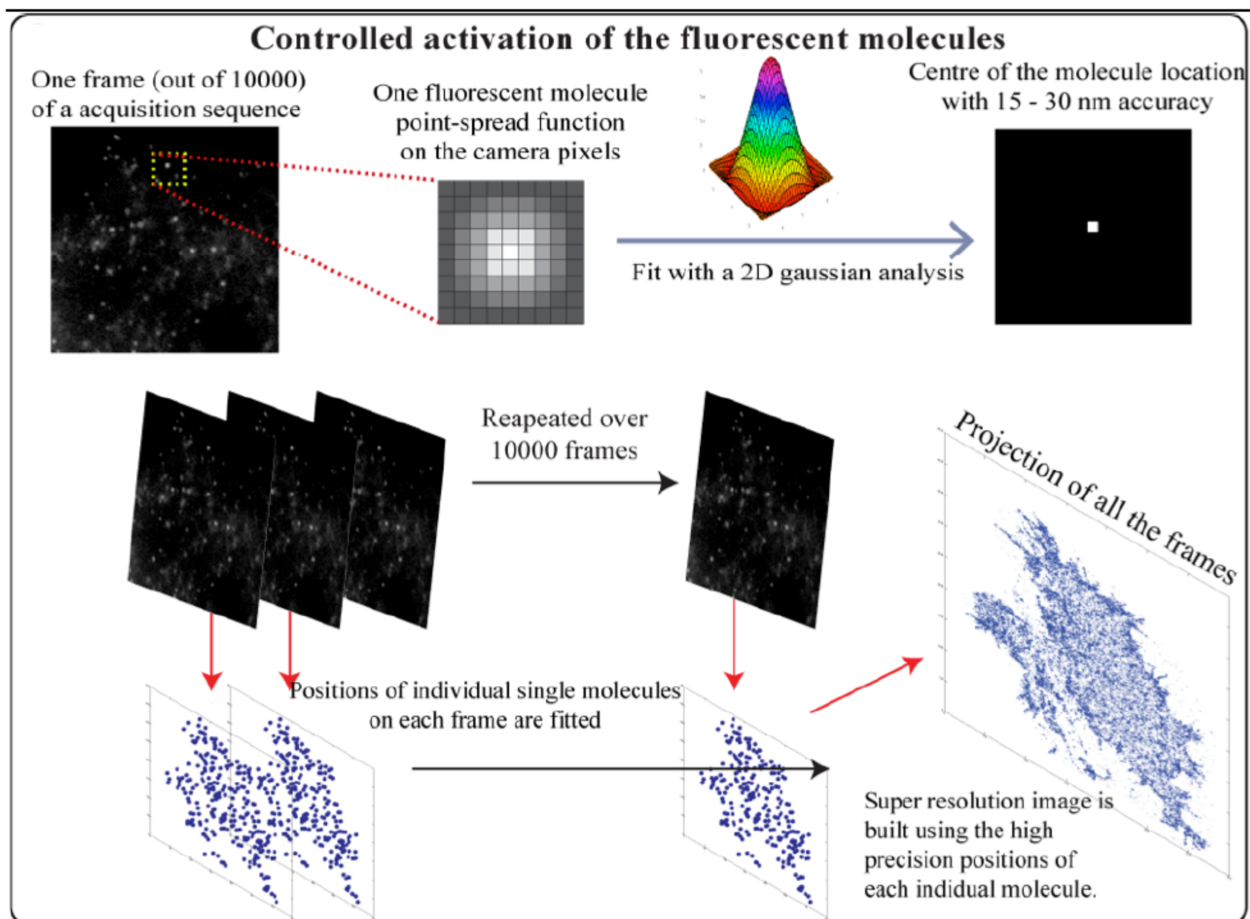


Figure 21: Flow Chart for Creating a High Resolution Image Using PALM Superresolution Microscopy

The key principles in the acquisition of localization-based super-resolution images are 1) the control of the excited state of specific probes and 2) the fitting of single molecules point-spread function (PSF) with 2D gaussian equation. By combining the high precision localizations of each molecule fitted on each frame of an image sequence (up to 10,000 and more) a super-resolution image can be reconstructed.

2.24.1. PALM Sample Preparation

RAW PAmCherry-Dectin-1 cells were plated on clean coverslips 24 hours prior to the experiment. The remaining experiment was done in condition of low or no ambient light stimulation to avoid possible activation of the PAmCherry fluorescent molecules. Ligand stimulation was performed for 10 min at 37°C as described previously using either HRPMI as non-stimulated control and HRPMI containing 100 µg/ml or 50 µg/ml of β-glucans. Following stimulation, cells were placed on ice and fixed in a solution containing 3% PFA (paraformaldehyde) and 0.2% Glutaraldehyde for 20 min on ice and 10 min at room temperature. The cells were then rinsed in PBS several times and imaged within the next 24 hours. The

coverslips were transferred to an imaging chamber (Quorum Technologies, Guelph, Ontario) containing PBS and placed on the stage of the TIRF microscope.

2.24.2. Total Internal Reflection Fluorescence (TIRF) Microscopy for Superresolution Imaging

TIRF microscopy was performed on an Olympus IX-81 motorized inverted microscope base installed by Quorum Technologies (Guelph, ON, Canada). Fixed cells on coverslips were illuminated as described above, except with coupling to a TIRF module. This module allowed manual adjustment of the angle of incident illumination such that total internal reflection was achieved at the coverslip-water interface. Images were acquired through a 100X (1.45 numerical aperture) objective lens with a Hamamatsu EM-CDD camera (ImageEM91013) using Volocity software. Imaging and photoconversion were performed simultaneously through the TIRF excitation fiber using a 405 nm laser (for photoactivation) and 561 nm laser (for excitation of the photoconverted fluorophores). Image sequences of up to 5000 frames were recorded and then exported to the computer workstation for further analysis.

2.24.3. Single Molecule Analysis of Dectin-1 Clusters

Computational Single Molecule Analysis of the Dectin-1 clusters, mainly using the Matlab software, as well as statistical analysis of the Dectin-1 clustering Data, was performed by Dr. Nicolas Touret and John Maringa (Ph.D. candidate in the lab of Dr. Nicolas Touret) in collaboration with Dr. Khuloud Jaqaman, UT Southwestern Medical Center (Dallas, Texas).

Briefly, each image of the sequence is processed using Matlab and fluorescent spots are fitted in a 2D gaussian using a PSF (point spread function) determined for our system. The fitting of all the fluorescent molecules of the image sequence provides the coordinates and intensities of most PAmCherry–Dectin-1 molecules.

The distribution of the molecules is then performed through our “Clustering Analysis” program in Matlab (Mathworks). In short, each image coordinate distribution is compared to a randomly distributed population of similar density. Statistical values of clustering (here derived from the Ripley’s K function) inform us on the type of distribution (random, or more or less clustered) of the Dectin-1 receptors. Areas of higher local density (value derived from the Ripley’s K function) are used to delineate clusters and are called heat maps (see Figure 69). A binary map is produced

from the local densities above those of randomized data to define cluster region and isolate the number of receptors and surface of each of these clusters (Figure 69).

Chapter 3. DECTIN-1 ACTIVATION BY SOLUBLE BETA-GLUCANS IS MEDIATED BY RECEPTOR CLUSTERING

This chapter has been written in preparation for a manuscript that is in progress to be submitted for high impact journals such as the "Journal of Cell Biology." Most of the data covered in this section is publishable and might go into one manuscript or two. All experiments have been completely finished for this publishable manuscript and are covered thoroughly in this chapter

3.1. Introduction

In the past two decades, invasive fungal infections have emerged as a major global threat to human health especially in immunocompromised patients, whose numbers are increasing due to the advent of HIV, organ transplantations, and the use of modern immunosuppressive therapies for autoimmune diseases and cancer. Recently, this increase in fungal disease burden has been highlighted as a more serious public health concern due to the emergence of novel pathogenic fungi, lack of fungal vaccines, and inefficiency of current antifungal therapeutics partly due to the emergence of drug resistance in fungi. It is therefore of compelling interest and a healthcare priority to understand at the molecular level the mechanisms that our immune system utilizes to fight fungal infections.

The initial step in mounting an effective anti-fungal immune response is the early recognition of the fungal pathogen by specialized pattern recognition receptors (PRR) present on the surface of innate immune cells. Several studies have identified Dectin-1 as the major PRR involved in fungal recognition, and as a C-type lectin receptor that plays a key role in antifungal immunity. Dectin-1 specifically recognizes (1,3) β-glucans, complex polysaccharides of glucose that comprise the major structural component/scaffold of fungal cell walls. By way of its β-glucan specificity, Dectin-1 recognizes a number of fungal species including *Candida, Pneumocystis,* and *Aspergillus.*

Dectin-1 upon ligand binding elicits a signaling cascade via its cytoplasmic ITAM-like motif (also known as hemITAM), leading to various cellular responses including phagocytosis, cellular maturation, generation of a respiratory burst, and the production of inflammatory cytokines and chemokines, all of which culminate to produce an effective immune response against the fungal pathogen. Ligand binding to Dectin-1 is followed by tyrosine phosphorylation of its hemITAM motif by Src family kinases (SFKs), which creates a docking site for the recruitment and activation of SYK (Spleen tyrosine kinase), via its SH2 domains. Unlike classical ITAM motifs, only phosphorylation of the membrane-proximal tyrosine residue within the hemITAM sequence is sufficient to mediate Dectin-1 signaling. The activation of SYK, a pivotal kinase in Dectin-1 signaling, further induces subsequent downstream signaling cascades, which involve the activation of NADPH oxidase, PLC-γ2, PKC-δ, MAPK cascades (Erk1/2, JNK and p38), and transcription factors NF-κB and NFAT. Most importantly, SYK activation triggers the formation of the CARD9/BCL10/MALT1 (CBM) adapter complex, which induces the NF-κB signaling pathway leading to cytokine production. Recently, activation of PKC-δ downstream of SYK has been shown to couple SYK activation to NF-κB signaling, where PKC-δ facilitates the proper assembly of the trimeric CBM scaffolding complex, which in turn activates the IKK complex required for the activation of both canonical (p65) and non-canonical (RelB) NF-κB subunits. Dectin-1 ligation also activates a second, SYK-independent, arm of signaling mediated by Raf-1 to modulate NF-κB activity, but the membrane-proximal mechanisms/events involved in this pathway are unclear. Raf-1 acts to promote phosphorylation of the canonical p65 subunit and repress non-canonical RelB activity. All of the signaling pathways downstream of SYK and Raf-1 form a large network that ultimately leads to the modulation of innate and adaptive immune responses essential for antifungal immunity, especially through the production of inflammatory cytokines that activate Th1 and Th17 cells.

β-glucans have been long known for their immunomodulatory properties, and are therefore used as nutritional supplements that provide immunotherapeutic benefits/ that boost the immune system. Although the discovery of Dectin-1 as the major β-glucan receptor has greatly enhanced our knowledge of the immunomodulatory effects of β-glucans, the precise/exact molecular mechanisms of how β-glucans activate Dectin-1 are still unclear and need to be elucidated/understood.

Many studies have demonstrated the immunomodulatory effects of polysaccharides, including β-

glucan, isolated from mushrooms, fungi, yeast, algae, lichens, and plants (Novak and Vetvicka, 2008). Recently, the molecular mechanism of fungal pathogen recognition by innate immune cells, such as macrophages and DCs, has become clearer with the identification of Dectin-1 as the major PRRs for β-glucans, which reside in the fungal cell wall. Most of the molecular mechanism data have been generated using live fungi and particulate β-glucan, whereas few studies have examined the mechanism of purified, soluble β-glucan, used as a biological response modifier to augment the host immune response (Tsoni and Brown, 2008a).

Previous studies have shown that the ability of β-glucans to induce immune responses is influenced by the size/molecular mass of the β-glucan (Batbayar et al., 2012; Brown et al., 2003; Lowe et al., 2001; Rice et al., 2005). Several studies have reported that laminarin, a small water-soluble β-glucan (6-8 kD), although capable of binding to Dectin-1, fails to mount any immune responses, and in fact it has been extensively used as a Dectin-1 blocking ligand. On the contrary, larger Dectin-1 ligands, including particulate and high-molecular weight soluble β-glucans, have been demonstrated to trigger Dectin-1 mediated signaling and immune responses. Several rodent and in vitro models have highlighted curdlan, a long linear (1, 3)-β-D glucan (161 kDa), as a potently bioactive β-glucan that targets Dectin-1 to induce immune responses, whether it is in solubilized or particulate form. Yeast cell wall extract, zymosan, and whole glucan particles (WGP) from *Sacchoromyces cerevisiae,* have been shown to induce Dectin-1 signaling, phagocytosis, and cytokine production. In a recent study it was reported that Dectin-1 signaling is only activated by particulate β-glucans, which cluster the receptor in synapse-like structures dubbed as 'phagocytic synapse' (Goodridge *et.al* Nature, 2011). Additionally, the crystal structure of Dectin-1 suggested that while individual Dectin-1 receptors can bind to β-glucans, cooperative binding of dimers might be more efficient (Brown et al., 2007). **Although, in vitro characterization of Dectin-1 in the presence of its natural ligand indicates higher-order complex formation between Dectin-1 and β-glucans, no in vivo evidence suggests that Dectin-1 oligomerizes.**

Based on the above evidence and studies, we propose a **'Clustering Model'** for Dectin-1 activation and signal-transduction **(**Figure 22). In this model increasing the size of the ligand would enhance its signaling capacity and immunomodulatory potential. **Accordingly, we speculate that, in addition to being recognized and bound by Dectin-1, the β-glucan molecule must be of sufficient size to cluster Dectin-1 receptors on the cell surface as a**

prerequisite for induction of biological activity; however, this has not been proven unequivocally.

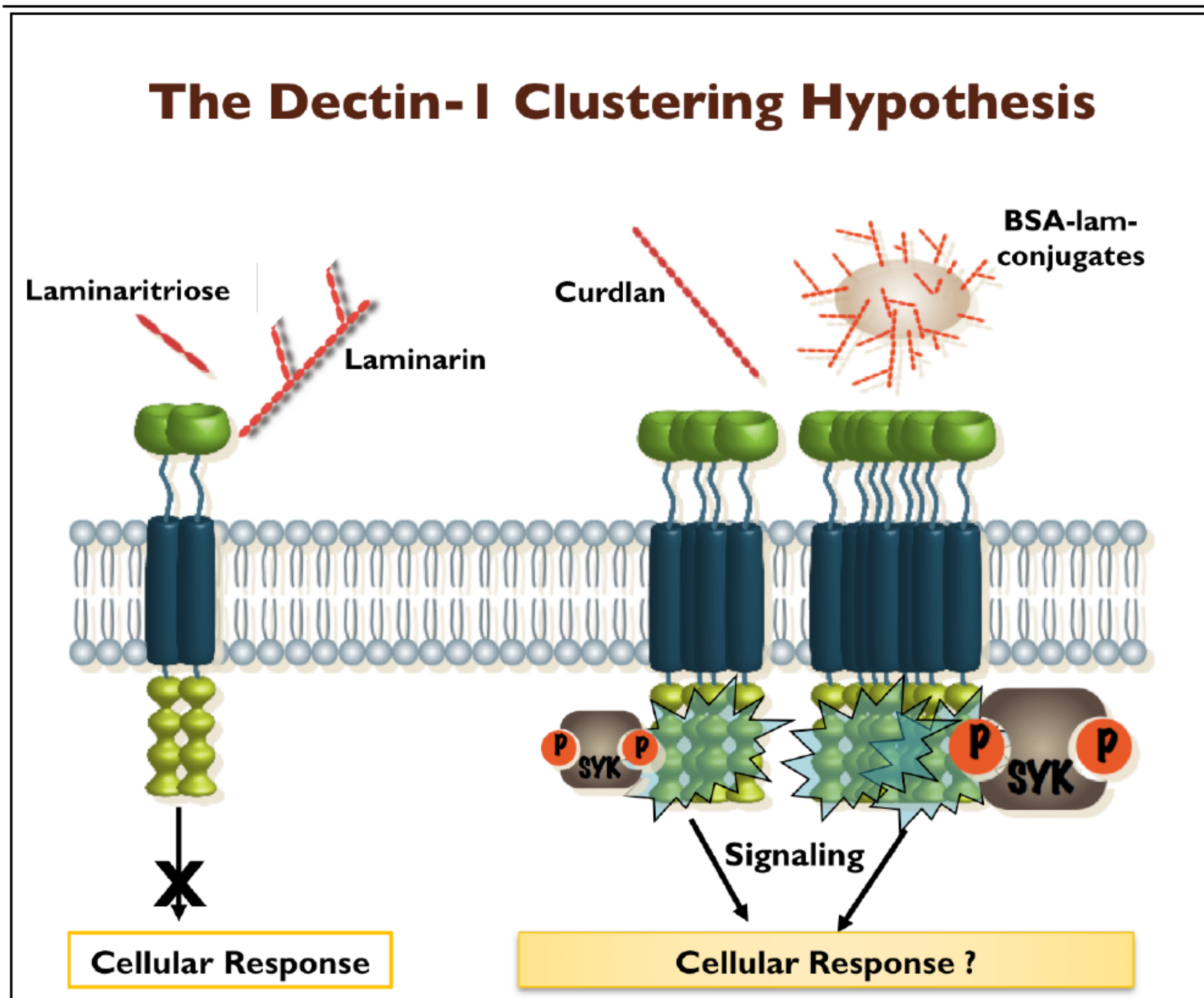


Figure 22: The Dectin-1 Clustering Hypothesis

The Dectin-1 Clustering Hypothesis proposes that Dectin-1 is clustered by ligands of larger size/molecular weight (such as Curdlan and BSA-laminarin conjugates, depicted in above figure) into large multimeric complexes, capable of inducing Dectin-1 signaling events and consequently Dectin-1–mediated cellular responses. On the contrary, ligands of smaller size/molecular weight such as laminarin are too small to promote Dectin-1 clustering and therefore fail to induce sufficient Dectin-1 signaling and cellular responses. Depicted in figure is SYK (spleen tyrosine kinase) the activation of which is a hall mark of Dectin-1 signaling. We propose that Dectin-1 clustering activates SYK above a certain threshold that is sufficient to trigger downstream signaling from Dectin-1.

Therefore, in this study we were interested to investigate why β-glucans of varying size have different immunomodulatory efficacies/biological activities. We specifically wanted to elucidate whether differences in β-glucan size affect Dectin-1 at the molecular level; in particular, its oligomeric organization on the plasma membrane and its capacity to activate signaling events. Our main goal here was to **determine whether Dectin-1 clustering by β-glucans is required**

for its activation. To test our clustering hypothesis, we analyzed the oligomerization state of Dectin-1 on the plasma membrane in response to ligands of different sizes using innovative superresolution imaging techniques coupled to high-level analysis of the molecule behavior. In parallel we examined the capacity of these ligands to induce Dectin-1 signaling. This enabled us to build a correlation between the effects of Dectin-1 ligands on receptor clustering and their ability to trigger Dectin-1 signaling. We also sought in this study to explore in detail whether ligands of variable size would affect various underlying Dectin-1 signaling pathways. Our findings provide the first evidence that Dectin-1 is activated by receptor clustering, and here present a **"Clustering Model"** for Dectin-1 activation and signal-transduction (see section **1.3.2**, *Chapter 1*). Finally, we demonstrate that the size of the ligand differentially and qualitatively affects downstream signaling from Dectin-1 **(**Figure 22**)**.

In this study for the purpose of testing the hypothesis that receptor clustering activates Dectin-1, we sought to determine the effect of ligands of varying size on the clustering of Dectin-1 and its ability to induce intracellular signaling. Accordingly, we first established different experimental conditions enabling various degrees of Dectin-1 clustering. Using different cell lines, we then determined the potential of each of these conditions in activating Dectin-1 via the monitoring of various signaling readouts known to be induced downstream of Dectin-1 ligation, e.g., activation of SYK and NF-kB activation. Moreover we investigated the formation of Dectin-1 clusters upon ligand binding using chemical cross-linking and single molecule superresolution microscopy techniques. Results form this study enabled us to closely examine the effect of ligand size on the capacity of Dectin-1 to induce various signaling events.

3.2. Results

3.2.1. Development of Different Probes to Induce Dectin-1 Clustering

In order to examine the effect of Dectin-1 clustering and activation, we sought to investigate the relationship between the ligand size and the capacity of Dectin-1 to induce signaling. For that purpose we exploited different experimental conditions enabling various levels of Dectin-1 clustering. It is worth-mentioning that there is a huge discrepancy in the Dectin-1 literature regarding whether soluble β-glucans are capable of activating Dectin-1, especially that a recent study by Goodridge *et al.* (2011) reported that only particulate glucans are capable of activating Dectin-1. Accordingly, in this study, in order to rule out the effect of the solubility of β-glucans on Dectin-1 activation we decided to investigate the effect of size differences on Dectin-1 activation using soluble β-glucans rather than particulate ligands (Batbayar et al., 2012; Goodridge et al., 2011). Unfortunately, completely soluble β-glucans are not commercially available, however, we were extremely privileged to obtain a panel of these highly soluble ligands, specially prepared for this study, by Dr. David Bundle's group (Department of Chemistry, U of A, Edmonton, Alberta). Various conditions inducing Dectin-1 clustering in cells *(ex-vitro)* were then established. These included Dectin-1 stimulation by a variety of natural and synthetic β-glucans of variable sizes, which are highly pure and soluble (mainly provided by the Bundle lab) (as described in **section 2.5**). Analyzing Dectin-1 mediated signaling responses in response to water-soluble β-glucans of variable size /molecular weight will give insight into how ligand size affects Dectin-1 activation and probably clustering of Dectin-1 at the plasma membrane. Moreover, another experimental approached that we exploited in order to understand the effects of Dectin-1 clustering on signaling was antibody cross-linking of Dectin-1 by Dectin-1 antibodies. Antibody cross-linking is a useful tool for the purpose of our study as it mimics the effects of Dectin-1 clustering by large-sized ligands. The different clustering probes used for this study are briefly described below and summarized in Figure 23**.**

(i) Naturally-derived Soluble β-glucans:

These are ligands of Dectin-1 that are derived from natural sources e.g., bacteria, fungi, yeasts and mushroom. Water-soluble forms of these ligands are not always commercially available. To achieve different levels of Dectin-1 clustering we used a panel of soluble β-glucans of progressively increasing size, including laminarin, scelero-glucan, curdlan, and soluble WGP

(Whole Glucan Particle) (these ligands are described in **Chapter 1, section 1.6.1**) Most of these ligands, especially curdlan, are not fully soluble and need to be chemically processed to achieve maximum solubility (as described in *materials and methods, Chapter 2*). The Bundle lab has assisted us in overcoming this problem and has provided us with soluble forms of these β-glucans. Curdlan is a high molecular weight linear polymer consisting of β-(1,3)-linked glucose residues. Curdlan is produced as a water-insoluble polysaccharide by the soil bacterium, *Alcaligenes faecalis*. Consequently, curdlan was phosphorylated by the Bundle lab to produce a water-soluble form of curdlan that we referred to as P-curdlan (Phospho-curdlan) during this study. P-curdlan has been extensively used in this study as a soluble Dectin-1 ligand (β-glucan) of large size and high molecular weight (~5000-10,000 kDa) (Curdlan described **section 1.5.2)** (Figure 23**)**.

(ii) BSA Laminarin Conjugates:

The Bundle group has also developed a panel of BSA (bovine serum albumin)-laminarin conjugates. In each of these conjugates a variable number of laminarin molecules are attached to a BSA protein carrier that acts as a scaffold The group has recently provided us with a series of these conjugates with different numbers of laminarin molecules ranging from 4-18 molecules per BSA protein. We also have a BSA-laminarin panel in which the laminarin has been labeled with the fluorescent dye Alexa Fluor (AF546 dye). This will enable us to visualize the binding of these ligands to Dectin-1 by imaging techniques. An advantage of using this BSA–laminarin conjugate research tool is that it mimics the Dectin-1 ligands exposed on the surface of a fungal pathogen. (see **section 1.5.2**) **(**Figure 23**)**.

(iii) Antibody Cross-linking of Dectin-1

Another alternative to the use of Dectin-1 ligands will be to induce clustering of Dectin-1 by means of antibody cross-linking (Figure 24). When used on live cells, the bivalent anti-human Dectin-1 antibody will have a mild cross-linking effect (only brings two receptors together). Subsequent incubation with a secondary anti-mouse antibody should induce the formation of larger clusters **(Figure 24).**

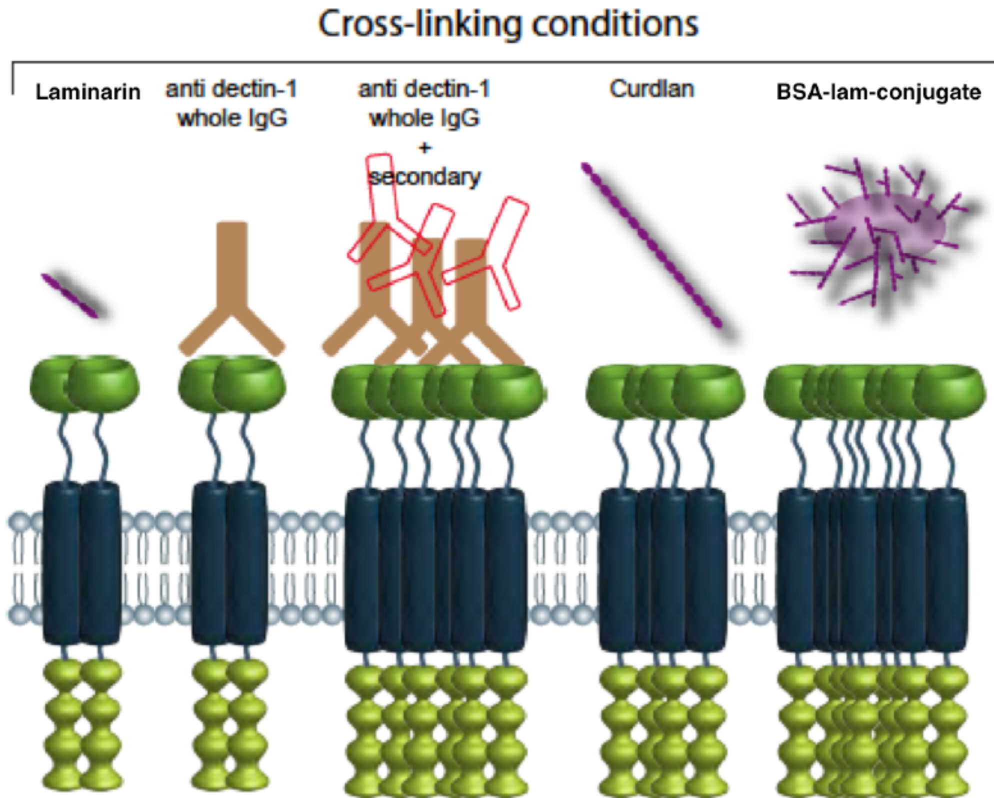


Figure 23: Conditions for Dectin-1 Clustering

Various levels of Dectin-1 clustering will be achieved by using diverse ligands of variable size or antibodies. Larger aggregates/clusters of surface Dectin-1 will be produced by using whole IgG with or without anti-mouse secondary antibodies, and complex ligands like curdlan or BSA-laminarin conjugates with variable number of laminarin Dectin-1 was staining performed after fixation of the cells, and either (a) stimulation with ligand or (b) treatment of cells with primary antibodies followed by secondary antibodies induced. Results from these immunofluorescent experiments showed that compared to control conditions higher clustering conditions by larger ligands and antibody cross-linking induced Dectin-1 clustering.

3.2.2. Generation of a RAW 264.7 Macrophage Cell Line Stably Expressing human Dectin-1 (RAW Dectin-1 Cells)

3.2.2.1. Exogenous Expression of Dectin-1 in RAW 264.7 Macrophages to Form RAW Dectin-1 Cells

Mouse Dectin-1, although endogenously expressed in the murine macrophage cell line known as RAW 264.7 cells, is expressed at very low levels. Studying Dectin-1 signaling in these cells is challenging, as the stimulation of Dectin-1 poorly expressed on the surface of these cells is not sufficient to induce Dectin-1 Signaling events. Therefore, to be able to test the effect of the various clustering conditions described above, we generated a stable murine macrophage cell line exogeneously expressing Dectin-1 by viral transduction and antibiotic selection (Materials and

Methods, *Chapter 2*). We are specifically interested in studying Dectin-1 signaling mediated by the human Dectin-1 (hDectin-1) isoform, which hasn't been well-studied in the literature and needs to be further characterized in order to have a better understanding of Dectin–mediated antifungal immunity in human hosts. Briefly, as described in *Materials and Methods* (Chapter 2), we expressed the human isoform of Dectin-1 into the mouse RAW 264.7 cells by retroviral transduction and G418 (neomycin) selection, and we named these cells 'Raw Dectin-1'. Wild type RAW 264.7 cells, not exogenously expressing human Dectin-1, were also maintained for several experiments performed in this study, and refered to as 'RAW WT' cells.

Immunoblotting of whole cell lysate (WCL) was performed on RAW Dectin-1 and RAW WT cells using goat anti-hDectin-1 Abs to determine the successful expression of Dectin-1 in these cells. Western Blotting of whole-cell lysates (WCLs) prepared from these cells demonstrated the existence of two bands on the gel (Figure 24), which were only present in the transduced cells 'RAW Dectin-1' cells but not in the wild-type cells 'RAW WT" cells'. The higher Mwt band (~40-50 kDa) migrated on the gel as a smear of bands between 40 and 50 kDa, and was shown to correspond to the mature complex glycosylated form of Dectin-1 as confirmed by glycosylation experiments (Data not shown), thereby proving that RAW Dectin-1 cells was appropriately folded and targeted to the cell membrane. The lower band corresponded to the core glycosylated immature (~28 kDa) form of the protein, which is not targeted to the membrane and is not of our interest in this study **(**Figure 24). Although the predicted molecular weight of the full-length human Dectin-1 is 27.6 kDa, these higher molecular weight bands of Dectin-1 detected on the gel represent mature, glycosylated forms of the receptor, which have an N-linked glycosylation site in their stalk region (Figure 12) (Kato et al., 2006). Treatment of RAW Dectin-1 lysates with PNGase F collapsed the Dectin-1-reactive bands to a smaller precursor ~37 kDa in size (Figure 24). Since this did not occur upon Endo H treatment, the results suggest Dectin-1 was expressed as a glycosylated and mature form of the receptor, and likely targeted to the plasma appropriate folding, targeting, and surface expression of Dectin-1 in the RAW macrophages.

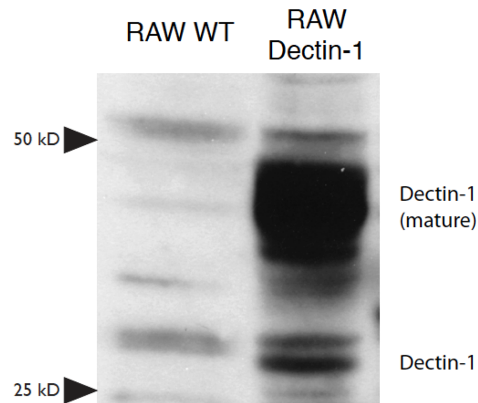


Figure 24: Expression of Human Dectin-1 Cells in RAW Dectin 1A Macrophages

RAW 264.7 were virally transduced with the pFB-neo plasmid (Stratagene, Agilent Technologies, Santa Clara, CA) containing the human Dectin-1 cDNA. Cells were selected using 1 mg/ml G418 (neomycin) in the culture medium. Expression and surface targeting of Dectin-1 in the RAW Dectin-1 cells were assessed. Western blotting of whole-cell lysate prepared from RAW WT- and RAW Dectin-1–expressing cells. For immunoblotting, the goat anti–Dectin-1 Ab (R&D Systems) was used at 1:1000, followed by an anti-goat IgG coupled to HRP (1:5000). In the RAW Dectin-1 lane, several bands appeared upon ECL treatment and film development. The upper and large bands correspond to the complex glycosylated form of the receptor, whereas the smaller one (30 kDa) corresponds to the core glycosylated (determined by PNGase and EndoH treatment; data not shown).

We next wanted to confirm the cell surface expression of Dectin-1 on the plasma membrane by immunofluorescent staining using specific Abs against human Dectin-1 (mouse anti-hDectin-1) as described in *Materials and Methods, Chapter 2* **(**Figure 25**)**. Human Dectin-1 (hDectin-1) was readily detected at a noticeable level at the cell surface of RAW Dectin-1 upon immunofluorescent staining of nonpermeabilized cells (Figure 25). The localization of Dectin-1 on the plasma membrane of these cells was much higher than the intracellular levels of Dectin-1 in the cytoplasm (Figure 26**)**, demonstrating that exogenous Dectin-1 was successfully and appropriately targeted to the plasma membrane. Furthermore, the specificity of the mouse anti-human Dectin-1 (manti-hDectin-1) antibody was confirmed by immunofluorescent staining of RAW Dectin-1 cells using a mouse IgG isotype control **(**Figure 26**)**. We also examined the expression of hDectin-1 in RAW WT controls and there was no detectable expression of hDectin-1 on the surface of these cells. Moreover, expression of mouse Dectin-1 was not observed in RAW Dectin-1 cells, as probed by an antibody against mouse Dectin1 (anti-mouse-Dectin-1, R &D Systems) **(**Figure 26**)**.

Clearly, the 'Raw Dectin-1' cell line that we developed for this study presents an excellent cell model system for studying human Dectin-1 signaling, as they are specialized innate immune cells

exogenously expressing hDectin-1 on the cell surface, and they possess all machinery required for Dectin-1 Signaling. Most importantly, Dectin-1–mediated signaling responses induced by Dectin-1-specific ligands can be efficiently compared to RAW WT controls that don't express hDectin-1, and therefore shouldn't be inducing Dectin-1 signaling. Finally RAW Dectin-1 cells cells express negligible levels of mouse Dectin-1, and consequently the Dectin-1 signaling responses initiated in these cells should be only attributed to human Dectin-1 and not to the mouse isoform.

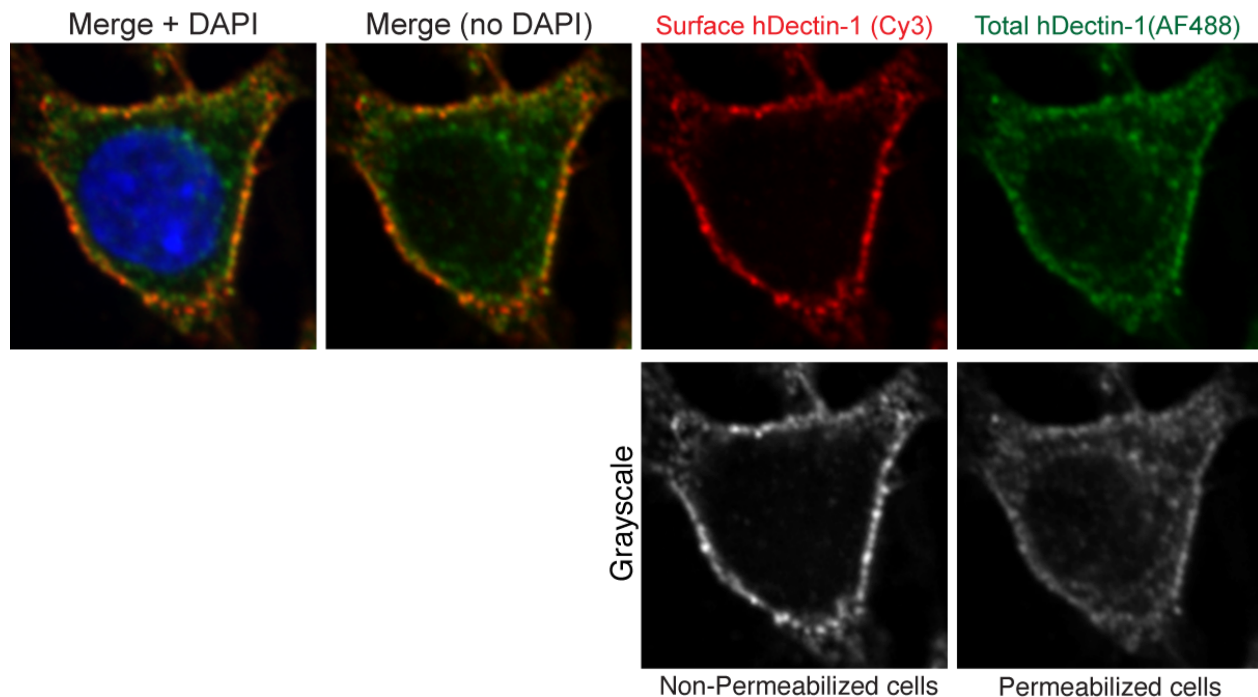
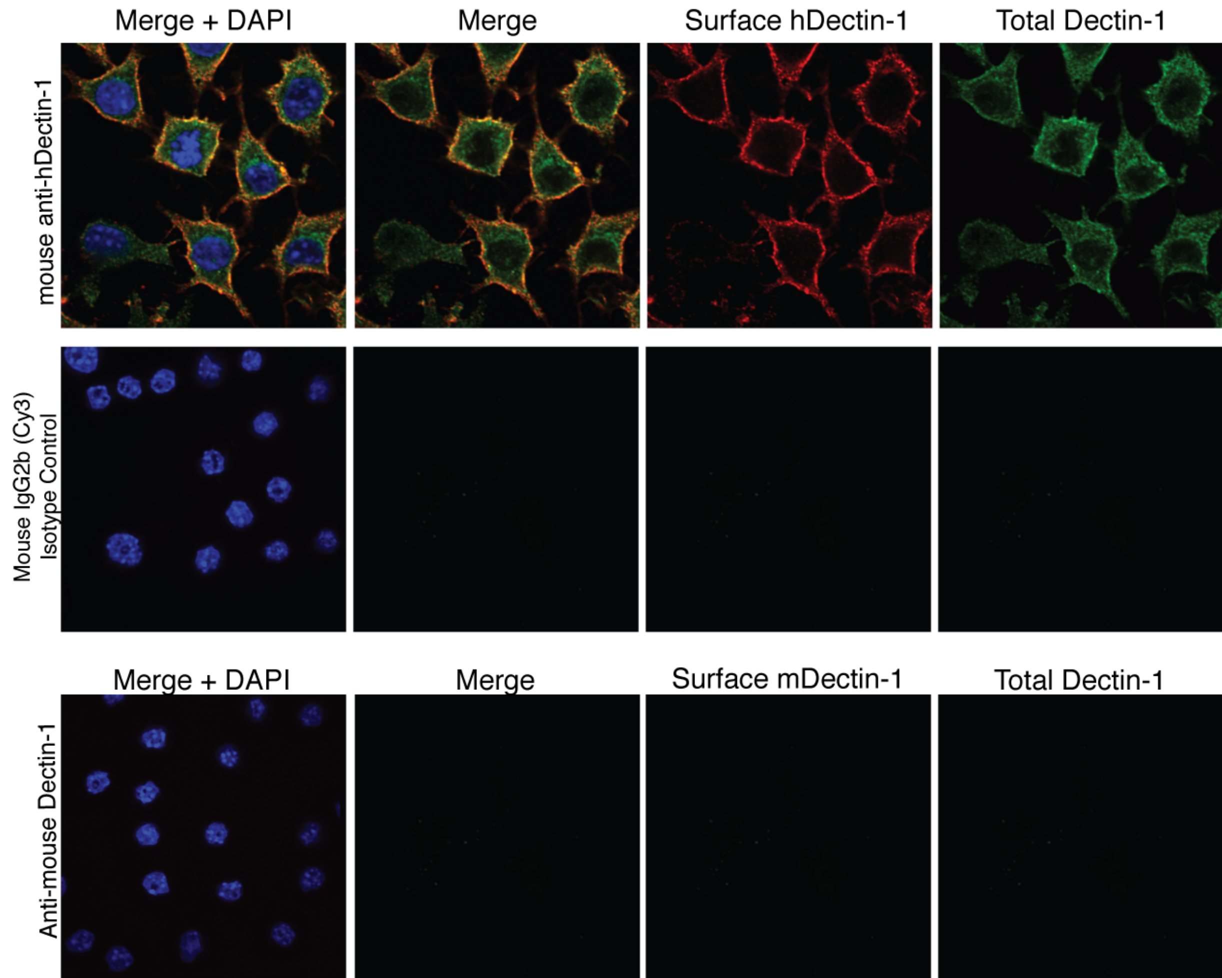


Figure 25: Generation of RAW Dectin-1 Cells Expressing Human Dectin-1 (hDectin-1)

RAW 264.7 macrophages were virally transduced with the pFB-neo plasmid (Stratagene, Agilent Technologies, Santa Clara, CA) containing the human Dectin-1 cDNA. Cells were selected using 1 mg/ml G418 in the culture medium. Expression and surface targeting of Dectin-1 in the RAW Dectin-1 cells was assessed by immunofluorescent staining on nonpermeabilized cells (surface Dectin-1) with a mouse anti-Dectin-1 Ab, followed by a donkey anti-mouse coupled to Cy3 (detected in red channel), which revealed appropriate membrane targeting of the receptor. Following permeabilization with 0.1% Triton X-100, the same cells were stained with the same Ab, except that the secondary was coupled to Alexa Fluor 488 (visualized in green channel), revealing internal structures. DAPI was used to localize the cells' nuclei. Data are representative of three independent experiments. Images were acquired by a spinning disc confocal microscope.



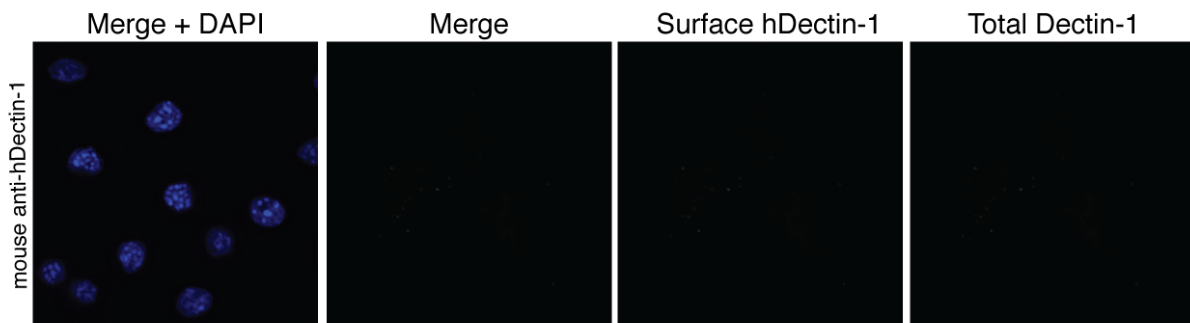


Figure 26: Characterization of Dectin-1 Expression in RAW Dectin1 Cells versus Control RAW WT Cells

To visualize expression of surface Dectin-1 on RAW Dectin-1 cells and RAW WT cells were washed twice with warm PBS followed by 10 min of 4%PFA (paraformaldehyde) fixation. Immunofluorescence on nonpermeabilized cells was then performed by adding mouse anti-human (h) Dectin-1 primary Ab or mouse IgG2b as a mouse isotype control to both RAW 1A Dectin-1 Cells (Panel A) and RAW WT cells (Panel B). This was followed by surface blocking with 5% donkey serum, followed by addition of the secondary antibody, donkey anti-mouse coupled to Cy3 antibody. After permeabilization with 0.1% Triton X-100, the same cells were stained with the same secondary Ab, except that it was coupled to Alexa Fluor 488, revealing internal structures in addition to surface Dectin-1 (Total Dectin-1) only with primary Ab and not mouse isotype control, and only in RAW Dectin-1 Cells. DAPI was used to localize the cells' nuclei. Images were acquired by confocal microscopy, which revealed the membrane targeting of the receptor and little intracellular expression of Dectin-1. Data are representative of five independent experiments. Images were acquired by confocal microscopy.

3.2.2.2. *Dectin-1 Exogenously Expressed on the Surface of RAW Dectin-1 Cells Binds to Dectin-1 Ligands Developed for the Study*

Having established the appropriate cell lines with surface-localized exogenous Dectin-1, as well as various probes and ligands required to induce Dectin-1 clustering, we then moved to examine the ligand binding capacity and functionality of the Dectin exogenously expressed on the surface of RAW Dectin-1 cells.

We first decided to confirm that the surface expression of Dectin-1 would confer RAW macrophages the capacity to bind to β-glucans, the cognate ligands of Dectin-1. We were particularly interested in testing whether the Dectin-1 ligands that we developed for this study would specifically bind to Dectin-1 expressed on the surface of RAW Dectin-1 cells that we generated as described above. For that purpose we established a ligand-binding assay based on an immunofluorescent approach that involves fluorescently labeled ligands. Therefore, the panel of BSA-laminarin conjugates were coupled to the fluorescent dye Alexa Fluor (AF)-546 [AF546] (prepared by the Bundle lab), which enabled us to visualize the ligands in the red channel on a confocal or epifluorescent microscope. To examine the specificity of the binding of these ligands to Dectin-1, the ligand-binding assay was performed on RAW Dectin-1 Cells, and in parallel on RAW WT control cells that do not express Dectin-1. Briefly, cells were incubated with the fluorescent ligands for 10 mins on ice, followed by washes with PBS and fixation with 4% PFA (paraformaldehyde). Immunofluorescent staining of Dectin-1 was then peformed, by labeling Dectin-1 with a different fluorescent probe than AF546 used for labeling the ligands. This enabled us to visualize the localization of Dectin-1 in the cells with respect to the ligands. Incubation of RAW macrophages with various AF546-labeled BSA laminarin conjugates with varying numbers of laminarin molcules, followed by immunofluorescent staining revealed that these fluorescently-labeled Dectin-1 ligands were able to bind to the surface of RAW Dectin-1 cells and not to that of RAW WT control cells, thereby demonstrating the specificity of the

binding of these ligands to Dectin-1 (Figure 27). To further confirm that these ligands specifically bind to Dectin RAW WT cells were transiently transfected with mEmerald Dectin-1 (mEmerald is a variant of the green fluorescent protein GFP). Again as described above, these cells were incubated on ice with selected AF546-labled BSA-laminarin conjugates with different numbers of laminarin, followed by fixation, and visualization by confocal microscopy. Remarkably, the fluorescently labeled BSA-laminarin conjugates (Red) localized to the surface of the RAW WT cells expressing mEmerald-Dectin-1 (Green) and strongly colocalized with Dectin-1 signal present on the plasma membrane (Figure 28). Similar to results described above and demonstrated in (Figure 27), binding of the fluorescently labeled Dectin-1 ligands was not detected in untransfected RAW WT cells, which lack exogenous and endogenous expression of Dectin-1 (Figure 26). Thus, the expression of mEmerald Dectin-1 in RAW WT cells mediates the ability of these cells to bind to Dectin-1 (Figure 28).

In conclusion, RAW Dectin-1 cells have a large proportion of the receptor expressed at the surface, which confers RAW macrophages (which do not express Dectin-1) the ability to specifically bind to β-glucans, the cognate Ligands of Dectin-1. This also confirms that in RAW WT macrophages there is no other receptor or molecule capable of binding to these polysaccharides.

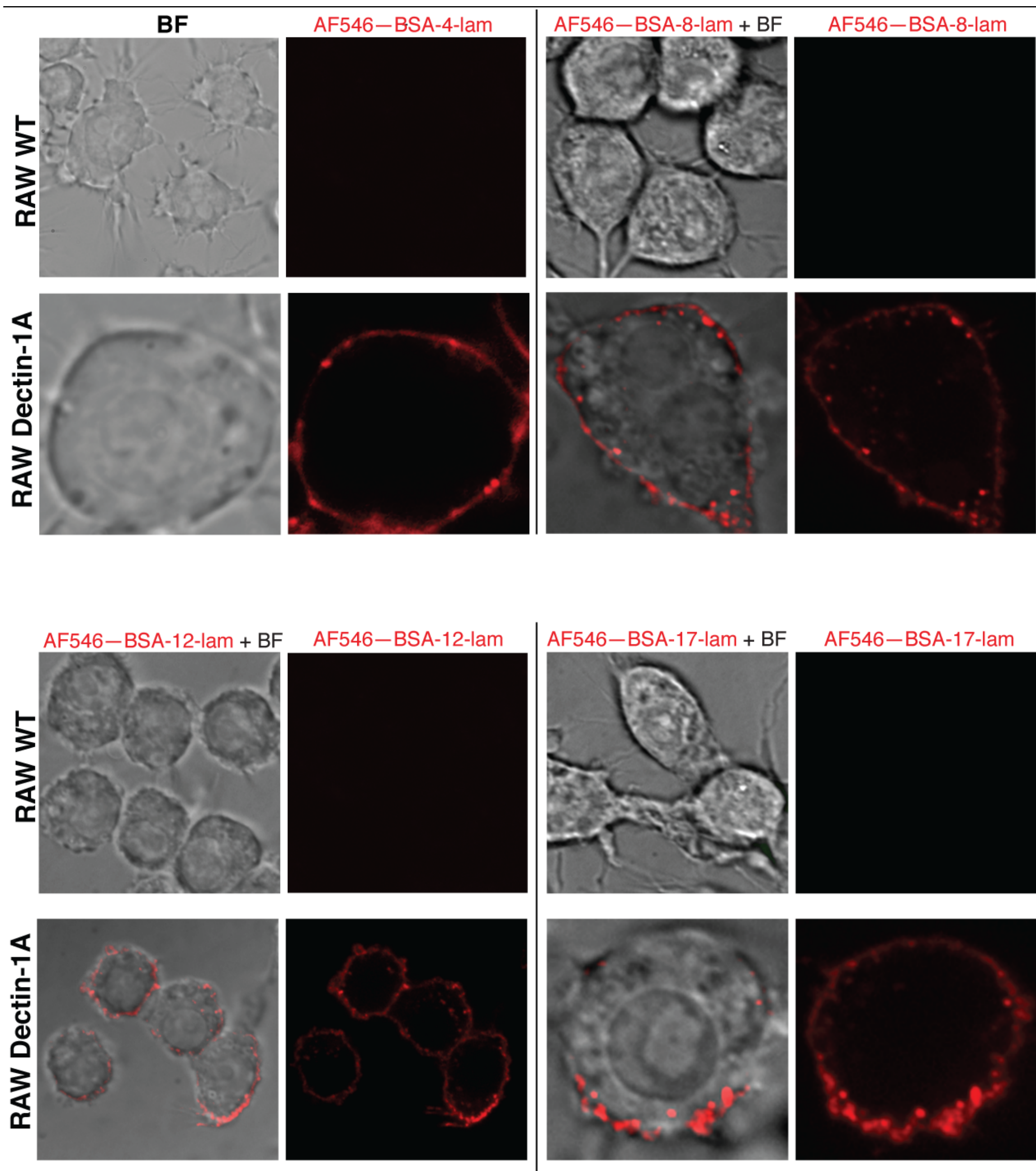


Figure 27: Binding Assay of Fluorescently Labeled Ligands (AF546–BSA-n-lam) to RAW Dectin 1 Cells

Binding Assay of fluorescently labeled (Alexa-Fluor 546) BSA-laminarin conjugates with n=4. 8, 12 and 17 number of laminarin molecules. RAW Dectin 1A and RAW WT cells were washed 2X with ice cold PBS and then incubated with each of the ligands the AF546 labeld BSA-n-laminarin conjugates (indicated in above figure) for 10 min on ice followed by washing with, fixation with 4% PFA and and then imaging by confocal microscopy and the same field of view was also acquired under Bright Field (BF) illumination to visualize cells in case of lack of ligand binding. Data is representative of four independent experiments.

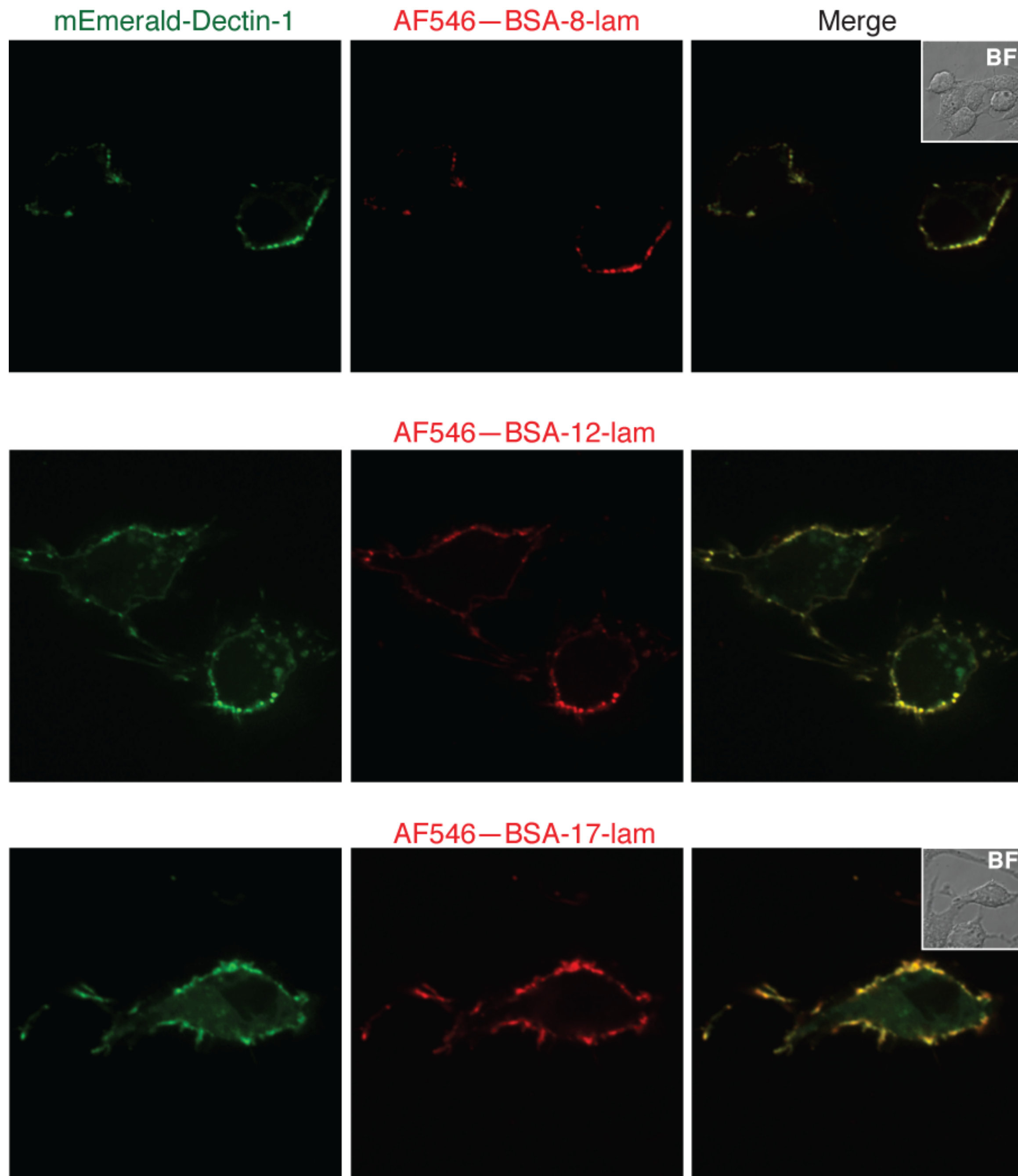


Figure 28: Binding Assay of Various Fluorescently-labeled BSA-laminarin Conjugates to RAW WT Cells Transfected with mEmerald–Dectin-1

RAW WT cells were transfected with mEmerald–Dectin-1 (green), and the following day cells were incubated for 10 mins on ice with 100μg/ml of labeled BSA-laminarin conjugates fluorescently-labeled with Alexa fluor (AF) 546 (red). Cells were then washed 5x with PBS, fixed by 4% PFA and visualized by confocal microscopy. BSA-laminarin conjugates can bind to Dectin-1 could be seen in the merge channel (row three) as surface colocalization

between AF546-BSA-lam in the red channel and Dectin-1 in the green channel. Insets show the same field of view acquired under brightfield (BF) illumination. Data is represantative of three independent experiments. Similar binding assays of AF546-labeled BSA-laminarin conjugates were performed in RAW WT control Cells and revealed no ligand binding (Data not shown).

3.2.2.3. Dectin Exogenously Expressed on the Surface of RAW Dectin-1 Cells is Functional

After confirming that Dectin-1 exogenously expressed in RAW Dectin-1 is successfully targeted to the cell surface and can bind to Dectin-1 ligands, we wanted to test if these cells are functional and can induce Dectin-1–mediated cellular responses **(see section 1.5.3)**.

As described in sections **1.5.3** & **1.5.4**, Dectin-1 is a phagocytic receptor that mediates phagocytosis of particulate Dectin-1 ligands such as zymosan, and is a well-established celluar function mediated by Dectin-1. Accordingly, in order to test the functionality of RAW Dectin-1 cells we chose to investigate the capacity of these cells to induce Dectin-mediated phagocytosis. A phagocytic assay was performed on these cells as described in **section 2.23** (*Material and Methods, Chapter 2*). A useful tool provided by the Bundle Lab for this phagocytic assay is FITC-labeled laminarin -beads **(**Figure 29**)**. These beads are comprised of laminarin molecules conjugated to 3μm polystyrene beads, which were in turn coupled to FITC (a green flourescent dye) to form 'FITC-laminarin beads' that could be visualized by a confocal/epifluorescent microscope in the green channel **(**Figure 29**)**. These beads are large enough to be phagocytosed (3μm) by cells and contain molecules of laminarin, a well-known Dectin-1 ligand, therefore they mimic Dectin-1 particulate ligands, and phagocytosis of these beads should be specifically mediated by Dectin-1. Accordingly, these beads are very specific for Dectin-1–mediated phagocytosis as compared to other Dectin-1 particulate ligands such as zymosan, which in addition to β-glucans contains non-β-glucan ligands that could stimulate phagocytosis by other phagocytic receptors present on the cell surface, therefore producing misleading results. For the phagocytosis assay, RAW Dectin and RAW WT cells were cultured on coverslips in a 12-well plate 24 hours prior to the experiment. The following day cells were treated with FITC-labeled laminarin beads (Green) for 30 minutes at 37°C, followed by synchronization of beads by centrifugation at 8000 rpm thereby allowing the beads to evenly distribute on the cell surface. The 12-well plate was then placed on ice and washed 3X with cold PBS, followed by fixation with 4% PFA (paraformaldehyde). Cells were then blocked in blocking buffer consisting of 5 % DS (Donkey Serum) in PBS. Blocking was then followed by labeling non-permeabilized cells

with a Cy5–labeled anti-FITC antibody (anti-FITC-CY5), at a dilution of 1:1000 in blocking buffer for 30 min. This enabled us to detect non-phagocytosed FITC-labeled laminarin beads in the CY5 channel (blue). Coverslips were then mounted with DAKO and visualized by confocal microscopy. Phagocytosed beads ingested inside the cells appeared in the merged images only in green colour, whereas non-phagocytosed beads outside of the cells were visualized as a merge of green (FITC) and blue (CY5) colours **(**Figure 29**)**. As shown in **(**Figure 29**)**, the phagocytic assay revealed that only RAW Dectin-1 cells were able to phagocytose the FITC-labled beads (green). On the other hand, in RAW WT control cells only a few beads (blue + green) were externally bound to the cell surface and remained outside of the cells unable to get in, which demonstrates that the phagocytosis of the FITC-labeled laminarin beads was Dectin-1–mediated.

This experiment confirms the phagocytic capacity of RAW Dectin-1 cells expressing surface Dectin-1, which further validates the functionality of Dectin-1 in these cells. Therefore, heterologous expression of human Dectin-1 on the surface of RAW macrophages appears to reproduce cellular functions mediated by native macrophages in the host that endogenously express Dectin-1.

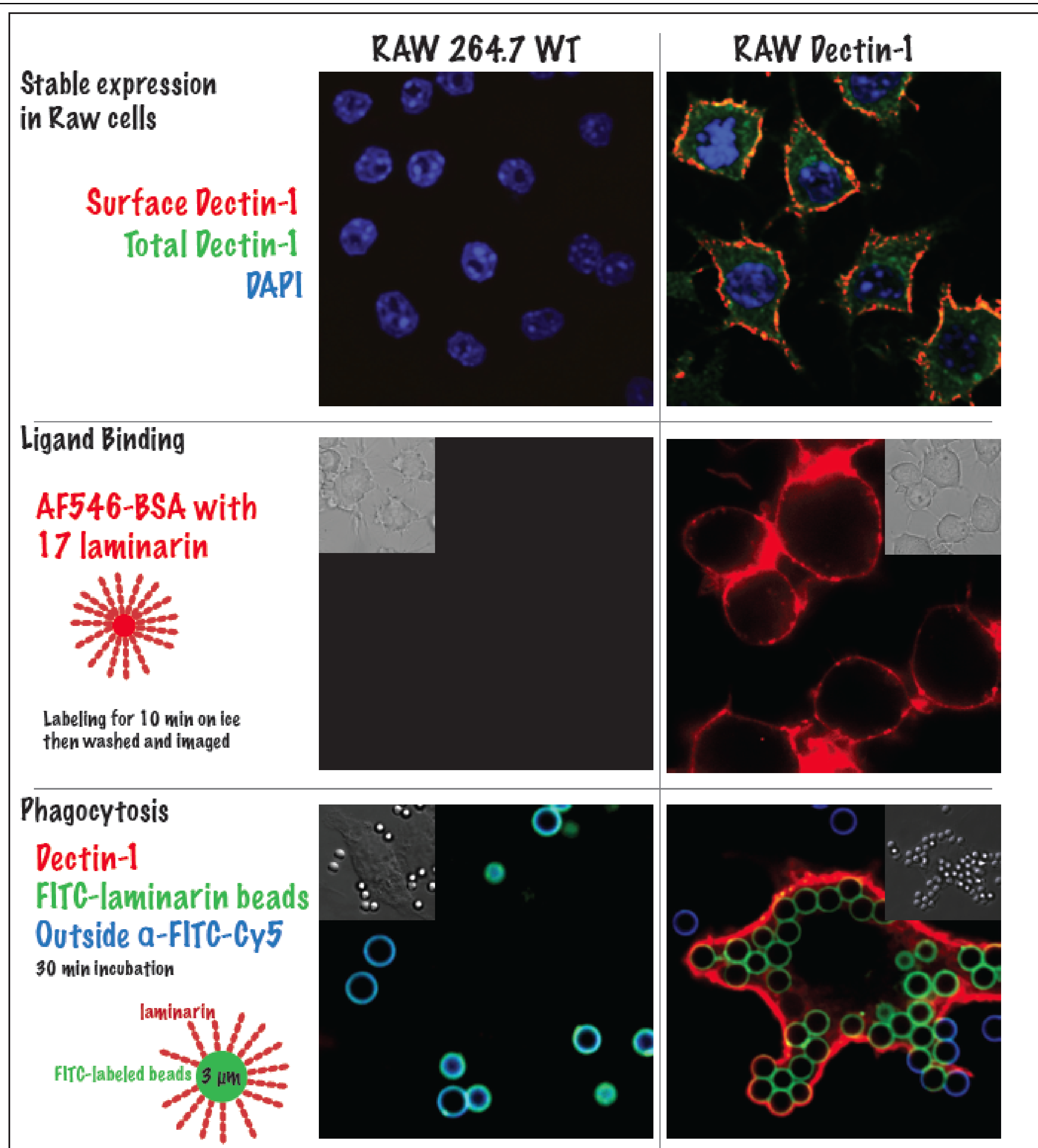


Figure 29: Characterizing the Functionality of Dectin-1 Exogenously Expressed on the Surface of RAW Dectin-1 cells

Above images were visualized by confocal microscopy as well as in bright field illumination of same field of view (insets). Panel 1 shows that RAW Dectin-1 cells, unlike RAW WT, successfully express Dectin-1 mainly on the surface (red signal). Panel 2 demonstrates that RAW Dectin-1 are able to bind to Dectin-1, as seen by the binding of these cells to AF546–BSA-17-lam (red signal), whereas RAW WT cells lacking Dectin-1 expression and are unable to bind to the fluorescent Dectin- ligand as seen by absence of red signal. After confirming Decti-1 expression and ligand binding capacity, the functionality of Dectin-1 in RAW Dectin-1 Cells for inducing Dectin-1 mediated functions was assessed by testing the phagocytic capacity of these cells as compared with RAW WT cells (3rd panel). For the phagocytosis assay cells were seeded on glass coverslips in 12 well plate one day before the experiment. The

following day cells FITC-labeled laminarin beads (green) were washed 3x with PBS centrifuged at 8000 rpm and then resuspended in PBS. Cells were treated with beads for 30 minutes at 37°C with Cells in the 12 well plate were synchronized by centrifugation at 8000 rpm to allow the beads to spread out homogenously on the cells, and then the plate was put back on ice washed 3X with cold PBS, followed by fixation for 10 min with 4% PFA (paraformaldehyde). Cells were then blocked in blocking buffer (5 % Donkey Serum) in PBS, followed by labeling non-permeabilized cells with an anti-FITC-Cy5 (blue) antibody in blocking buffer (1:1000) for 30 min. Coverslips were then mounted with DAKO and visualized by confocal microscopy. As seen in figure non-phagocytized beads outside the cells are labeled in blue + green, whereas internalized beads are labeled only in green. All above Data is representative of 3 independent experiments.

3.2.2.4. Dectin Exogenously Expressed in RAW Dectin-1 Cells Can Induce Signaling Upon Binding of P-curdlan and BSA-laminarinCconjugates

After confirming that Dectin-1 exogenously expressed on the surface in RAW Dectin-1 is functional and can bind to particulate Dectin-1 ligands to induce cellular esponses such as phagocytosis, we decided to examine whether the P-curdlan and BSA-laminarin conjugates that we developed (Bundle Lab) could activate well characterized Dectin-1 signaling readouts in these cells.

SYK is a pivotal tyrosine kinase activated upon Dectin-1 ligation, and its activation is a hallmark of Dectin-1 Signaling **(see section 1.5.4.1)**. SYK is one of the best-characterized signaling effectors activated downstream of Dectin-1 stimulation, and is considered a very characteristic and specific signaling readout for examining Dectin-1 Signaling. Thus, to further validate the function and signaling capacity of Dectin-1 stably expressed in RAW cells we decided to examine SYK activation upon treatment of RAW Dectin-1 and RAW WT cells with or without ligands

We first wanted to confirm that RAW Dectin-1 cells could induce signaling upon stimulation with the water-soluble P-curdlan prepared for this study. Although curdlan is well recognized in the Dectin-1 literature as a potent ligand of Dectin-1, the curdlan used in most of these Dectin-1 studies was mainly a macroparticulate form of curdlan that is largely insoluble. Therefore it was of particular interest, before examining the effect of our panel of different-sized water-soluble ligands on Dectin-1 activation, to ensure that the phosphorylation modification introduced to curdlan (generating the highly soluble P-curdlan) in order to improve its poor solubility wouldn't affect curdlan's well-known capacity for inducing Dectin-1 signaling **(see section 1.6,** *Chapter 1 & Material and Methods, Chapter 2***)** (Li et al., 2014; Novak and Vetvicka, 2008; Popescu et al., 2013). Upon SYK activation, the tyrosine residues Y525 and Y526 (numbered according to their position in human SYK) located in the catalytic activation loop of SYK are phosphorylated

(Mocsai et al., 2010). Therefore to examine the ability of P-curdlan to induce Dectin-1 signaling, we performed immunoblotting of cell lysates from RAW WT or RAW 1A (expressing Dectin-1) with or without P-curdlan (Figure 30) and probed for SYK phosphorylation at the residues Y525 & Y526 (Y525/Y526) using a phosphospecific antibody detecting SYK phosphorylation at these residues [phospho-(Y525/Y526)]. It is important to note that the cells were serum-starved for 4-6 hours before the experiment in order to reduce the background signal of SYK pkhosphorylation. As seen in Figure 30, treatment of RAW Dectin-1 cells with P-curdlan for 10 mins stimulated a significant increase in SYK phosphorylation at Y525/Y526 as compared with unstimulated cells. Interestingly, SYK phosphorylation at these sites was not detected in RAW WT cells upon treatment with P-curdlan, indicating that the signaling response of SYK activation induced in RAW Dectin-1 cells is Dectin-1–dependent (Figure 30). Interestingly, P-curdlan, a highly soluble β-glucan, was able to induce Dectin-1 signaling in the RAW macrophage cell line developed by us, despite several reports stating that Dectin-1 is not activated by soluble ligands and is only activated by particulate ligands (Batbayar et al., 2012; Brown and Gordon, 2003; Goodridge et al., 2011).

Therefore Dectin-1 exogenously expressed on the plasma membrane of RAW Dectin-1 cells is capable of inducing Dectin-1 signaling events. Furthermore, P-curdlan, despite the introduction of a phosphorylation modification, is still capable of inducing the critical, upstream Dectin-1 signaling event of SYK phosphorylation (Figure 30).

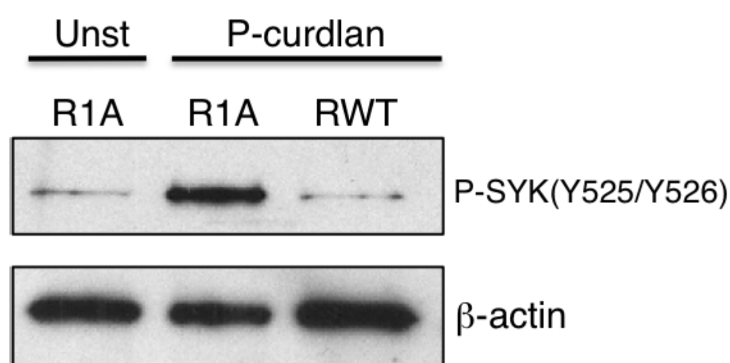


Figure 30: Dectin-1 Exogenously Expressed on the Surface of RAW Dectin-1 cells is Capable of Activating Dectin-1 Signaling in Response to P-curdlan Stimualtion

RAW Dectin-1 or RAW WT cells were serum starved for 4-6 hours and then washed 3x with PBS followed by treatment with P-curdlan at 100μg/ml in PBS for 10 min at 37°C. Cell lysates were prepared, separated by SDS-PAGE, transferred onto nitrocellulose membrane, and blotted with Abs against phospho-(Y525/Y526) Syk and against β-actin as loading controls. Data are representative of three similar experiments.

SYK is a cytosolic protein that is recruited to the cytosolic side of the plasma membrane upon engagement of its SH2 domains on phosphorylated ITAM or hemITAM domains, which is a crucial requirement for SYK activation **(see section 1.3.4).** We thus sought to investigate the recruitment of SYK in response to treatment with BSA-laminarin conjugates, as an alternative signaling readout for SYK activation in response to Dectin-1 stimulation. The recruitment of SYK was visualized by live cell imaging of cells on a spinning- disc confocal microscope. A day before the experiment RAW Dectin-1 and RAW WT cells were transiently transfected with a plasmid encoding SYK-GFP. The following day cells were treated on ice for 5 minutes (at 4˚C to avoid ligand uptake by endocytosis) with the fluorescently labeled BSA-laminarin conjugate, AF546–BSA-12-lam (Figure 31). Ligands were then washed off thoroughly to remove excess in bound ligand, then the recruitment of SYK, as well as ligand binding were simultaneously monitored at room temperature for 10 minutes by confocal microscopy at different time points indicated (Figure 31).

Upon treatment of RAW Dectin-1 cells with AF546–labeled BSA-laminarin conjugate, the ligand gradually bound to the cell surface. Ligand binding over the time course of the experiment was accompanied with a progressive recruitment of SYK-GFP to the cell membrane, which colocalized with the red signal of the fluorescently labeled ligand. In contrast, RAW WT cells until the end of the duration of the experiment (as demonstrated by the image of a cell acquired at the timepoint of 10 minutes at the end of the experiment; see Figure 31) revealed no obvious ligand binding, nor SYK recruitment to the plasma membrane. This proved that the BSA-laminarin ligands binding to Dectin-1 on the cell surface of RAW Dectin-1 are capable of inducing SYK recruitment to the plasma membrane, which is an essential prerequisite for SYK activation.

In conclusion, RAW Dectin-1 cells are able to bind β-glucans, unlike RAW WT cells, which lack Dectin-1 expression. Binding of β-glucans is correlated with the recruitment of SYK to the plasma membrane.

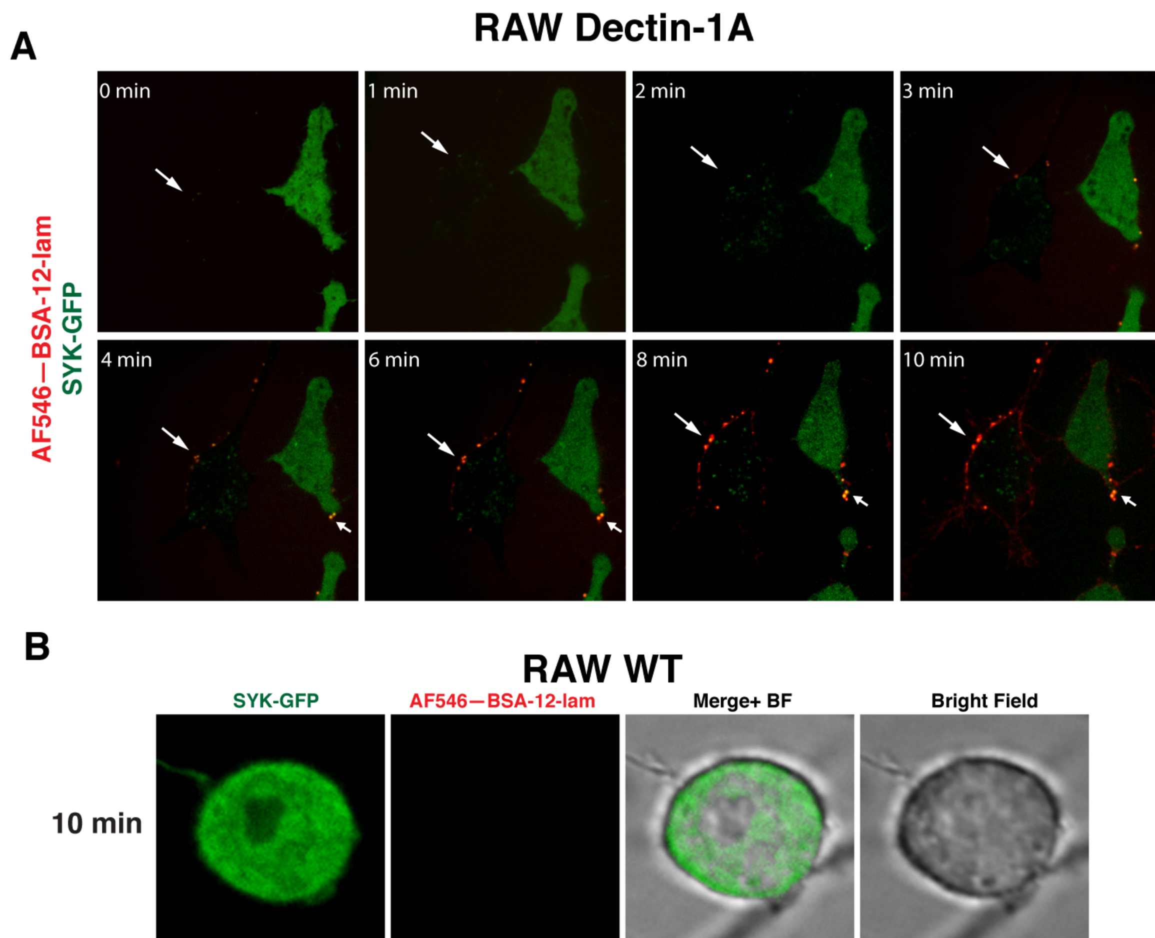


Figure 31: Time-lapse of Live cell Imaging Simultaneously Monitoring Ligand Binding and SYK Recruitment

RAW Dectin-1 and RAW WT Cell were gently washed 3 times with PBS after incubation with fluorescently-labeled ligand AF546–BSA-12-lam for and imaged live at room temperature on a confocal microscope for a time course of 10 minutes at different timepoints labeled in white in the above figure. Ligand binding was monitored through the red channel (AF546) and SYK-GFP recruitment via the green channel. (A) Some of the cells transfected with SYK-GFP were oversaturated due to high levels of expression SYK-GFP, thus the laser intensity of the green (GFP) channel was geatly reduced to facilitate to monitor SYK recruitment. One of the cells with a faint GFP signal (cell on the left) was easy to follow for ligand binding and SYK recruitment. White arrows indicate colocalization sites of ligand binding and SYK recruitment to the plasma membrane. (B) A selected image of RAW WT transfected with SYK-GFP cell at the 10 min time point at the end of the experiment revealed abscence of red signal indicating failure of ligand binding. Also cells showed no recruitment of SYK-GFP to the cell surface. Data represents one of three replicates.

Overall, experiments described in this section, and the previous section **(3.2.2.3)**, demonstrate the functionality and signaling capacity of Dectin-1 exogenously expressed on the surface of RAW Dectin-1 Cells. Hence, heterologous expression of human Dectin-1 in RAW macrophages most likely reproduces normal conditions and functionality of native macrophages endogenously

expressing Dectin-1.

3.2.2.5. Dectin-1 is Endogenously Expressed on the Surface of BMDMs and BMDCs and is able to Bind to Fluorescently-labeled Dectin-1 Ligands

To further validate our model of the 'RAW Dectin-1' macrophages exogenouly expressing Dectin-1, we decided to additionally utilize primary immune cells, endogenously expressing Dectin-1, for selected experiments of this study. This would give us the advantage of comparing our results from the RAW macrophage cell line to those provided by native immune cells of the host naturally expressing Dectin-1. We therefore generated primary Bone Marrow-derived Dendritic cells (BMDCs) from mice, as well as primary mouse Bone Marrow-derived Macrophages (BMDMs) (described above in *Materials and Methods, Chapter 2*). Next, we decided to examine the expression of Dectin-1 in these primary cells and test their ability to bind to fluorescently labeled β-glucans.

First, expression of mouse Dectin-1 in BMDCs and BMDMs was determined by immunofluorescence. BMDCs were cultivated as described in *Materials and Methods, Chapter 2.* Following fixation of non-permeabilized BMDCs with 4% PFA, surface Dectin-1 was immunostained using rat anti-mouse Dectin-1 antibody followed by an anti-rat antibody coupled to Cy3 (a red fluorescent dye). A strong red fluorescent signal was captured on the spinning-disk confocal microscope, which illustrated the membrane expression of Dectin-1 in primary BMDCs **(**Figure 32**)**. Similarly, immunofluorescent (IF) staining of non-permeabilized BMDMs followed by confocal microscopy demonstrated the surface expression of Dectin-1 in these cells, as seen by a strong red signal of the plasma membrane (Figure 33**)**. An isotype control was also performed in parallel to the immunostaining of Dectin-1 in BMDMs to ensure the specificity of Dectin-1 detection. Briefly, in the IF protocol described above, a mouse isotype control (mouse IgG2b) was used instead of the rat anti-mouse Dectin-1 antibody, followed by a Cy3-labeled secondary Ab (red). Visualization of these cells by confocal microscopy revealed no red signal on the cell membrane, thereby confirming the specificity of the anti-mouse Dectin-1 antibody used to mouse Dectin-1 expressed on the surface of these cells (Figure 33**)**.

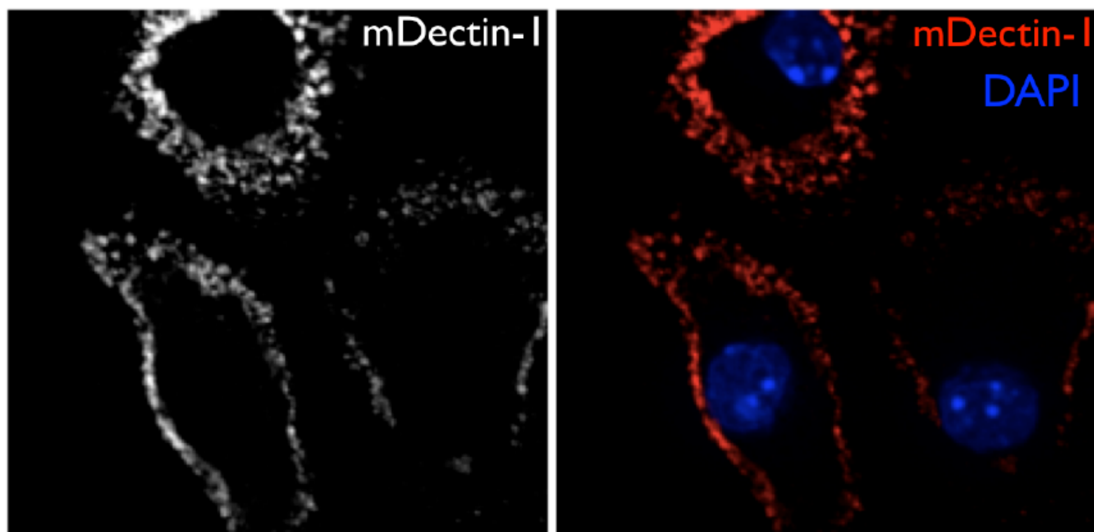


Figure 32: Mouse Bone-Marrow-Derived Dendritic Cells (BMDCs) Endogenously Express Dectin-1

The surface expression of endogenous mouse Dectin-1 in BMDCs was confirmed by immunofluorescence (IF). The cells were fixed with 4% PFA and immunostained with rat anti-mouse Dectin-1, mouse anti-human Dectin-1, followed by detection with the appropriate Cy3-coupled secondary antibodies (shown in green). Cell nuclei were stained with DAPI (blue). Images were visualized on a spinning disc confocal microscopy. Data is representative of five independent experiments.

Western blotting of cell lysates prepared form BMDCS and BMDMs further confirmed the endogenous expression of Dectin-1 in these cells (Figure 34). Briefly, SDS-PAGE of cell lysates was performed, followed by immunoblotting to probe for Dectin-1 expression using a rat anti-mouse Dectin-1 antibody with subsequent incubation with (HRP)-coupled secondary antibody. ECL treatment and film development of the western blot revealed two bands: a higher molecular weight band between 37 and 50 kDa corresponding to the mature complex glycosylated form of Dectin-1, and another lower molecular weight band between 25 and 37 kDa of Dectin-1 at the predicted molecular weight of full length Dectin-1 (28 kDa). As mentioned in section **1.5.1**, Dectin-1 is a type II transmembrane protein and the expression of the complex glycosylated form of the Dectin-1 in BMDMs and BMDCs as revealed by western blotting confirms the appropriate folding and surface targeting of Dectin-1 in these cells, which is in accordance with immunofluorescent experiments demonstrating the surface expression of endogenous Dectin-1 (Figure 32 & Figure 33).

Next, after confirming endogenous expression of mouse Dectin-1 in BMDMs and BMDCs, we

wanted to test whether or not these cells are capable of binding to Dectin-1 ligands. With the fluorescently labeled BSA-laminarin conjugates we had a unique reagent, which allowed us to test the β-glucan binding capacity of Dectin-1 in primary immune cells. The same ligand-binding assay, as described above in **section 3.2.2.2**, was performed on BMDCs, BMDMs in addition to RAW WT cells, which were used as control cells that do not bind to Dectin-1.

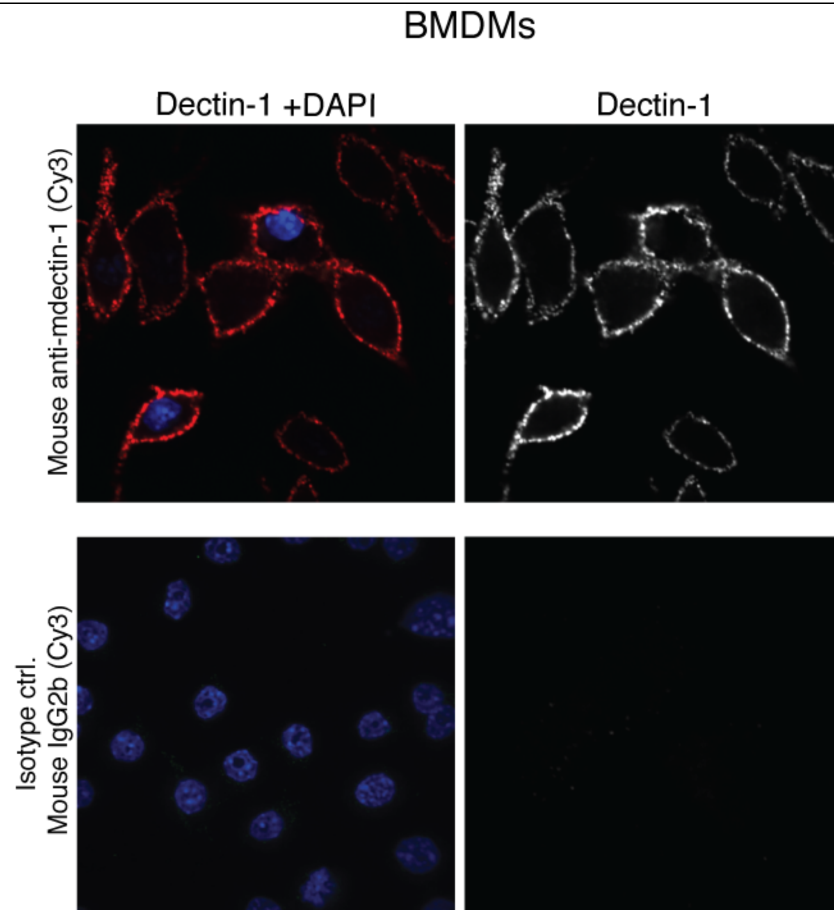


Figure 33:Dectin-1 Expression in Mouse Bone-Marrow-Derived Macrophages BMDMs

The surface expression of endogenous mouse Dectin-1 in was probed by immunofluorescence (IF). The cells were fixed with 4% PFA and immunostained with rat anti-mouse Dectin-1, mouse anti-human Dectin-1, or mouse IgG2b isotype control antibodies, followed by detection with the appropriate Cy3-coupled secondary antibodies (shown in green). Cell nuclei were stained with DAPI (blue). Images were visualized by confocal microscopy. The lack of labeling of BMDMs with the isotype control demonstrates the specificity of the anti-mouse Dectin-1 antibody to its Dectin-1 antigen. Data is representative of three independent experiments.

Cells were incubated with AF546 labeled BSA-laminarin conjugates (red) for 10 minutes on ice, followed by fixation of non-permabilized cells, and subsequent immunofluorescent staining of Dectin-1 in green. Visualization of cells by confocal microscopy revealed binding of fluorescently labeled Dectin-1 ligands to Dectin-1 expressed on the surface of BMDMs and BMDCs, but not RAW WT cells, as seen by remarkable colocalization between the green signal

representing plasmalemmal Dectin-1 and the red signal of the AF546-labeled BSA-laminarin conjugates (Figure 35).

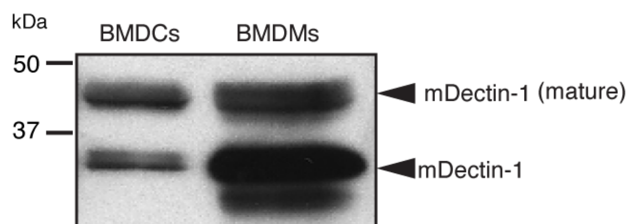


Figure 34: Western blot of Expression of Dectin-1 in BMDMs and BMDCs

The expression of endogenous mouse Dectin-1 in Bone Marrow-Derived Macrophages (BMDMs) and Bone Marrow-Derived DCs (BMDCs) as probed by western blotting. SDS-PAGE of cellular lysates, followed by immunoblotting was performed to detect expression of endogenous expression of mouse Dectin-1 using rat anti-mouse antibody against Dectin-1 (1:1000) in BMDMs) and subsequent incubation with anti-rat IgG HRP-coupled secondary (1:5000). ECl treatment followed by film development revealed two bands; a higher band corresponding to mature Dectin-1 (glycosylated form) and the lower band corresponding to the predicted Molecular weight of full length Dectin-1 (~28 kDa). The positions of molecular weight standards, kiloDaltons (kDa), are indicated to the left of the immunoblots. This western blot is represantative of three independent experiments.

Therefore both immunoblotting and immunofluorescence (IF) experiments reveal that Dectin-1 is endogenously expressed on the cell surface as a glycosylated and mature form of the receptor, that is targeted to the plasma membrane, and which is able to specifically bind to Dectin-1 ligands.

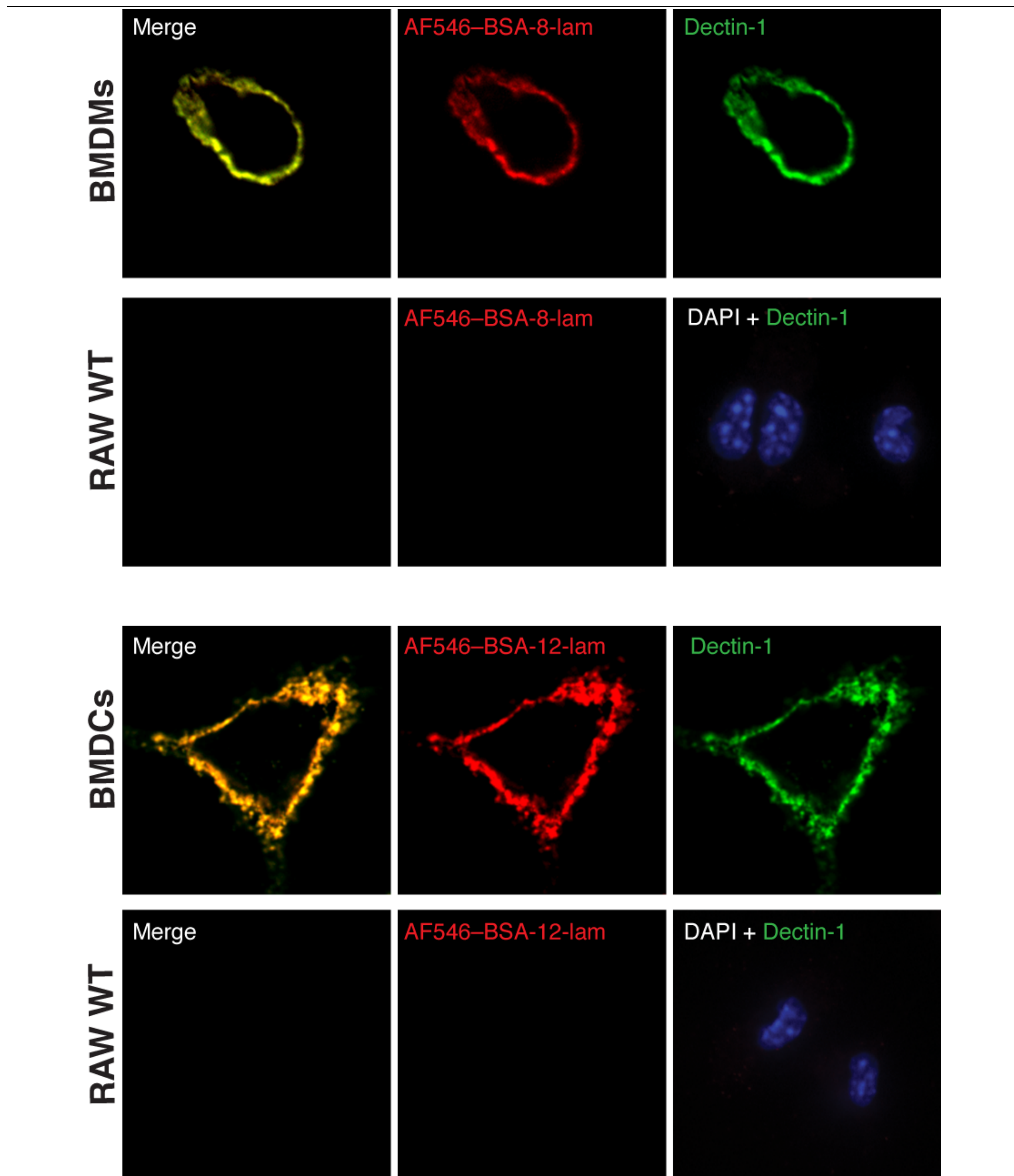


Figure 35: Ligand Binding Assay of BSA-laminarin Conjugates to BMDMs and BMDCs

Ligand binding assay was performed for BMDMs, BMDCs or RAW WT cells as described in Figure 28. Briefly, cells were incubated on ice with AF546-coupled BSA-laminarin conjugates dissolved at 100 µg/mL in serum free hRPM ice-cold culture medium. Cells were then washed 5x with ice cold PBS, and fixed for 10 mins at room temp. with 4% PFA. Non-permeabilized cells were then immunostained for surface Dectin-1 expression (green channel). Ligand binding to Dectin-1 was then visualized by imaging cells on a confocal microscope. All images shown are representative results from three independent experiments.

3.2.3. The Effect of Ligand size on Dectin-1–induced Upstream Signaling

After establishing the cell lines and different probes for inducing Dectin-1 clustering, we then moved forward to investigate if the size of the β-glucan correlates with the magnitude of the induced signaling events. This investigation was performed through immunoblotting and immunofluorescence experiments.

3.2.3.1. Effect of Ligand Size on Dectin-1 Upstream Signaling Events in Raw Dectin-1 Cells

Experiments described above, in **section 3.2.2,** demonstrate that the RAW Dectin-1 cell line that we developed is a convenient and appropriate model to study Dectin-1 signaling in an innate immunity context. Most of the known molecular machinery involved in Dectin-1 signaling is reproduced in the context of RAW Dectin-1 cells. Having access to the appropriate cell system and water-soluble Dectin-1 ligands, we confidently moved on to address the main research question of this study, which is examining the effects of ligand size on the induction of Dectin-1–mediated signaling events. We first decided to investigate the effect of the ligand size on early, upstream signaling events triggered by Dectin-1 stimulation, such as activation of Src, SYK, PKCδ and PLC-γ2 **(see section1.5.4)**.

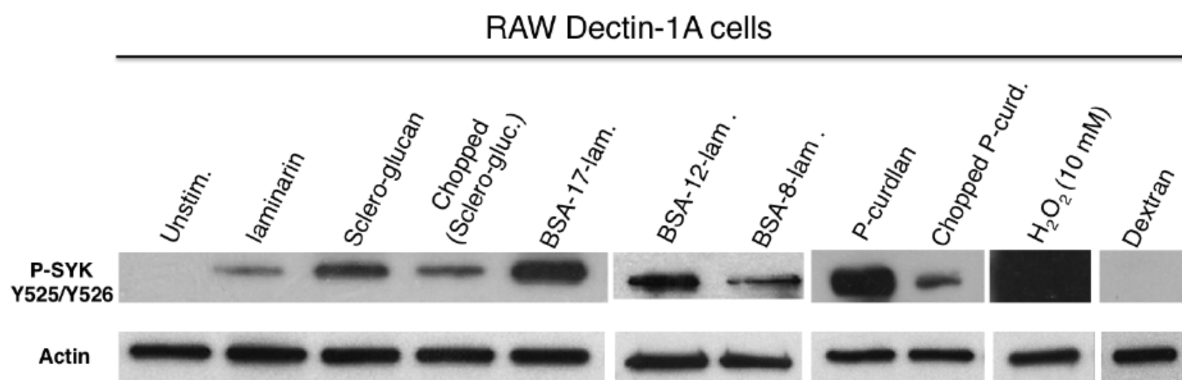


Figure 36: SYK Phosphorylation as a Function of Ligand Size

SYK phosphorylation a hallmark of Dectin-1 signaling was assessed by immunoblotting in RAW Dectin-1 Cells in response to a variety of naturally-derived Dectin-1 ligands of varying size, as well as to a panel of BSA-laminarin conjugates with increasing numbers of laminarin. Cells were serum-starved for 4-6 hours, and then stimulated with ligands at a concentration of 100 μg/mL in serum-free sterile culture medium at 37°C. Cells were promptly washed 5x with PBS to wash off the ligands, promptly lysed, processed by SDSPAGE, and immunoblotted for the presence of phosphorylated SYK at Y525/Y526 residues, as a readout for SYK activation. β-actin was also probed in the immunoblot as protein loading control. Stimulation with H_2O_2 was used as a positive control for SYK phosphorylation and dextran, a non-Dectin-1 sugar ligand, was used as a negative control of Dectin-1 stimulation. Results from this western blot are representative of three similar experiments.

As mentioned above in **section 3.1**, soluble β-glucans are deemed by some as biologically

inactive ligands that are incapable of activating the Dectin-1 receptor, unlike particulate ligands (Brown, 2006; Brown et al., 2003; Goodridge et al., 2011). Therefore it was particularly important for our study to rule out the effects of β-glucan solubility, or inhomogeneity of the β-glucan preparation, on their capacity to induce Dectin-1 signaling, in order to accurately attribute the observed signaling responses to merely the size of the β-glucan. We thus considered assessing via immunoblotting SYK phosphorylation in RAW Dectin cells treated with soluble β-glucans of variable size (Figure 36). Naturally-derived ligands, of increasing size/molecular weight were used for this experiment: P-curdlan>Scleroglucan>laminarin; as well as, a panel of BSA-laminarin conjugates with an increasing number of laminarin molecules: 8, 12 and 17 (Figure 36). The Bundle lab also generated smaller fragments of P-curdlan and scleroglucan (a branched water-soluble β-glucan), by hydrolysis (rendering 'Chopped P-curdlan' and 'Chopped Scleroglucan'), which gave us a further advantage to assess size effects of β-glucan on Dectin-1 activation. Cells were also treated with H_2O_2 (10mM), which is a positive control for induction of SYK phosphorylation. Additionally, RAW Dectin-1 cells were treated with dextran, a non-β-glucan sugar that is not a Dectin-1 ligand. Dextran was therefore used as a negative control that doesn't induce Dectin-1 signaling. Figure 36, shows the western blot of cell lysates prepared from RAW Dectin-1 cells stimulated by the above-mentioned followed and probed for phosphorylation of SYK at Y525/Y526. Remarkably, as revealed by the immunblot, progressive increase in ligand size induced a corresponding increase in the levels of SYK phosphorylation in the following order P-curdlan>scleroglucan>laminarin and BSA-17-lam>BSA-12-lam>BSA-8-lam (Figure 36). Also intriguingly, generating smaller fragments of Sceleroglucan, 'chopped sceleroglucan and smaller fragments of 'P-curdlan', Chopped P-curdlan' induced lower levels of SYK phosphorylation than their undegraded, full molecular weight, counterparts, i.e., P-curdlan>Chopped P-curdlan, and Sceleroglucan>Chopped Sceleroglucan (Figure 36). It is hard though, to compare the size effects of the BSA-laminarin panel with the naturally-derived β-glucans, thus, to avoid confusion, the size effect of these two sets of Dectin-1 ligands was assessed separately. As explained in **section 1.6.6**, laminarin, a small β-glucan of low molecular weight, is described as a blocker (inhibitor) of Dectin-1 that is incapable of inducing Dectin-1 signaling events (Batbayar et al., 2012; Brown and Gordon, 2003; Goodridge et al., 2011). Interestingly in this experiment, laminarin induces SYK phosphorylation in RAW Dectin-1 at a level higher than that of unstimulated cells (Figure 36). Furthermore laminarin stimulates the

lowest level of SYK phosphorylation in comparison to all of the other larger-sized ligands used for this western blot experiment (Figure 36).

As predicted the positive control H_2O_2 induced intensive SYK phosphorylation in accordance with its potent ability to induce SYK activation. On the other hand, the negative control dextran, as expected, didn't induce any Dectin-1 signaling in the RAW Dectin-1 Cells, demonstrating that SYK activation in these cells is mediated via β-glucan ligand stimulation of Dectin-1 (Figure 36).

To further validate that the observed signaling responses induced by these ligands in RAW Dectin-1 cells were specific to Dectin-1 stimulation. We therefore performed a western blot experiment similar to the one described above where, we stimulated RAW WT control cells in parallel to RAW Dectin-1 cells with the selected ligands P-curdlan, laminarin and the BSA-12-lam conjugate. Phosphorylation of SYK did not occur in RAW WT controls indicating that SYK activation observed in RAW Dectin-1 cells is Dectin-1–dependent (Figure 37).

In conclusion, increasing the size or molecular weight of the soluble Dectin-1 ligand seems to increase the magnitude of SYK phosphorylation, a key signaling event of Dectin-1 upstream signaling.

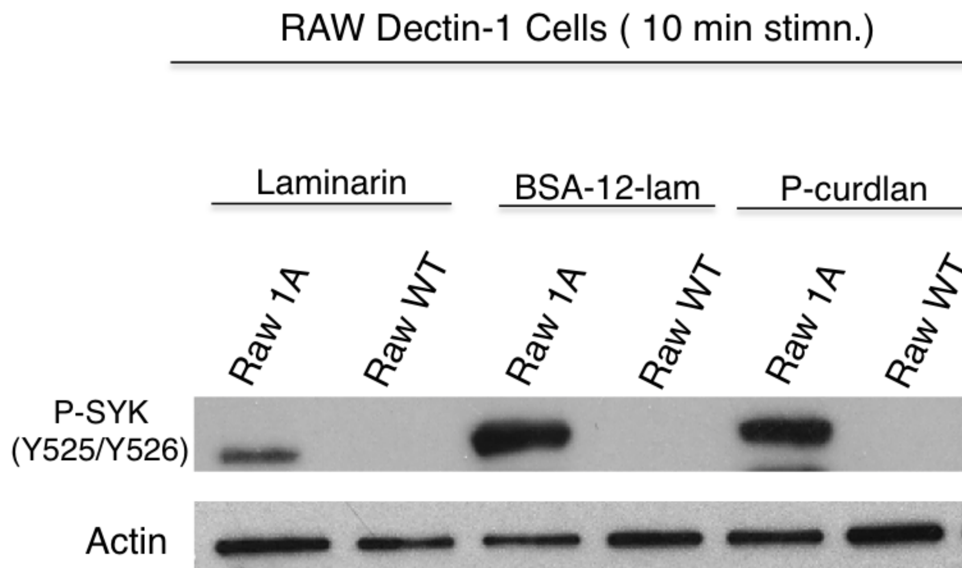


Figure 37: SYK Phosphorylation in RAW Dectin-1 cells versus RAW WT cells in Response to Dectin-1 Ligands of Different Size

SYK phosphorylation was probed by immunoblotting in RAW 1A cells versus RAW WT cells. Cells were serum starved for 4-6 hours and then treated with ligands indicated in figure at a concentration of 100 µg/mL in serum-free sterile culture medium at 37°C for 10 min at 37°C. Cell lysates were prepared, separated by SDS-PAGE, transferred on nitrocellulose membrane, and blotted with Abs against phospho-(Y525/Y526) SYK and against β-actin as protein loading control. Data is representative of five independent experiments.

Since some of the naturally-derived ligands in the previous experiment are not precisely characterized in terms of size and molecular weight, a better approach to examine the effect of β-glucan size differences on Dectin-1 activation, is to use multivalent ligands of Dectin-1 with varying numbers of the ligand exposed on a gel platform (e.g., polyacrylamide gel) or conjugated to a protein carrier such as BSA (Bovine Serum Albumin). Accordingly, as mentioned above in **section 3.2.1,** we generated, in collaboration with the group of Dr. Bundle, a series of BSA protein conjugates containing increasing numbers of the β-glucan laminarin molecules. By adjusting the coupling of laminarin and through estimation of the conjugate molecular weight by SDS-PAGE, we obtained a panel of BSA-laminarin conjugates, consisting of the BSA carrier protein covalently coupled to variable numbers of laminarin molecules (from 4 to 20 laminarin molecules). Indeed as shown in Figure 36, BSA-laminarin conjugates were capable of stimulation of SYK in RAW Dectin-1 cells. Even the small β-glucan laminarin showed minimal SYK activation, but increasing, the valency on the BSA protein potentiated the response several fold.

As explained in **section 1.5.4**, Dectin-1 signaling requires phosphorylation of its cytosolic hem-ITAM motif on tyrosine 15 by Src family kinases (SFKs), supposedly Src, allowing the phospho-ITAM–like motif to recruit SYK to mediate further downstream signaling (Batbayar et al., 2012; Brown, 2006a; Goodridge et al., 2009b). According to this current model of activation of the Dectin-1 hemITAM motif, Dectin-1-dependent activation of Src family kinases (mainly the Src isoform) is upstream of SYK activation resulting in SYK recruitment to the plasma membrane and subsequent activation of SYK via phosphorylation of key tyrosine residues (Goodridge et al., 2012; Kerrigan and Brown, 2010).

Therefore, to further verify that increasing the number of laminarin molecules coupled to the BSA protein carrier could indeed promote Dectin-1–signaling, we next sought to investigate the ability of these conjugates to stimulate various levels of the key upstream event of Src family kinase activation in analogy to SYK phosphorylation induced in these cells as demonstrated in Figure 36. We stimulated RAW Dectin-1 cells with the BSA protein conjugates containing 4, 9 or 17 laminarin molecules, in addition to the water-soluble naturally-derived β-glucans, laminarin and P-curdlan **(** Figure 38**)**. We then probed for SYK phosphorylation (Y525/Y526) and SFK/Src phosphorylation (Y416) by immunblotting using an anti-phospho (Y416) Src Ab that globally reports SFK activation. As seen in the western blot in Figure 38, a remarkable parallel can be seen between the number of β-glucan laminarin molecules on the BSA conjugates and the level

of SFK phosphorylation, which also closely follows the trend of SYK phosphorylation. Moreover BSA protein carrier with the highest number of laminarin, BSA-17-lam, induced Src and SYK activation at a comparable level to that induced by P-curdlan. This confirms that increasing the valency of the Dectin-1 ligand correlates with the magnitude of the upstream signaling event induced by Dectin-1. Therefore, in conclusion increasing the valency of the laminarin molecules on the BSA protein carrier, increases the Dectin-1 signaling capacity for inducing key upstream mediators of the Dectin-1 signaling pathway, namely SFKs and SYK.

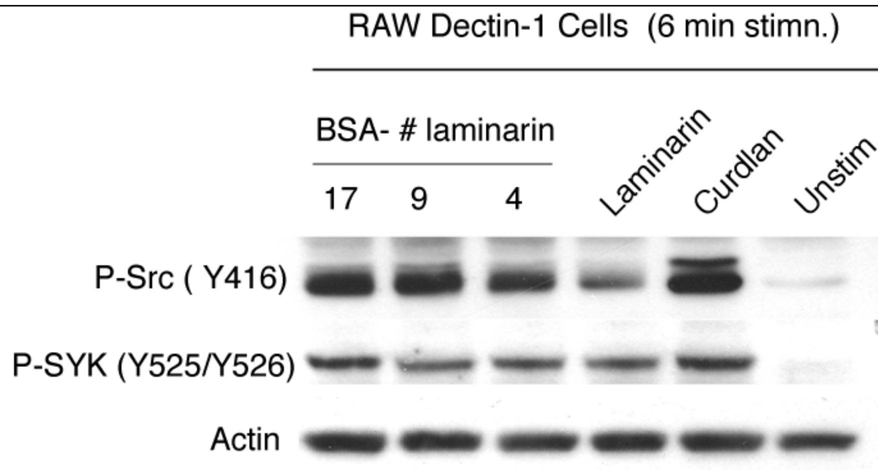


Figure 38: SYK and Src Phosphorylation in RAW Dectin-1 cells in Response to a Panel of BSA-Laminarin Conjugates and Different-sized ligands

SYK phosphorylation was probed by immunoblotting in RAW 1A cells versus RAW WT cells. Cells were serum starved for 4-6 hours and then left unstimulated, or treated with laminarin, P-curdlan and a panel of BSA-laminarin conjugates (with 4, 9 and 17 laminarins), as indicated in figure at a concentration of 100 µg/mL in serum-free sterile culture medium at 37°C for 10 min. Cells were then washed with PBS, and Whole Cell lysates were prepared and analyzed by immunoblotting for the presence of phospho-SYK (Y525/Y526), or Phospho Src (Y416) and β-actin as protein loading control. Data is representative of five independent experiments.

This significant relationship between ligand size and the level of Dectin-1–mediated activation of SFKs (Src) and SYK prompted us to question whether the phenomena was limited to these two most membrane-proximal Dectin-1 signaling partners. Therefore in order to further test the effect of ligand size on the strength of signaling events activated at the upstream level within the Dectin-1 signaling cascade, we sought to investigate the activation of selected signaling players induced downstream of SYK, such as PKCδ and PLC-γ2 (Figure 39). Western-blots were performed to probe for activation of PLC and PKC, in response to a variety of ligands of variable size (Figure 39). WGPsol (Soluble whole glucan particle) and BSA-17-lam were selected as ligands of molecular weight higher than that of laminarin (Figure 39). Laminarin, a small soluble β-glucan is estimated to have a molecular weight of 7.7 kDa (Mueller et al., 2000). BSA-17-

laminarin, then, would contain ~130 kDa of β-glucan since it is seventeen laminarin molecules coupled covalently to a BSA molecule. WGPsol was measured in the Bundle laboratory as a polydisperse mixture of β-glucan molecules at 30, 80, and 800 – 1,000 kDa in size. Remarkably, the larger ligands, WGPsol and BSA-17-lam were able to stimulate higher levels of phosphorylation of PKCδ and PLC-γ2 than the much smaller β-glucan laminarin and unstimulated controls (Figure 39). We also probed in parallel for SYK and SFK phophorylation, and interestingly they followed the same pattern of PKCδ and PLC-γ2 phosphorylation, with more activation induced by the larger ligands versus laminarin and unstimulated controls (Figure 39). Again, laminarin, a low Mwt β-glucan, largely decribed as an inhibitor of Dectin-1 signaling, was intriguingly able to induce activation of PKCδ and PLC-γ2, although minimal compared with the other ligands of higher molecular weight (Figure 39).

Therefore, both WGPsol and BSA-17-laminarin are capable of inducing higher levels of Src family kinase, SYK, PLCγ2, and PKCδ phosphorylation than laminarin, consistent with their larger size. This demonstrates that ligands of larger size are indeed able to induce various effectors upstream of the Dectin-1 signaling pathway (Figure 39).

To conclude our study so far, differences in the size or valancy of the β-glucan ligand result in corresponding differences in the magnitude of upstream signaling events activated by these ligands.

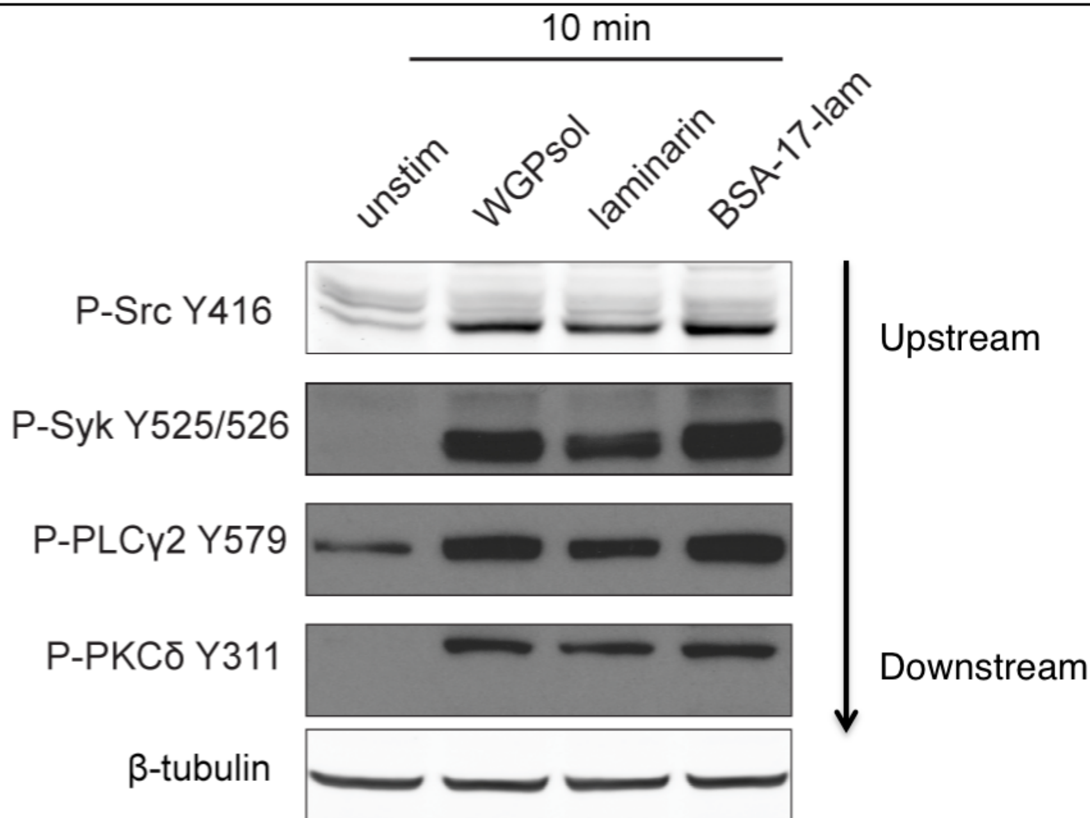


Figure 39:Activation of Upstream Signaling Events in Response to Ligands of Different Size

RAW Dectin-1 cells were serum-starved for 4-6 hours before the experiment. RAW Dectin-1 cells were stimulated with ligands of variable molecular weightin order to probe for activation of key upstream effectors of the Dectin-1 signaling pathway, such as Src, SYK, PLCγ2 and PKCδ. Briefly, 100 μg/mL laminarin, WGPsol, BsA-17-laminarin, or left unstimulated for 10 minutes at 37°C, after which the cells were immediately wshed to get rid of ligands, lysed, processed by SDS-PAGE transferred to nitrocellulose membrane, and immunoblotted for the presence of phosphorylated Src family kinases [anti-P-Src-(Y416) or equivalent on SFKs), phosphorylated Syk [anti-P SYK-(Y525/Y526], phosphorylated PLCγ2 (on residue Y579), phosphorylated PKCδ (on residue Y311), or β-tubulin (as a loading control). The arrow on the right hand side of the figure indicates the sequence of signaling events from upstream to downstream. Immunoblots presented are representative of three independent experiments.

After biochemically investigating the effect of ligands of different size/molecular weight on upstream signaling events of the Dectin-1 signaling pathway, we were interested in examining whether ligand size differentially affects the cellular and plasma membrane localization of key effectors activated upstream of Dectin-1 signaling. These key cellular events stimulated upon Dectin-1 ligation could only be investigated through immunofluorescent approaches, which we therefore exploited for our experiments.

As explained above, upon ligand binding, Dectin-1 is phosphorylated by SFKs, presumably Src, at tyrosine 15 of the hemITAM motif of its intracellular tail. This single tyrosine phosphorylation of the hemITAM is sufficient to cause recruitment of SYK to the cytosolic side of the plasma membrane via engagement of both of its SH2 domains to individually phosphorylated

hemITAMs, which is a crucial requirement for SYK activation downstream of Dectin-1 ligation (Gantner et al., 2003; Rogers et al., 2005) **(see sections 1.3.4 and 3.2.2.4)**. Therefore we first sought to examine SYK recruitment to the plasma membrane in response to AF546-labeled BSA-laminarin conjugates that were shown from previous experiments to induce Dectin-1 activation of SYK. For that purpose, we performed a live cell imaging experiment similar to the one described in **section 3.2.2.4**, in which RAW Dectin-1 and RAW WT cells were transfected with the SYK GFP-tagged fusion protein, SYK-GFP (green), and the localization of SYK-GFP was monitored over time during ligand stimulation by live confocal imaging **(**Figure 40**)**. Cells transfected with SYK-GFP were incubated with the AF546 fluorescently labeled ligands, AF546–BSA-17-lam and AF546-BSA-8-lam, for 5 minutes on ice (to avoid internalization of the ligand by endocytosis). Ligands were then immediately washed off, so that only bound ligand remained on the cell surface, and cells were promptly imaged live at room temperature on a Spinning Disc Confocal microscope. Interestingly, SYK-GFP was cytoplasmic when the cells were left unstimulated, but addition of the AF546–BSA-laminarin conjugates, altered its distribution, with SYK being profoundly recruited to the plasma membrane accumulating to form punctate structures at distinct sites of bound ligand **(**Figure 40**)**. Interestingly, AF546–BSA-17-lam induced more SYK recruitment and formation of ligand puncta than AF546-BSA-8-lam, which has a lower number of laminarin molecules. This observation is consistent with our western blot results for these two ligands (Figure 36). Indeed, treatment of cells with the BSA-17-laminarin conjugate with a higher valency of laminarin molecules, stimulated stronger recruitment of SYK-GFP to the plasma membrane and led to the formation of SYK-GFP puncta with higher intensity, which perfectly colocalized with regions of intensive ligand binding at the cell membrane **(**Figure 40 & Figure 41**)**. Moreover, the lack of recruitment of SYK-GFP, as well as ligand binding, in RAW WT cells, demonstrates that the observed cellular event of SYK recruitment was specific to Dectin-1 activation **(**Figure 40**)**. The colocalization of SYK puncta with fluorescently labeled ligand, suggests that SYK most probably binds to the tyrosine-phosphorylated, cytosolic hemITAM motif of the Dectin-1 receptor expressed at the cell surface (this will be later investigated as demonstrated further below) **(**Figure 40 & Figure 41**)**. In this experiment, SYK recruitment was monitored over a time course of 15 minutes **(**Figure 41**)**. Both SYK puncta and fluorescent ligand were apparent at the plasma membrane as soon as 5 minutes after the start of imaging (t=0). Interestingly, the intensity and formation of these puncta persistently increased

during the time course of live cell imaging, until the time point of 15 minutes, after which the ligand started to internalize (data not shown) **(**Figure 41**)**.

In conclusion, RAW Dectin-1 cells are able to bind β-glucans, unlike RAW WT cells., and binding of β-glucans to these cells is accompanied with the recruitment of SYK to the plasma membrane. However, binding of BSA-lam-conjugates of higher ligand valency to RAW Dectin-1 cells is associated with more recruitment of SYK to the plasma membrane, as well as formation of SYK puncta of higher intensity at sites of ligand binding. This in accordance with the ability of BSA-laminarin conjugates with increasing numbers of laminarin to induce higher levels of SYK phosphorylation, as demonstrated above by western blotting (Figure 36, Figure 37, & Figure 38**)**.

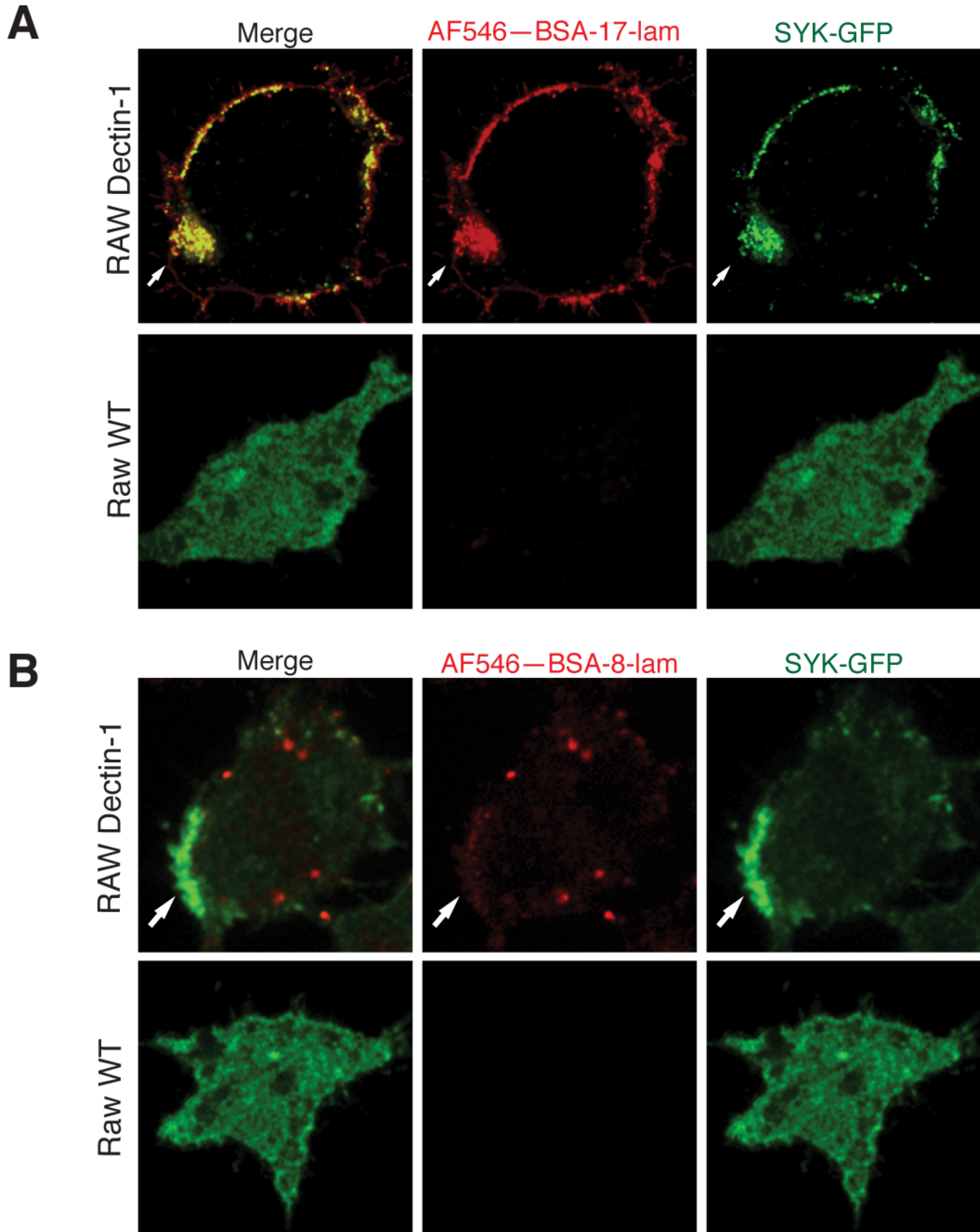


Figure 40: Monitoring Ligand Binding and SYK Recruitment in Live Cells in Response to BSA-laminarin Conjugates with Variable Numbers of Laminarin

RAW Dectin-1A and RAW WT cells were transfected with SYK-GFP. The following day, after 5 min incubation of cells on ice with fluorescently labeled ligand (AF546–BSA-n-lam). Ligand was washed off the cells 5x with cold PBS and then hRPMI culture medium was replaced with PBS after the final wash. Cells were then immediately

visualized live at room temperature for 20 minutes on a confocal microscope, with images acquired every 2 minutes. Above figure shows representative images of cells acquired at a timepoint of 10 minutes. White arows indicate regions of pronunced SYK recruitment and formation of intense puncta. White arrows also indicate sites of intensive ligand binding. Data is representative of three independent experiments.

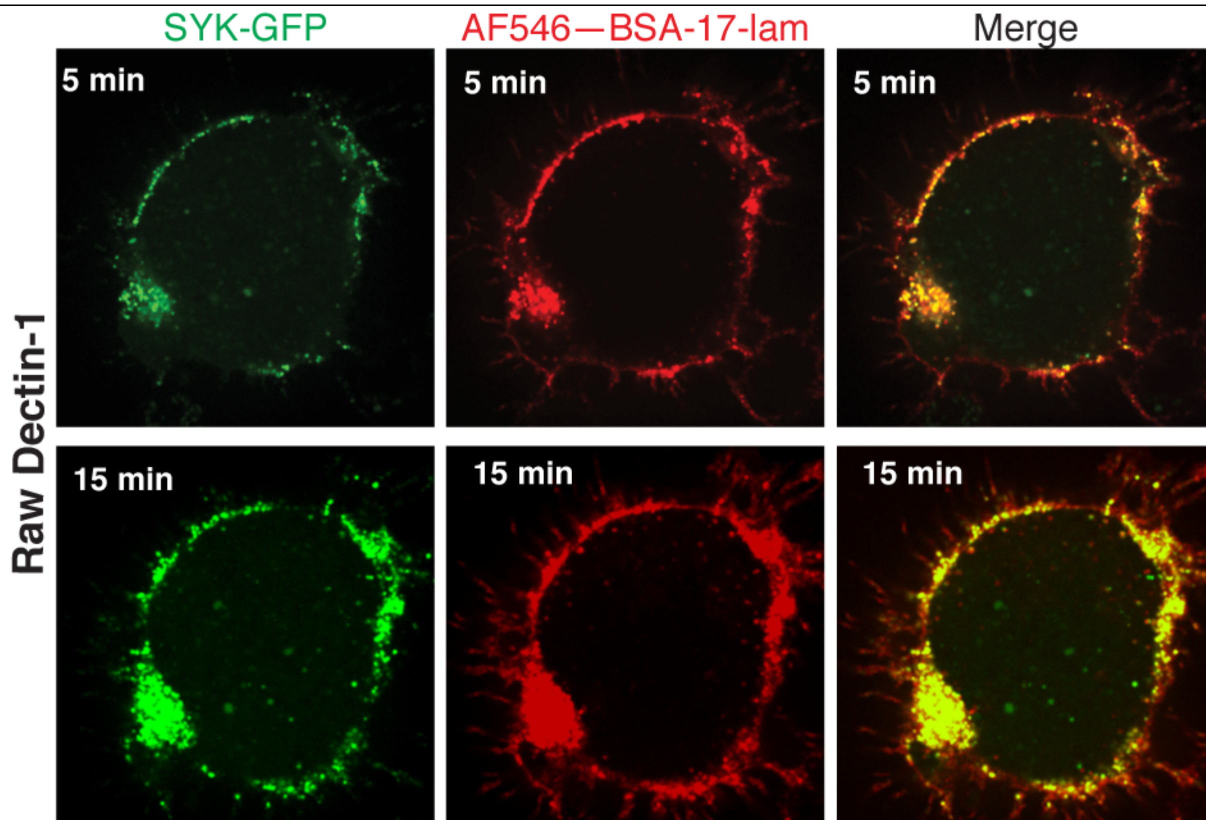


Figure 41: Time Course for SYK Recruitment and AF546–BSA-17-lam Binding to RAW Dectin-1 Cells

The RAW Dectin-1 cell represented in the top panel of (Figure 40) was transfected with SYK GFP and stimulated with 100 µg/mL AF546–BSA-17-lam. This cell was monitored for the whole time-course (20 minutes) of the live cell imaging experiment as described in (Figure 40) The above figure represents two images of this cell acquired at the timepoints of 5 mins and 15 mins. The timepoint of 15 mins shows much higher recruitment of SYK-GFP and formation of SYK puncta of higher intensity that perfectly colocalize with dense regions of ligand binding at the cell membrane. Data is representative of three independent experiments.

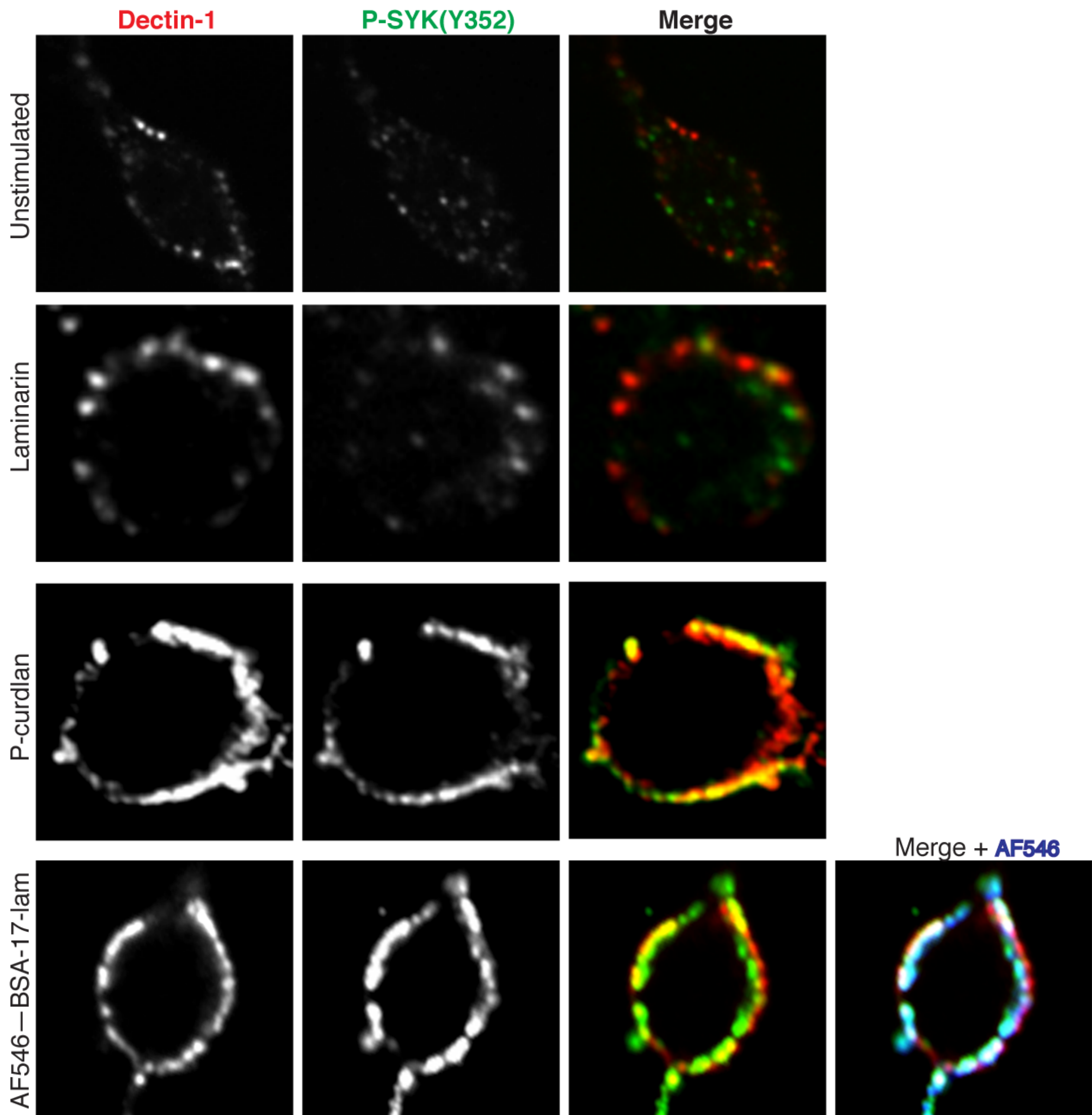
As mentioned above, the intense colocalization of SYK puncta with fluorescently labeled ligand (Figure 35), at the plasma membrane suggests that SYK, via its SH2 domain, presumably binds to the phosphorylated hemITAM motif of the cytoplasmic tail of the Dectin-1 receptor at the cell surface (see schematic in Figure 43). This prompted us to determine the membrane localization of activated phospho-SYK with respect to Dectin-1 expressed on the plasma membrane. We also wanted to further examine the effect of soluble ligands of variable sizes on the membrane recruitment and activation/phosphorylation of SYK.

We thus performed an immunofluorescent experiment in which we treated RAW Dectin-1 Cells with three soluble ligands of different size (laminarin, P-curdlan and AF546–BSA-17-lam) followed by dual immunostaining of surface Dectin-1 (using an anti-human Dectin-1 Ab, immunolabeled in red) and total phospho-SYK [using anti-P-SYK (Y352) Ab, immunolabeled in green]. Following stimulation with ligands for 10 minutes at 37°C, cells were fixed, immunofluorescently stained for Dectin-1 (labeled in red), permeabilized and immunofluorescently stained for phospho-SYK at Y352 (an activatory phosphorylation site of SYK) (labeled in green).

Visualization of cells by confocal microscopy revealed profoundly higher levels of SYK phosphorylation and membrane recruitment upon stimulation with P-curdlan and AF546–BSA-17-lam, as compared with laminarin, a much smaller β-glucan, and unstimulated controls **(**Figure 42**)**. Intriguingly, these high molecular weight ligands not only induced the formation of intensive phospho-SYK (P-SYK) puncta at the plasma membrane, but also induced the formation of high intensity Dectin-1 puncta at the cell membrane, which remarkably colocalized with the phosph-SYK puncta **(**Figure 42**)**. Moreover, AF546–BSA-17-laminarin (blue signal) also strongly colocalized with the Dectin-1 and P-SYK puncta **(**Figure 42**)**. Although we did not stain for the presence of P-curdlan due to technical difficulties, this high molecular weight β-glucan gave similar results to cells treated with AF546–BSA-17-lam. Therefore these results are greatly indicative that the observed intense Dectin-1 punctae could possibly be Dectin clusters that are promptly formed at the plasma membrane upon binding to ligands of larger size, such as P-curdlan and BSA-17-lam, which is in accordance with our clustering hypothesis explained above **(see section 3.1,** Figure 22**)**. Furthermore, the intense colocalization of SYK puncta with sites of the membrane rich in Dectin-1 and fluorescently labeled ligand suggests, as predicted above, that SYK most probably binds to the cytoplasmic, tyrosine-phosphorylated hemITAM motif of the Dectin-1 receptor at the plasma membrane. This in turn presumably leads to the high activation and phosphorylation levels of SYK induced by larger ligands **(**Figure 42**)**.

Again, laminarin did not induce much SYK phosphorylation and demonstrated poor recruitment to the plasma membrane. The intensity of the green signal of P-SYK produced in response to laminarin stimulation was very low compared to the other higher molecular weight ligands, P-curdlan and the multivalent ligand, AF546–BSA-17-lam, with 17 laminarin molecules, AF546–BSA-17-lam **(**Figure 42**)**.

In conclusion, the size of the β-glucan that binds to Dectin-1 correlates with the recruitment of SYK to the plasma membrane at sites of Dectin-1 staining. Ligands of larger size and increased valency resulted in pronounced recruitment of SYK (appearing as intense puncta) to regions of intense Dectin-1 Staining. Furthermore, stimulation with β-glucans of larger size leads to enhanced phosphorylation of the SYK kinase as demonstrated by this immunofluorescence experiment, as well as western-blot experiments demonstrated above using anti-Phospho-SYK (Y525/Y526).


A
RAW Dectin-1
Dectin-1
P-SYK(Y352)
Merge
Unstimulated
Laminarin
P-curdlan
Merge + AF546
AF546—BSA-17-lam

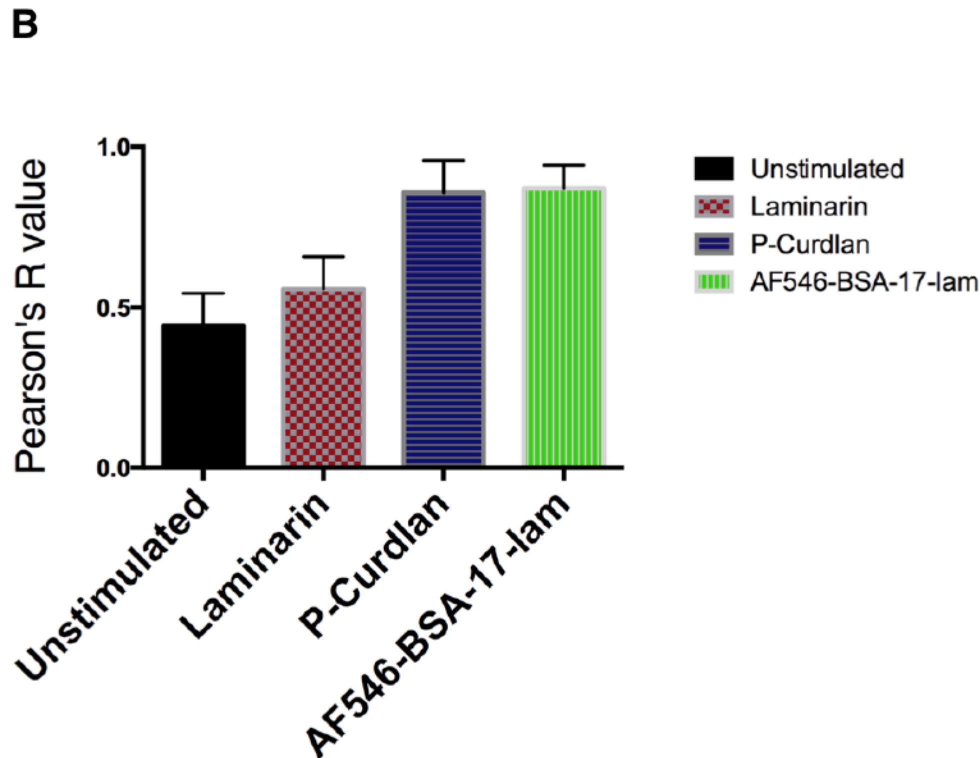


Figure 42: SYK Activation and Recruitment in Response to Different-sized Ligands

(A) RAW Dectin-1 macrophages were incubated with 100 µg/mL of P-curdlan, AF546–BSA-17-lam, laminarin or left untreated for 10 min at 37°C before fixation with 4% PFA. Cells were washed and Dectin-1 was immunostained in nonpermeabilized macrophages with the mouse anti–Dectin-1 and CY5-conjugated anti-mouse secondaryAbs. SYK recruitment and activation were observed using a rabbit anti-phospho SYK (Tyr352) and an anti-rabbit AF488. Cells were then visualized by confocal microscopy. Dectin-1 was acquired in the Cy5 channel and the aquired images were pseudocolored in red. In contrast the images of AF546-labeled BSA-lam conjugate were acquired in the Cy3 channel and pseudocolored in blue to avoid interference with the Dectin-1 signal (red). Images are representative of 5 independent experiments. **(B)** To estimate the degree of colocalization between the Dectin-1 signal and the P-SYK signal at the cell surface Pearson's Colocalization Analysis was perfomed (using Fiji) for 3 independent experiments similar to that shown for the above immunofluorescent images in (A). Briefly, colocalization between Dectin-1 and P-SYK signals at the cell membrane of multiple cells in unstimulated state and in response to different-sized ligands, was analyzed using Pearson's Correlation Analysis. Pearson's correlation coefficient (R) values were calculated from three independent experiments (Each experiment had 2-3 cells analyzed for colocalization) using the 'Coloc2' plugin in Image J/Fiji and plotted with Prism software as illustrated in Figure 42B. Error bars represent SEM for the 3 different IF experiments performed.

As mentioned in **section 1.5.4**, SFKs, presumably the Src isoform, phosphorylate the hemITAM motif of the Dectin-1 cytoplasmic tail to result in SYK recruitment to the plasma membrane and subsequent activation of SYK (see schematic in Figure 43). Accordingly, Dectin-1–dependent activation of Src family kinases (SFKs), of which the Src isoform is the prototype, is upstream of SYK activation, thus activation of SFKs represents an ideal target to examine Dectin-1 signal transduction in response to β-glucan stimulation.

Therefore, we next decided to investigate if the recruitment of phosphorylated SYK to Dectin-1

dense areas, as observed in the above experiment (Figure 42), was in fact correlated to increased Dectin-1 signaling capacity presumably via enhanced activation of SFKs that lead to phosphorylation of the Dectin-1 hemITAM motif (see chematic of Figure 43). In order to answer this question we sought to examine by immunofluorescence the cellular/membrane localization pattern and phosphorylation of SFKs in response to Dectin-1 ligation by the multivalent Dectin-1 ligand, AF546–BSA-12-lam. We had already observed through western botting that SFK phosphorylation in response to various ligands followed a trend to similar to that of SYK phosphorylation (Figure 38), so it was of great interest to determine if SFKs would follow a cellular localization pattern identical to that observed for SYK recruitment to the plasma membrane (Figure 42). We thus, performed immunofluorescent confocal imaging on RAW-Dectin-1 cells and RAW WT control cells stimulated with AF546–BSA-12-lam to visualize the localization and activation of SFKs via a phospho-specific antibody against Src, anti-phospho Src (Y416) antibody fluorescently labeled with AF488 (green). This antibody recognizes the active phsophorylated form of most of the Src family kinases (SFKs). As shown in Figure 43, treatment of RAW Dectin-1 cells with AF546–BSA-12-lam leaded to recruitment of Src to the plasma membrane, which was not observed in RAW WT cells lacking Dectin-1. Interestingly, membrane proximal areas rich in the β-glucan conjugate were also enriched in the active form of Src (Figure 43). This indicates that Src is activated in response to Dectin-1 ligation by ligands and is presumably recruited to areas of the membrane rich in Dectin-1 leading to phosphorylation of its hemITAM, which promotes its signaling capacity, including the key membrane-proximal signaling event of SYK activation. Indeed the pattern of SFK recruitment to the plasma membrane (Figure 43), seems to follow that observed for SYK in the above-described experiments (Figure 40, Figure 41, & Figure 42). This prompted us to next test if activated SFKs were recruited to sites of the plasma membrane rich in Dectin-1, in a similar fashion to the recruitment of SYK to high intensity Dectin-1 puncta on the membrane, upon stimulation with larger-sized ligands (Figure 42).

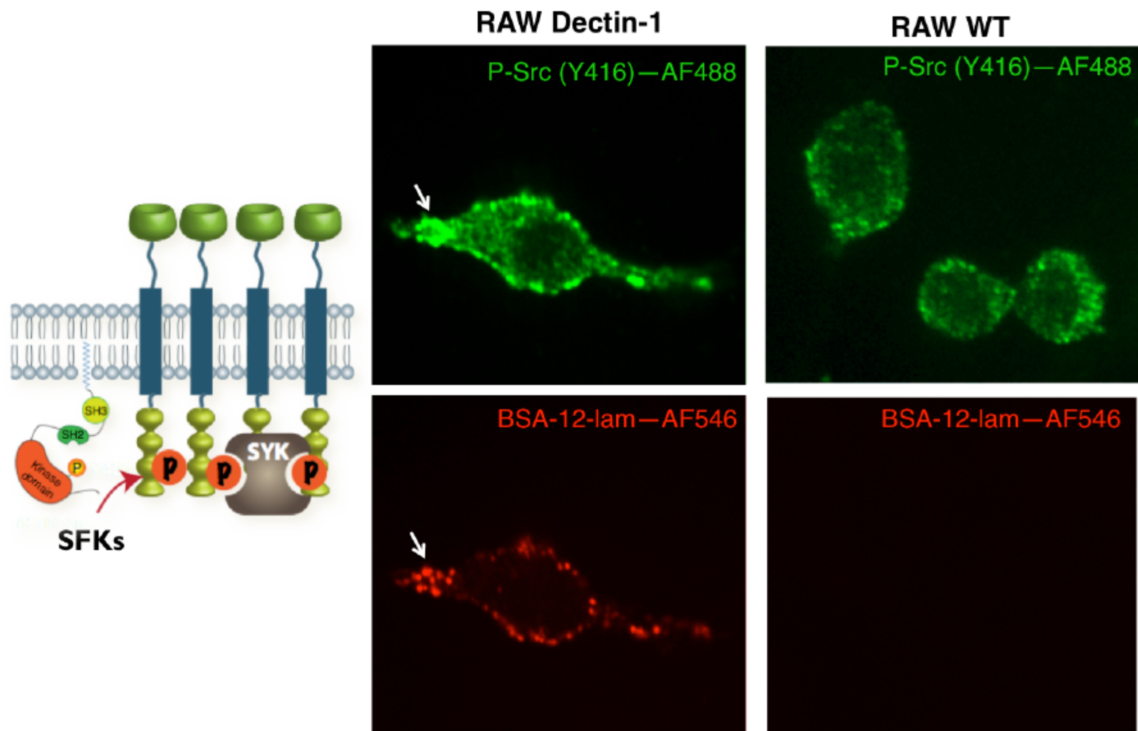


Figure 43: Src Activation and Recruitment by Large Ligands in Raw Dectin-1 versus RAW WT Cells

RAW Dectin-1 and RAW WT cells were stimulated with 100 µg/mL of AF546–BSA-12-lam for 5 minutes or left unstimulated. Non-permeabilized cells were then fixed, followed by immunostaining for Dectin-1 using a mouse anti-human Dectin-1 Ab and a Cy3-conjugated anti-mouse secondary Ab (red) Cells were then permeabilized, blocked with blocking buffer, and activation/phosphorylation of SFK was visualized using rabbit anti-phospho Src (Y416) coupled to AF488 (green) (Invitrogen). Cell images were then acquired on a spinning disc confocal microscope. White arrows indicate sites of intense Src recruitment to the membrane (top panel, left) and membrane – proximal areas enriched with ligand (bottom panel, left). Experiment is representative of three independent replicates. Also depicted in the above figure is a schematic of Src-mediated phosphorylation of the hemITAM of the intracellular tail of Dectin-1, leading to SYK recruitment and activation.

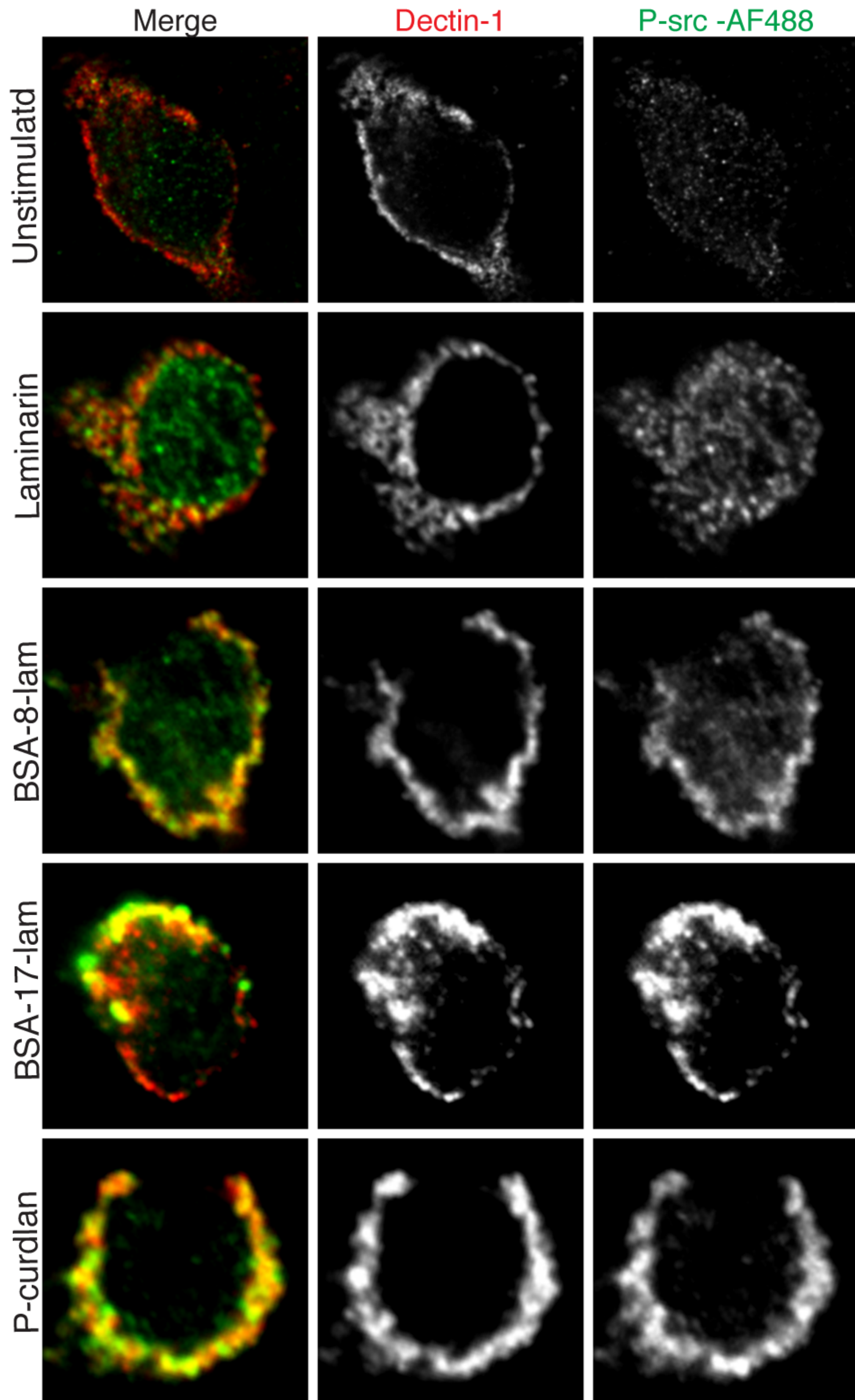
We therefore performed an immunofluorescent experiment, similar to the previous experiment described above, to probe for SFK activation in parallel to surface Dectin-1, upon treatment with a variety of soluble Dectin-1 ligands of variable size as depicted in Figure 44. Images in Figure 44, demonstrate a gradual increase in Src activation and recruitment punctate structures at the plasma membrane with the progressive increase in ligand size/molecular weight as follows: P-curdlan> BSA-17-lam>BSA-8-lam>laminarin>unstimulated. Interestingly, in alignment with the magnitude of laminarin-induced SYK-phosphorylation and membrane recruitment (Figure 42 **&** Figure 38**)**, laminarin showed limited membrane recruitment and stimulation of SFK phosphorylation, which is higher than that observed for unstimulated conditions, yet still much lower than that induced by other larger-sized ligands used in the experiment (Figure 44). Remarkably, in parallel to the progressive increase in the SFK signal in the green channel, as well

as the gradual recruitment of SFK to the plasma membrane, Dectin-1 also appears to coalesce into denser and brighter areas at the cell surface, which colocalize with membrane sites rich in P-src punctae (Figure 44). Indeed, this pattern of SFK activation and recruitment to the cell membrane is similar to that observed for SYK in response to ligand binding This suggests that SYK recruitment to sites of Dectin-1 puncta could be following SFK recruitment to areas of the membrane rich in these puncta, which are probably Dectin-1 clusters **(**Figure 42**)**. SFK recruited to Dectin-1 clusters would predictably promote phosphorylation of the Dectin-1 hemITAM and SYK recruitment, which initiates the Dectin-1 signaling cascade (see schematic of Figure 43). This provides another argument reinforcing the hypothesis of receptor clustering as a means to improve signal transduction.

We were then keen to confirm these results, by repeating the same immunofluoresecnt experiment using the more accurate system of Dectin-1 ligands of variable size, namely, the panel of BSA-laminarin conjugates with increasing valency of laminarin molecules: BSA-lam conjugates with 4, 8, 12, or 17 laminarins **(**Figure 45**)**. This would also give further insight into the effect of increasing the valency of the Dectin-1 ligand on the signaling capacity of Dectin-1. As depicted in Figure 45**,** RAW Dectin-1 cells were stimulated for 5 minutes with a panel of BSA-laminarin conjugates with variable numbers of the small β-glucan, laminarin. Increasing the valency of laminarin in the BSA-laminarin conjugates, indeed, resulted in a parallel increase of SFK phosphorylation and membrane recruitment **(**Figure 45**)**. Similar to results presented in (Figure 44), strong colocalization was observed between the P-src signal (green) and the Dectin-1 puncta formed on the membrane (labeled in red), the magnitude of which increases the valency of laminarin in BSA protein conjugate **(**Figure 45**)**. In analogy to results described in the previous experiment (Figure 44), this indicates that activated SFK is recruited to regions of the plasma membrane rich in Dectin-1 puncta, which again are probably represent Dectin-1 clusters, (see schematic of Figure 43). Therefore, it seems that BSA-laminarin conjugates with higher numbers of laminarin may induce more Dectin-1 clustering, which in turn, could facilitate and enhance SFK activation proximal to the plasma membrane, and subsequently recruitment and activation of SYK, thereby triggering the underlying Dectin-1 signaling pathway.

In conclusion, the above immunofluorescent experiments that examine the localization and activation of SYK and SFK, demonstrate that binding of Dectin-1 to ligands of higher molecular weight and larger size, promotes the formation of high intensity Dectin-1 punctae, which are

most likely Dectin-1 clusters that presumably facilitate phosphorylation of the Dectin-1 hemITAM motif by SFK. This results in SYK recruitment to the tyrosine phosphorylated hemITAM motif of Dectin-1 at the cytosolic side of the plasma membrane leading to SYK activation as observed by potent phosphorylation of SYK (as seen by both western blotting and immunofluorescence) and formation of intense SYK puncta at the cell surface.



A
RAW Dectin-1
Merge
Dectin-1
P-src -AF488
Unstimulatd
Laminarin
BSA-8-lam
BSA-17-lam
P-curdlan

B

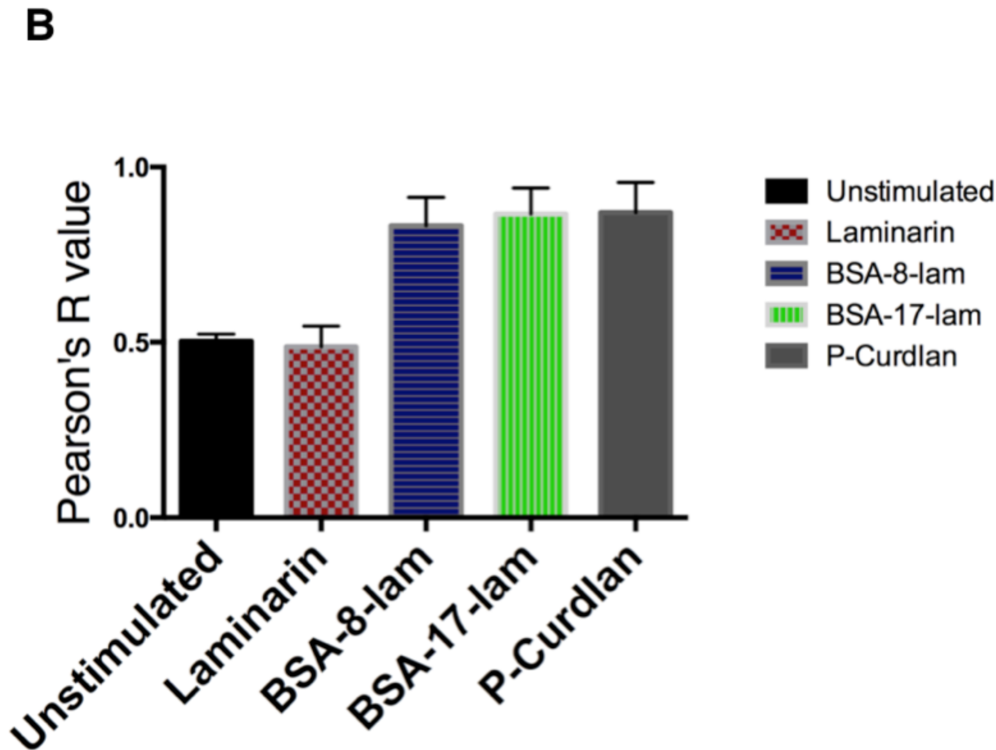
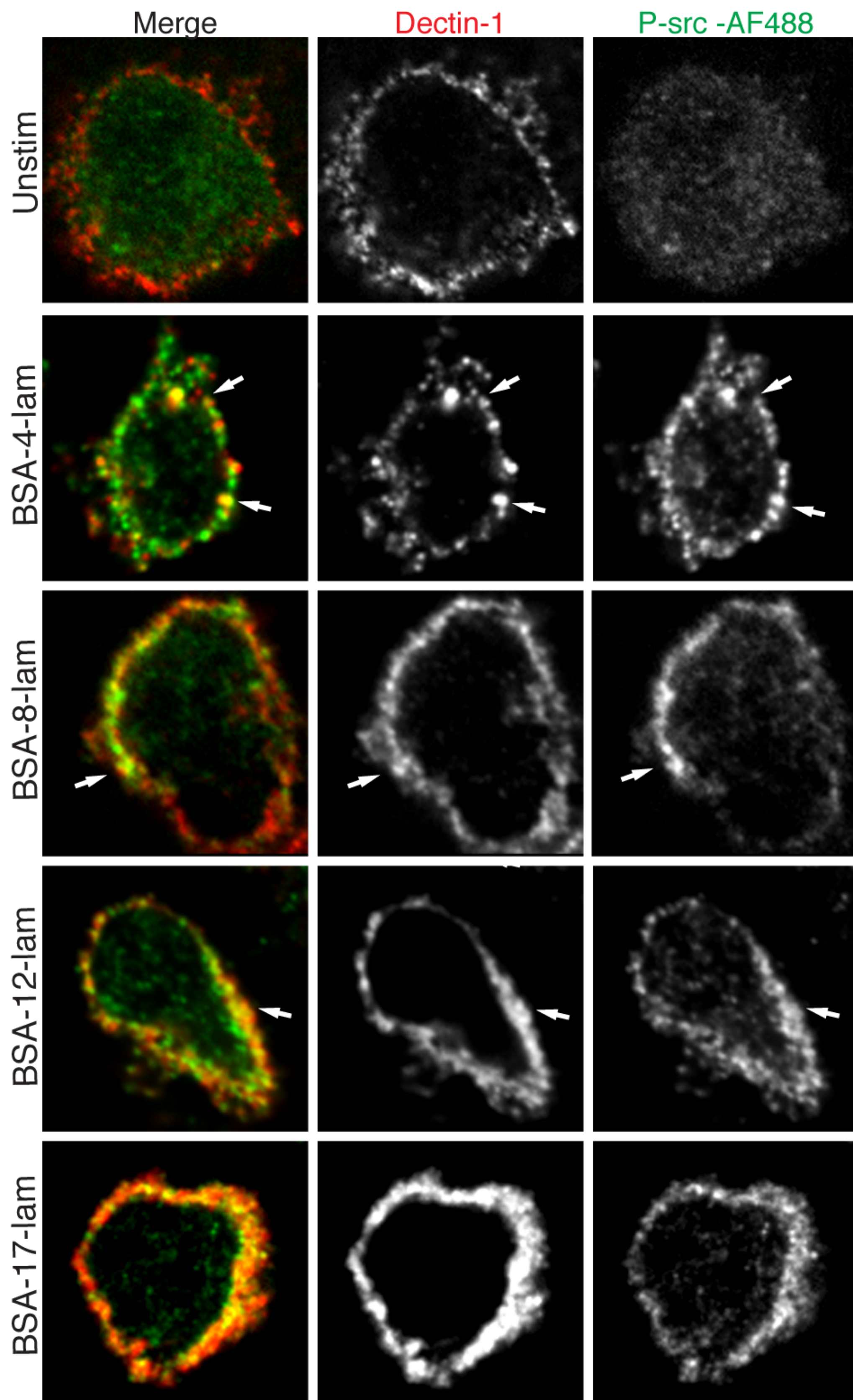


Figure 44: Effect of Ligand Size on the Activation and Recruitment of SFKs in RAW Dectin-1 Cells

(A) RAW Dectin-1 cells were serum-starved for 4 hours before the experiment. Cells were then treated with water-soluble ligands depicted in above figure or left untreated for 15 minutes at 37°C. The cells were then fixed and labeled with mouse anti-Dectin-1 Ab and a Cy3-coupled secondary antibody to identify surface Dectin-1 (red signal). For activated SFK detection cells were permeabilized and immunostained with a rabbit anti-phospho Src (Y416) coupled to AF488 (green) (Invitrogen. Visualization of cells was performed on the confocal microscope. Images are representative of three independent experiments. **(B)** To estimate the degree of colocalization between the Dectin-1 signal and the P-SFK signal at the cell surface Pearson's Colocalization Analysis was perfomed (using Fiji) for 3 independent experiments similar to that shown for the above immunofluorescent images in (A). Briefly, colocalization between Dectin-1 and P-SFK signals at the cell membrane of multiple cells in unstimulated state and in response to different-sized ligands, was analyzed using Pearson's Correlation Analysis. Pearson's correlation coefficient (R) values were calculated from three independent experiments (Each experiment had 2-3 cells analyzed for colocalization) using the 'Coloc2' plugin in Image J/Fiji and plotted with Prism software as illustrated in Figure 42B. Error bars represent SEM for the 3 different IF experiments performed.


A
RAW Dectin-1
Merge
Dectin-1
P-src -AF488
Unstim
BSA-4-lam
BSA-8-lam
BSA-12-lam
BSA-17-lam

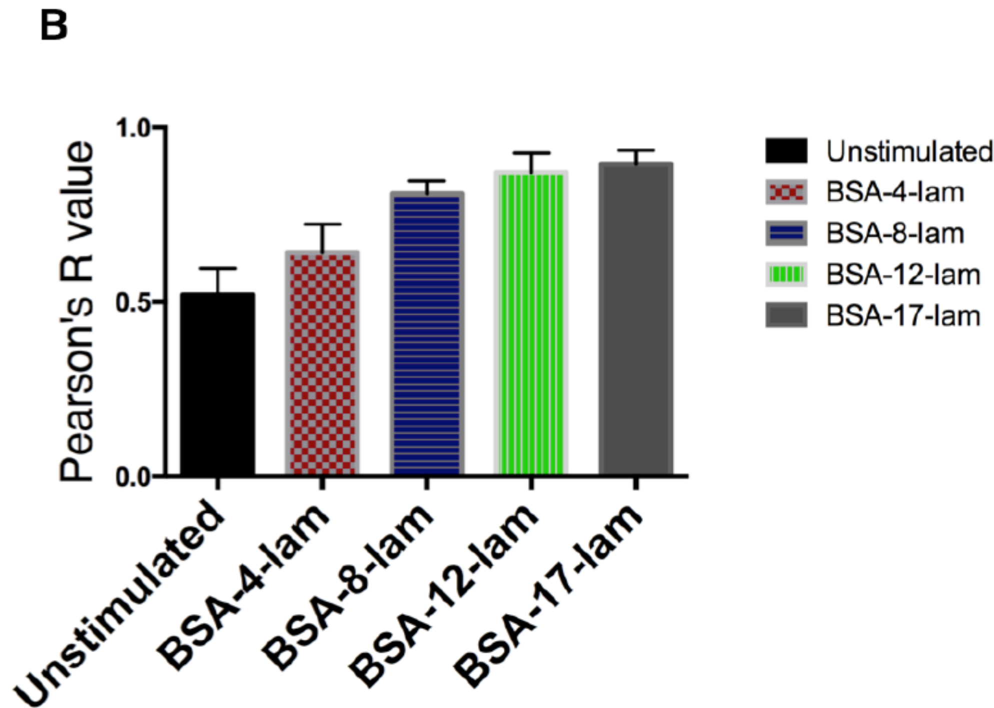


Figure 45: Activation and Recruitment of SFK in Response to a Panel of BSA-laminarin Conjugates of Increasing Valency

(A) RAW Dectin-1 cells were serum-starved for 4 hours. Cells were then treated with a panel of BSA-laminarin conjugates with an increasing number of laminarins (8, 12 and 17 lamianarins) or left untreated for 15 minutes at 37°C. The cells were then fixed and labeled with mouse anti-Dectin-1 Ab and a Cy3-coupled secondary antibody to identify surface Dectin-1 (red signal). For activated SFK detection cells were permeabilizd and immunostained with a rabbit anti-phospho Src (Y416) coupled to AF488 (green) (Invitrogen). White arrows indicate selected sites of intense Dectin-1 puncta and SFK recruitment. Cells were the imaged on a confocal microscope. Images are representative of three independent experiments. To estimate the degree of colocalization between the Dectin-1 signal and the P-SFK signal at the cell surface Pearson's Colocalization Analysis was perfomed (using Fiji) for 3 independent experiments similar to that shown for the above immunofluorescent images in (A). Briefly, colocalization between Dectin-1 and P-SFK signals at the cell membrane of multiple cells in unstimulated state and in response to different-sized ligands, was analyzed using Pearson's Correlation Analysis. Pearson's correlation coefficient (R) values were calculated from three independent experiments (Each experiment had 2-3 cells analyzed for colocalization) using the 'Coloc2' plugin in Image J/Fiji and plotted with Prism software as illustrated in Figure 42B. Error bars represent SEM for the 3 different IF experiments performed.

We were next curious to see if other upstream, membrane–proximal effectors activated downstream of SYK, and known to be involved in Dectin-1 early signaling events (such as PKCδ, PLC-γ2, and the formation of CARD9/BCL-10/ MALT-1; ***see section 1.5.4.1***), were also recruited to the Dectin-1 punctae observed upon stimulation with larger-sized ligands (e.g. P-curdlan).

As explained in **section 1.5.4.1**, the phospholipase PLC-γ2 acts at the plasma membrane where it cleaves PIP_2 into DAG and IP_3. This implies that if Dectin-1 indeed activates PLC, it should be

occurring in domain proximal to Dectin-1. A construct encoding the PH (plekstrin homology) domain (PIP_2 binding domain) of PLC-γ2 and fused to GFP (green), named PLC-γ-PH–GFP, was transiently transfected in RAW Dectin-1 cells (Figure 46**)**. Treatment of RAW Dectin-1 cells P-curdlan resulted in obvious membrane recruitment of the PLC-γ-PH–GFP, in addition to the formation of high intensity Dectin-1 puncta at the plasma membrane (Figure 46). In contrast, the distribution of PLC-γ-PH–GFP in unstimulated cells, was largely cytosolic with some PLC-γ-PH–GFP homogenously located on the entire cell membrane. Also surface Dectin-1 in these cells didn't coalesce into the intense Dectin-1 punctae observed in RAW Dectin-1 cells (Figure 46). Remarkably, strong colocalization of the PLC-γ-PH–GFP probe with Dectin-1–rich structures at the plasma membrane could be seen in RAW Dectin-1 cells, and not in unstimulated cells **(**Figure 46). This indicates that PIP_2, the susbtrate of the phospholipase is enriched in membrane domains rich in high intensity Dectin-1/β-glucan domains. This predicts that the signaling machinery involved in Dectin-1–induced early signaling events, is membrane–proximal, and most likely localized in areas of the cell membrane enriched in Dectin-1 puncta that we think are Dectin-1 clusters.

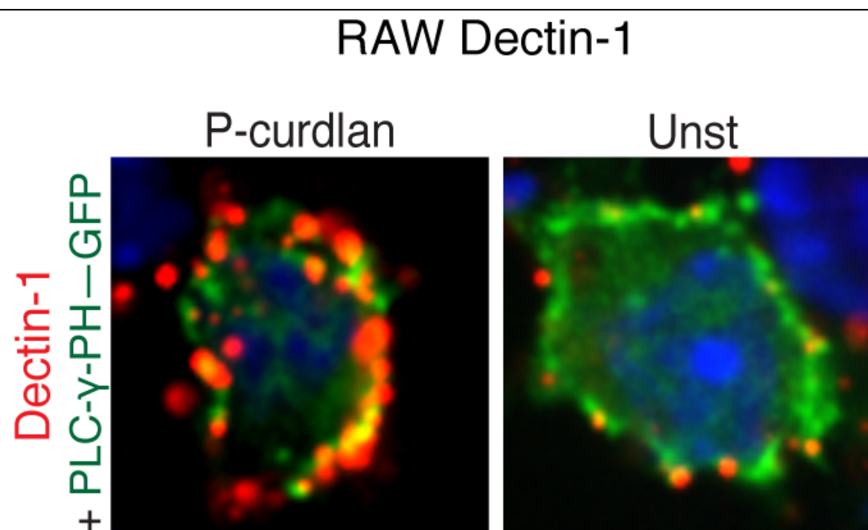


Figure 46: PLC-γ-PH (Plekstrin homology) Recruitment & Dectin-1 Puncta Formation in Response to P-curdlan treatment

A day before the experiment, RAW Dectin-1 cells were transiently transfected with a DNA construct encoding the fusion protein PLC-γ-PH–GFP (in green). Cells were then stimulated with 100 µg/mL of P-curdlan for 10 minutes, or left unstimulated, at 37°C. After their fixation, surface-localized Dectin-1 was detected on non-permeabilized cells via mouse anti-hDectin-1 antibodies, followed by labeling by a Cy3-conjugated secondary antibody (in red). DAPI staining (in blue) was used to localize the cells' nuclei. All images were acquired on the confocal microscope and are representative of the results from three independent experiments.

We then continued our investigations of the activation and membrane-localization status of SYK-

activated effectors present upstream of the Dectin-1 signaling pathway. The CARD-9/BCL-10/MALT-1 **(CBM)** scaffolding complex formed downstream of SYK activation is one of the upstream, key players involved in early Dectin-1 signaling events (described in detail in section 1.5.4.1 of *Chapter 1*). Interestingly, as mentioned in the introduction (section 1.5.4.1 of *Chapter 1*), the formation of the CBM complex at sites of the endosomal membranes containing Dectin-1 is thought to be required for its activation. Therefore, were sought to analyze the cellular /membrane localization of the CARD-9 and BCL-10 components of the CBM complex, in response to large-sized β-glucan (e.g., P-curdlan), in order to determine, whether, or not, they would be recruited to areas of the plasma membrane rich in Dectin-1. Using transient transfection of RAW Dectin-1 cells with plasmids encoding CARD9-myc or BCL-10-FLAG (obtained from Addgene), we examined the change of the distribution of these fusion proteins following treatment of with P-curdlan (Figure 47). Images in this figure illustrate results of a typical experiment, which shows that upon stimulation of cells with P-curdlan, both CARD9 and BCL-10 become enriched in plasma membrane domains where Dectin-1 is intensively localized. On the contrary, unstimulated control cells revealed no formation of Dectin-1 high intensity punctate structures at the plasma membrane, nor recruitment of CARD9 and BCL-10 to these puncta (Figure 47).

In conclusion, larger-sized, and multivalent, ligands of Dectin-1, in contrast to smaller ligands, are more capable of inducing the formation of high-intensity Dectin-1 puncta (presumably clusters Dectin-1 clusters) at the plasma membrane, which are enriched with key membrane-proximal effectors of upstream Dectin-1 signaling, including, SFKs, SYK, PLC-γ2, CARD9 and BCL-10 (Figure 47).

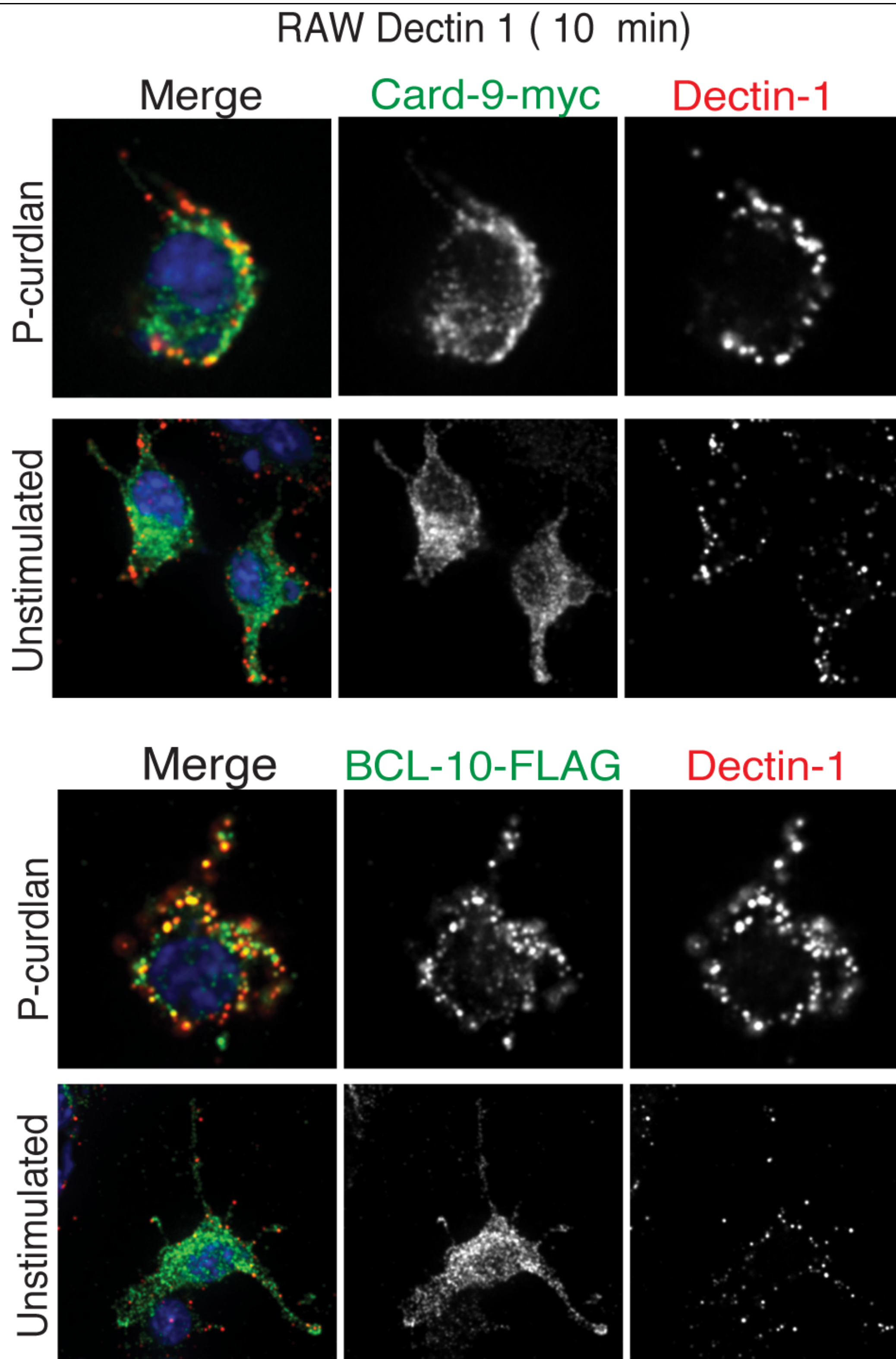


Figure 47: CARD9 and BCL10 I Recruitment to Dectin-1 Puncta upon P-curdlan Stimulation (10 min)

RAW Dectin-1 cells were transiently transfected with the contructs CARD9-myc or BCL-10-FLAG 24 hours before the experiment. Cells were then stimulated with 100 µg/mL P-curdlan for 10 minutes at 37°C. After their fixation,

surface-localized hDectin-1 was detected by incubation with anti-hDectin-1 antibodies (R&D), followed by labeling with a Cy3-conjugated secondary antibody (in red). Subsequent permeabilization, and incubation with an anti-myc antibody, or and anti-FLAG antibody, led to the labeling of CARD9-myc or BCL-10-FLAG with a secondary antibodies coupled to AF488 (in green). Nuclei were stained with DAPI (in blue). All images were acquired on the confocal microscope, and are representative results from three independent experiments.

Next we were curious to determine if the initiation of Dectin-1 signaling in general was required for the formation of the high intensity Dectin-1 puncta formed in response to stimulation by higher molecular weight ligands, as detected by immunofluorescence. As explained above, the initial, most upstream and crucial step required for Dectin-1 activation is activation of Src family kinases (SFKs) (see schematic of Figure 48). Accordingly, we decided to test if the formation of the Dectin-1 puncta is affected at all by this key early signaling event of the Dectin-1 signaling pathway.

For this purpose, we used the Src Family Kinase (SFK) inhibitor PP2, to determine the effect of Src phosphorylation on Dectin-1 puncta formation. PP2 is a well-known pharmacological inhibitor of SFKs, including the prototype Src isoform. First, we wanted to check that the SFK inhibitor PP2 was effective in inhibiting activation of SFKs in our cell model system of RAW Dectin-1 macrophages. Therefore we performed a western blot experiment, to probe for the effect of PP2 on Src phosphorylation using the anti-phospho-Src/SFK (Y416) antibody. Briefly, serum-starved RAW Dectin-1 Cells were stimulated with the potent Dectin-1 ligands/agonists, water-soluble P-curdlan, and insoluble curdlan for six minutes, in the absence or presence of PP2 (Figure 48). Treatment of cells with P-curdlan and insoluble curdlan in the presence of PP2, greatly diminished the level of Src phosphorylation induced by these ligands. Interestingly, PP2 even reduced the basal level of phospho-Src in unstimulated cells. Therefore the SFK inhibitor PP2, was efficiently able to inhibit SFK activation in our model cells of RAW Dectin-1 macrophages. Of interest is that phosphorylation of the Y416 residue of Src is autophosphorylated by Src itself, which is in accordance with our results presented this western blot (Figure 48).

We then confidently moved on to perform our immunofluorescent experiment testing the effect of PP2 on Dectin-1 puncta formation at the plasma membrane, with parallel investigation of SYK activation and membrane recruitment. We chose to examine the phosphorylation of SYK, as it is well known to be activated downstream of SFK phosphorylation (see Figure 49 & schematic of Figure 48). In this experiment, RAW Dectin-1 cells were treated with the large Dectin-1 ligands, BSA-17-laminarin or soluble P-curdlan, for 6 minutes, in the absence or presence of the SFK

inhibitor PP2 (Figure 49). Since laminarin doesn't induce the formation of intense puncate of SYK and Dectin-1, cells were also stimulated with the small Dectin-1 ligand, laminarin in the absence of PP2, as a non-clustering control for Dectin-1 (Figure 49).

As illustrated in Figure 49, PP2-induced inhibition of Src family kinases in cells stimulated with P-curdlan greatly suppressed SYK phosphorylation, membrane recruitment, and consequently the formation of high intensity SYK puncta at the membrane (Figure 49). This result is as expected since activation of SFKs is upstream of SYK phosphorylation and activation (see schematic of Figure 48). Interestingly, SFK phosphorylation disrupted the formation of Dectin-1 punctate structures formed at the plasma membrane. Remarkably, the pattern of the Dectin-1 signal on the surface of these cells is greatly resembled that detected on the surface of RAW Dectin-1 cells treated with laminarin, a small Dectin-1 ligand incapable of inducing the formation Dectin-1 high intensity puncta (Figure 49).

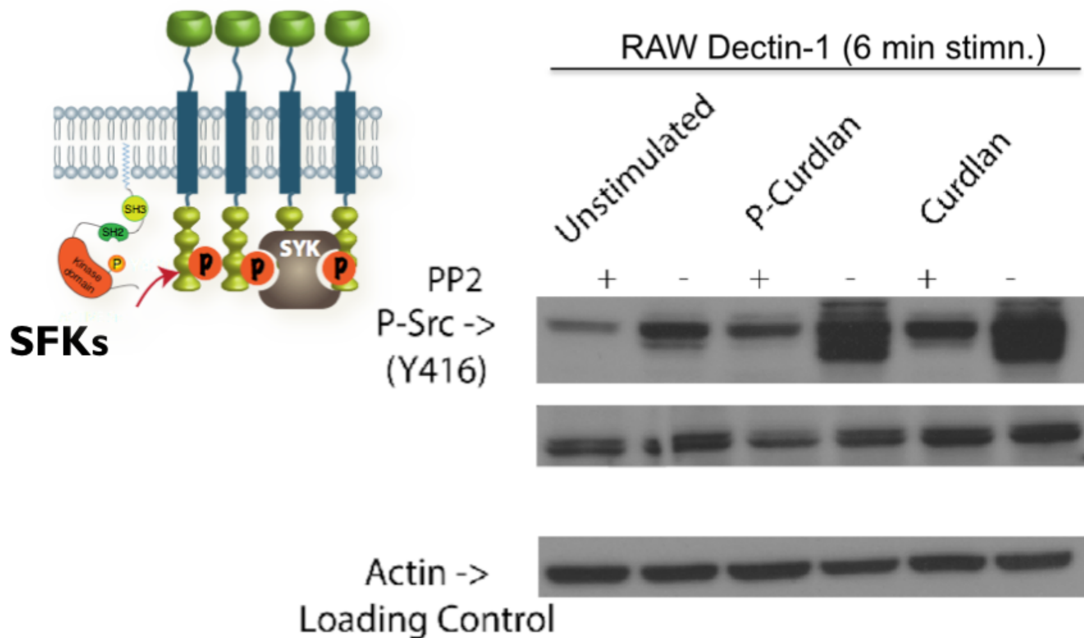


Figure 48: Effect of the Src Inhibitor PP2 on Dectin-1–induced Src Activation

RAW Dectin-1 cells were serum-starved for 4-6 hours before the experiment. Cells were then pre-treated with the SFK (Src family kinase) inhibitor PP2 (10µM), or vehicle control (0.1% DMSO) for 30 minutes at 37°C. Cells were left unstimulated, or stimulated with 100 µg/mL of soluble P-curdlan, or insoluble curdlan, in the absence or presence of PP2 (10µM). The cells were then promptly lysed, processed by SDS-PAGE, and immunoblotted for the presence of phosphorylated Src family kinases (on residue Y416 or equivalent), or actin (as a loading control). Immunoblots presented are results from two experiments. Presented on the left-hand side is the schematic demonstrating Src phosphorylation of the single tyrosine present in the cyoplasmic Dectin-1 hemTAM motif, leading to SYK recruitment and activation.

In conclusion, Src phosphorylation is required for the formation of these Dectin-1 puncta structures at the plasma membrane, which in turn suggests that Src activation could be an essential requirement for Dectin-1 clustering in response to larger Dectin-1 ligands.

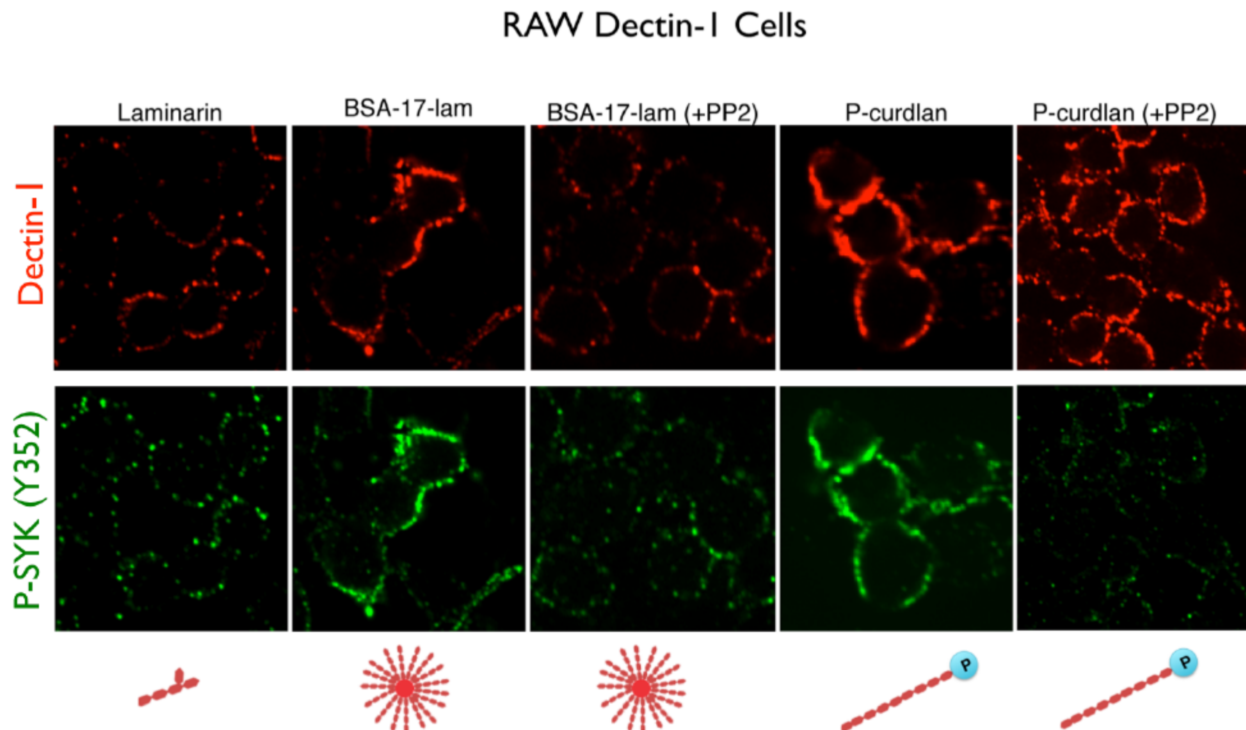


Figure 49: Effect of Inhibition of SFKs on Dectin-1 Puncta Formation and SYK Membrane Recruitment & Activation

This figure determines the effect of inhibiting SFK phosphorylation on the formation of Dectin-1 puncta. RAW Dectin-1 cells were serum-starved for 4 hours and then pre-treated with the SFK (Src family kinase) inhibitor PP2 (10μM), or vehicle control (0.1% DMSO) in serum free culture medium for 30 minutes at 37°C. Cells were left unstimulated, or stimulated with 100 μg/mL of soluble P-curdlan, or the BSA-17-laminarin conjugate, in the absence or presence of PP2 (10μM). Cells were also treated with 100 μg/mL of laminarin but in the absence of PP2, which was used as a non-clustering control. Next, the cells were fixed, then surface-localized hDectin-1 was immunostained by incubation with anti-hDectin-1 antibodies (R&D), followed by labeling with a Cy3-conjugated secondary antibody (in red). Next, the cells were permeabilized, and immunostained for phosphorylated SYK (at residue Y352) before visualization on the confocal microscope. Images shown are representative of 3 independent experiments. Below the images are schematic drawing of ligands used in the experiment.

Overall, in this section using a set of ligands of increasing size, as well as a panel of BSA-laminarin conjugates with different valencies of β-glucan, we determined their capacity to proportionally activate upstream Dectin-1 signaling events. In summary, larger ligands have a higher capacity of inducing Dectin-1 upstream signaling.

While these experiments were done numerous times (more than 4 times) and demonstrated that the RAW Dectin-1 macrophages represent an excellent model to analyze Dectin-1 signaling

events, we next decided to confirm some of these results in primary innate immune cells.

3.2.3.2. Ligand Size Effects on Dectin-1 Upstream Signaling in BMDCs and BMDMs

We wanted to make sure that the results obtained in the RAW Dectin-1 cell line that we developed, which exogenously and largely express Dectin-1 on the cell surface, could be replicated in a more native cell system closer to innate immune cells that normally exist in the host and which endogenously express Dectin-1. Accordingly, we repeated some of the experiments described above in section 3.2.3.1, in primary immune cells, namely BMDCs (Bone marrow-derived dendritic cells) BMDMs (Bone marrow-derived macrophages), which as confirmed by us in section 3.2.2.5**,** endogenously expresses Dectin-1 on the cell surface.

First, we wanted to determine of key upstream signaling events such as SYK and Src were also increasingly activated by ligands of larger size. Therefore we stimulated BMDCs with the soluble ligand small soluble β-glucan, laminarin, and the high molecular weight β-glucan, P-curdlan, and analyzed Src and SYK and activation by immunoblotting of phospo-SYK (at Y525/Y526) and phospho-Src (Y416). As illustrated in Figure 50 in analogy to what was observed for RAW Dectin-1 cells (Figure 36 & Figure 38), ligands of increasing size induced higher levels of SYK and Src phosphorylation: P-curdlan> laminarin> unstimulated. Therefore we were able to successfully reproduce the same correlation between ligand size and levels of SYK/SFK activation in the primary immune cells, BMDCs, as observed for the model cell line RAW Dectin-1 that we established.

We next wanted to examine whether SYK is recruited to the membrane upon treatment with larger ligands in a fashion similar to that observed in RAW Dectin-1 Cells. An immunofluorescent experiment was performed to probe for the recruitment and phosphorylation of SYK in response to the same Dectin-1 ligands used for the western blot described above, namely P-curdlan and laminarin (Figure 53). Remarkably, P-curdlan stimulation of BMDCs led to enhanced phosphorylation and SYK recruitment to the plasma membrane, as compared to the smaller ligand laminarin. This resulted in the formation of high intensity P-SYK puncta at the plasma membrane, which strongly colocalized with areas of intense Dectin-1 staining at the cell surface. On the other hand the magnitude of the P-SYK signal was very weak in BMDCs treated with laminarin that was slightly higher than that detected in unstimulated cells. Furthermore

laminarin-treated BMDCs showed poor recruitment of SYK to the plasma membrane, and the formation of high intensity Dectin-1 puncta at the cell membrane was hardly detectable in these cells

In conclusion, we were able to observe a pattern of SYK phosphorylation, SYK recruitment, and Dectin-1 surface staining, which was similar to that detected in RAW Dectin-1 cells in response to β-glucans of variable size.

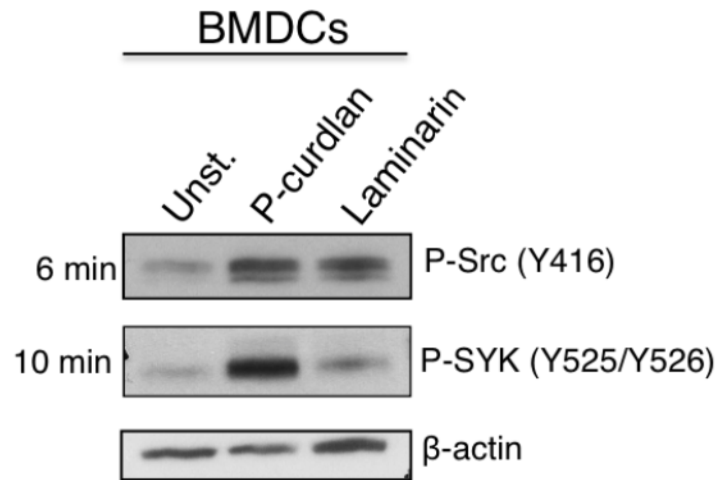


Figure 50: Src and SYK Phosphorylation in BMDCs in Response to ligands of Variable Size

SYK phosphorylation was probed by immunoblotting in BMDMs. On day 10 of primary culture, BMDCs were serum starved for 4-6 hours and then left unstimulated, or treated with laminarin, P-curdlan and a panel of BSA-laminarin conjugates (with 4, 9 and 17 laminarins), as indicated in figure at a concentration of 100 µg/mL in serum-free sterile culture medium at 37°C for 6 or 10 min. Cells were then washed with PBS, and whole cell lysates were prepared and analyzed by immunoblotting for the presence of phospho-SYK (Y525/Y526), or Phospho Src (Y416), and β-actin as protein loading control. Data is representative of three independent experiments.

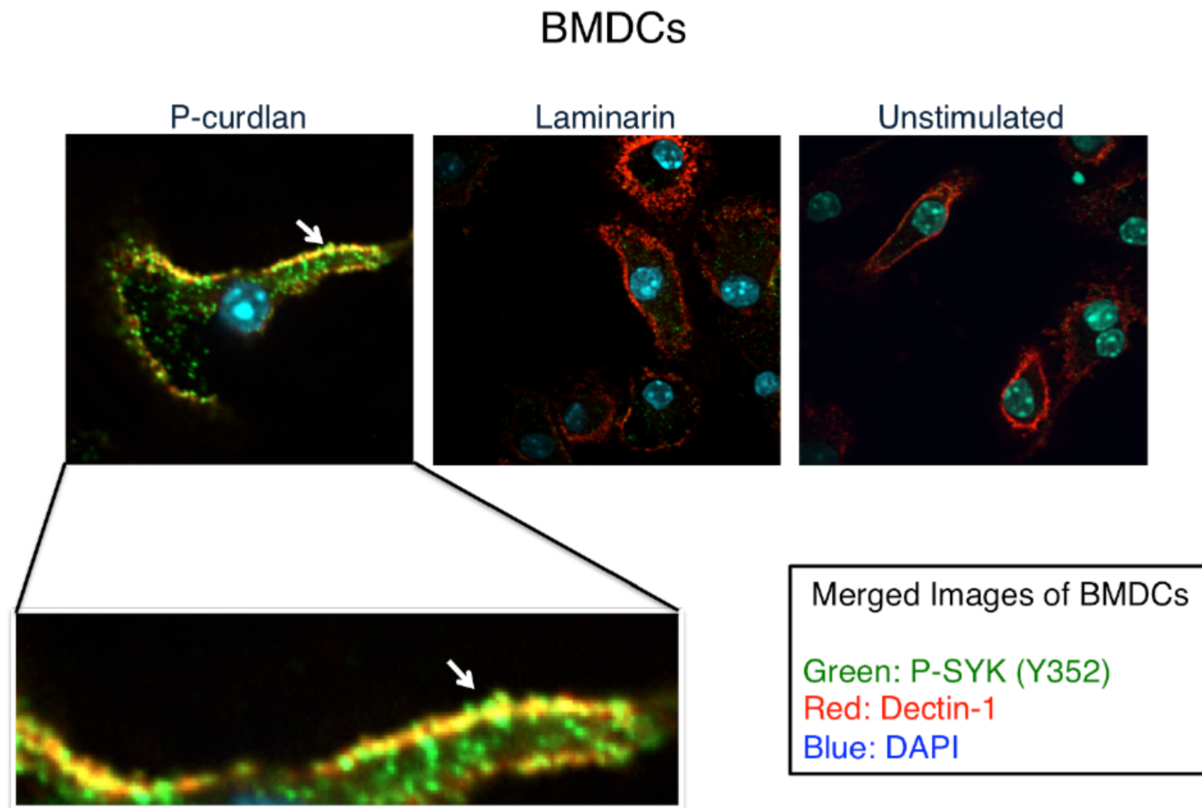


Figure 51: SYK Activation and Recruitment in BMDCs in Response to β-glucans of Variable size

Phosphorylation and recruitment of SYK as well as Surface Dectin-1 was observed in response to soluble β-glucans of increasing size. Primary BMDCs at day 10 of cell culture were serum starved for 5 hours. Cells were then treated at 37°C for 10 mins with 100 μg/mL of laminarin or P-curdlan in sterile serum-free culture medium, or cells were left unstimulated. Cells were then fixed, followed by immunostaining of surface-localized mouse Dectin-1 by incubation with rat anti-mouse Dectin-1 antibodies (R&D) and subsequent labeling with a Cy3-conjugated secondary antibody (in red). Next, the cells were permeabilized, and immunostained for phosphorylated SYK (at residue Y352) before visualization on the confocal microscope. Nuclei were stained with DAPI (in cyan blue). A 'zoom in' of a confocal image of a cell treated with P-curdlan is depicted in the above figure in order to highlight sites of SYK recruitment to the plasma membrane and colocalization with sites of intense Dectin-1 staining/ puncta. A white arrow demonstrates one of these sites with potent SYK recruitment and colocalization with Dectin-1 puncta at the cell membrane. All images shown are representative of 3 independent experiments.

After confirming SYK activation and recruitment to the plasma membrane, as well as the formation of high intensity Dectin-1 puncta in response to large ligands, we were next interested in investigating whether SFKs activated upstream of SYK colocalize with regions of immense Dectin-1 staining. To answer this question we performed an immunofluorescent experiment (similar to the one described in **section 3.2.3.1.)** to analyze phosphorylation of SFKs. As illustrated in Figure 52, in this experiment BMDCs were treated with soluble ligands of different size or increasing β-glucan valency: laminarin<P-curdlan; and BSA-8-lam<BSA-17-lam. This was followed by dual immunostaining of surface Dectin-1 (using a rat anti-mouse Dectin-1 Ab) and total phospho-SFK [using an anti-P-Src (Y416) Ab coupled to AF488]. Following

stimulation with ligands for 6 minutes at 37°C, cells were fixed, immunostained for surface Dectin-1 (in red), permeabilized and stained for phospho-SFK (in green) (Figure 52). Amazingly, induction of BMDCs by ligands of larger size resulted in a corresponding progressive increase in the levels of SFK phosphorylation, as well as more apparent recruitment of SFK to the plasma membrane that colocalized with sites of high intensity Dectin-1 staining (Figure 52). Increasing the size and number of laminarin on the BSA protein-carrier enhanced the formation of high intensity Dectin-1 puncta at the cell membrane (Figure 52). Indeed, P-curdlan–treated BMDCs revealed immense Dectin-1 staining on the entire plasma membrane, which perfectly colocalized with the P-Src/SFK-AF488 signal creating an intense rim of yellow colour at the cell surface (Figure 52). In contrast, the P-Src/SFK-AF488 signal was largely cytoplasmic in unstimulated and laminarin-treated cells, and was poorly localized at the cell membrane (Figure 52). Additionally, the surface staining of Dectin-1 in these cells was much lower than that detected in cells treated with larger-sized ligands such as P-curdlan and BSA-17-laminarin (Figure 52).

In conclusion, the pattern observed for SFK recruitment and activation, as well as Dcetin-1 puncta formation in BMDCs, greatly resembles that observed in RAW Dectin-1 cells treated with ligands of progressively increasing size or β-glucan valency.

Overall, the imunofluorescent and western blot experiments performed in BMDCs demonstrate that the 'ligand size'/'Dectin-1 activity' relationship determined for selected upstream signaling events, such as activation of SYK and SFKs, largely follows that observed in our cell model of RAW Dectin-1 macrophages (see description of results in **section 3.2.3.1.**) This validates that our results detected in the RAW Dectin-1 cell line that we developed for this study, are reproducible in primary BMDCs.

Next we want to further confirm that our immunfluorescent results visualized in BMDCs could be reproduced in primary BMDMs (Bone marrow-derived macrophages).

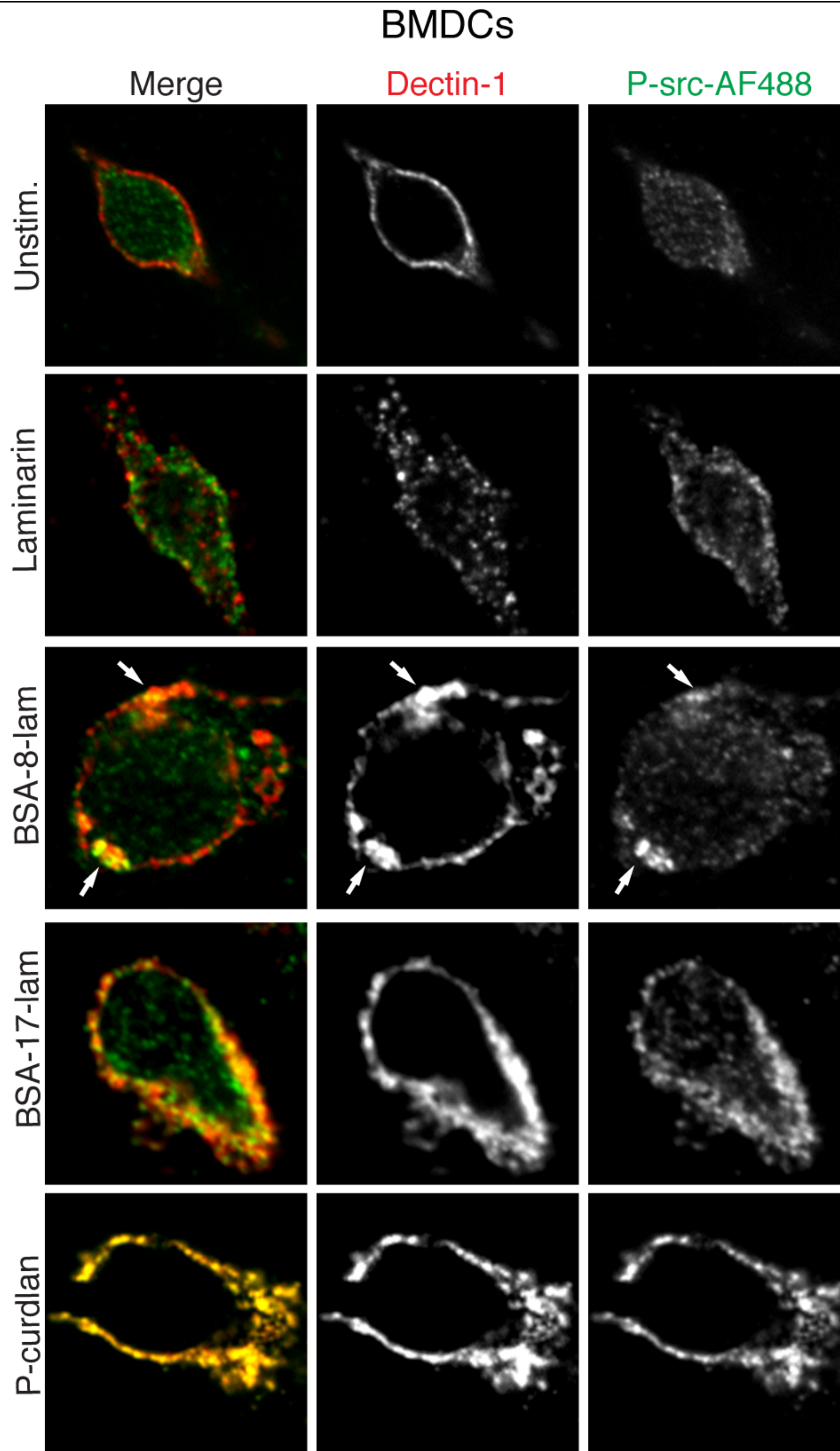

BMDCs
Merge
Dectin-1
P-src-AF488
Unstim.
Laminarin
BSA-8-lam
BSA-17-lam
P-curdlan

Figure 52: Ligand Size Effects of Soluble β-glucans on SFK Membrane Recuitment and Dectin-1 Puncta Formation in BMDCs

BMDCs from day 10 of primary culture were serum-starved for 4 hours. Cells were then stimulated with 100 µg/mL of the ligands indicated in above figure for 5 minutes or left unstimulated. Non-permeabilized cells were then fixed, followed by immunostaining for Dectin-1 using a rat anti-mouse Dectin-1 Ab and a Cy3-conjugated anti-mouse secondary Ab (in red). Cells were then permeabilized, blocked with blocking buffer, and activation/phosphorylation of SFK was visualized using rabbit anti-phospho Src (Y416) conjugated to AF488 (in green) (Invitrogen). Cell images were then acquired on a spinning disc confocal microscope. White arrows in the image of the cell stimulated by BSA-8-laminarin, indicate two sites of intense SFK recruitment to regions of the plasma membrane enriched in Dectin-1. Experiment is representative of three independent replicates.

We performed a P-SFK immunfluorescent similar to the one described above for BMDCs, to probe for SFK membrane recruitment and activation. Briefly, BMDMs were treated for 6 minutes at 37°C, with soluble Dectin-1 ligands of increasing molecular weight different size or increasing valency of laminarin: BSA-8-lam<BSA-17-lam and laminarin<P-curdlan (Figure 53**).** This was followed by a dual immunostaining of surface Dectin-1 (in red) and total phospho-Src/SFK (in green). As seen in Figure 53**,** we could reproduce the results observed for SFK membrane recruitment and activation observed in both RAW Dectin-1 cells, as well as BMDCs. Moreover, in a similar fashion to RAW Dectin-1 cells and BMDCs, intense Dectin-1 puncta were formed upon stimulation with ligands of higher molecular weight or higher laminarin valency. For instance, as illustrated in Figure 53, P-curdlan and the BSA-17-laminarin conjugate resulted in marked activation and recruitment of P-Src/SFK to the plasma membrane, as well as pronounced formation of cell surface Dectin-1 puncta (red signal) that strongly coalesced with membrane regions of intense P-Src signal (green) **(**Figure 53**)**.

This validates that results demonstrated by BMDCs and RAW Dectin-1 cells, regarding the relationship between ligand size, P-SFK activation/localization, and Dectin-1 puncta formation, could be successfully replicated in BMDMs.

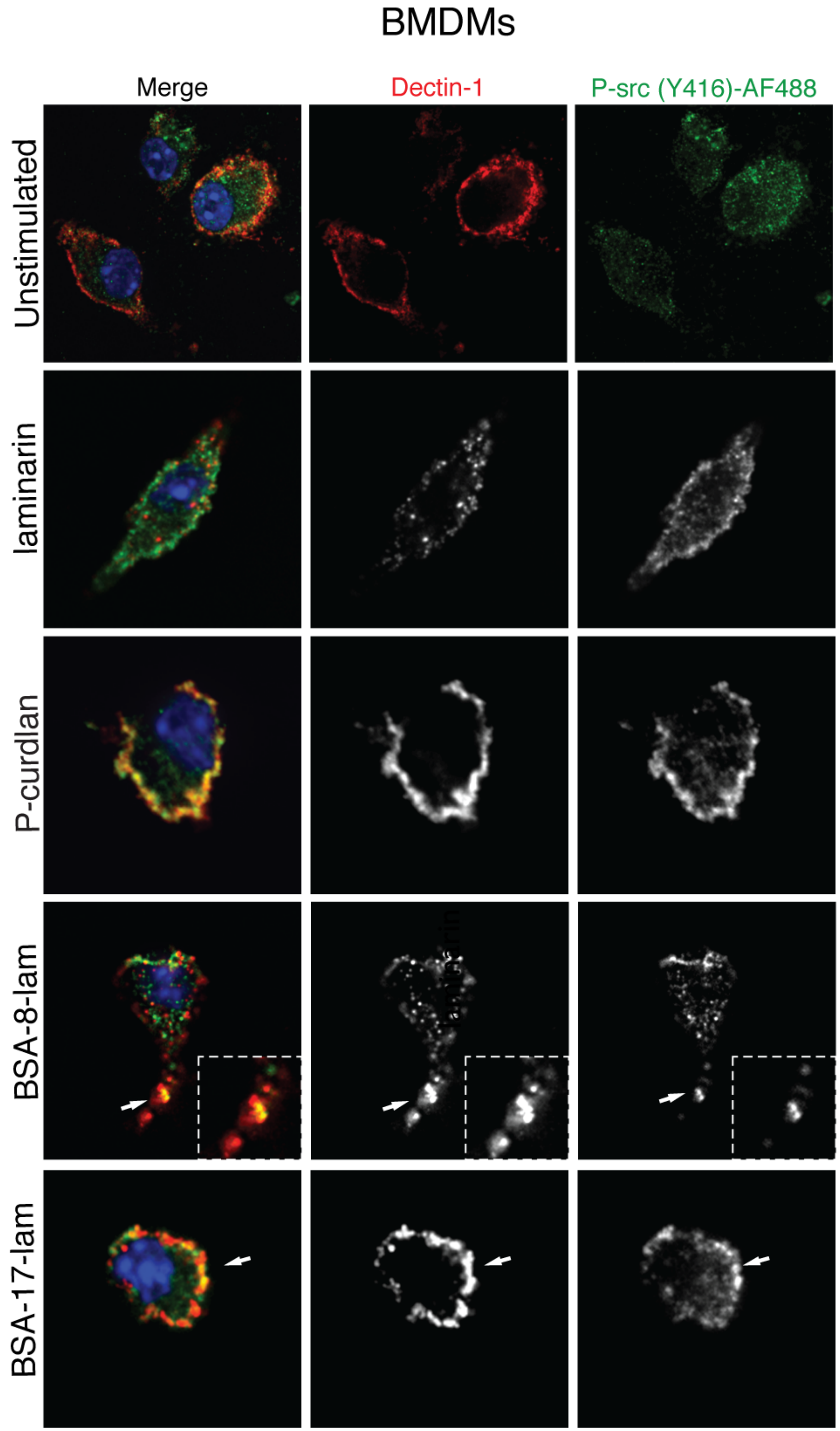


Figure 53: Src Activation and Recruitment in BMDMs in Response to Different-sized Dectin-1 ligands

BMDMs were serum-starved for 4 hours. Cells were then stimulated with 100 µg/mL of the ligands indicated in the above figure for 5 minutes or left unstimulated. Non-permeabilized cells were then fixed, followed by

immunostaining for Dectin-1 using a rat anti-mouse Dectin-1 Ab and a Cy3-conjugated anti-mouse secondary Ab (in red). Cells were then permeabilized, blocked with blocking buffer, and phosphorylation of SFK was visualized using rabbit anti-phospho Src (Y416) conjugated to AF488 (in green) (Invitrogen). Cell images were then acquired on a spinning disc confocal microscope. White arrows indicate sites of intense SFK recruitment to regions of the plasma membrane enriched in Dectin-1 puncta. Inset in 3rd panel demonstrates a 'zoom-in' of a region of the cell membrane with intense colocalization between surface Dectin-1 (red signal) and P-SFK (green signal) Experiment is representative of three independent replicates.

After examining the effect of ligand size on SFK activation and localization we moved on to investigate the effect of ligand size on the activation and recruitment of the crucial Dectin-1 signaling mediator SYK that is activated downstream of SFKs.

Again we repeated a typical immunofluorescent experiment in BMDMs, similar to the one described above in **(see section 3.2.3.1)** for BMDCs and RAW Dectin-1 macrophages. In this experiment BMDMs were treated with the same panel of different-sized soluble ligands of different size (laminarin, P-curdlan, BSA-8-lam, and BSA-17-lam) used for the previous P-Src experiment (Figure 54**)**. In summary, cells were serum-starved for 4 hours and stimulated with ligands for 10 minutes, followed by dual immunostaining of surface Dectin-1 (using an anti-rat Dectin-1 Ab, immunolabeled in red) and total phospho-SYK [using anti-P-SYK (Y352) Ab, immunolabeled in green] (Figure 54**)**. Visualization of cells by confocal microscopy revealed markedly higher levels of SYK phosphorylation and membrane recruitment upon stimulation with P-curdlan and BSA-17-lam, as compared with laminarin, a much smaller β-glucan, and unstimulated controls (Figure 54**)**. These larger-sized ligands not only induced the formation of intensive phospho-SYK (P-SYK) puncta at the plasma membrane, but also induced the formation of areas of intense Dectin-1 staining at the cell membrane, which remarkably colocalized with the phospho-SYK puncta in a similar fashion to that observed in BMDCs and RAW Dectin-1 cells (Figure 54**)**. This pattern of SYK recruitment and phosphorylation detected in BMDMs in response to different-sized ligands is largely identical to that observed for SFK recruitment/activation in the same primary cells (Figure 53).

In general, the relationship between SYK recruitment/activation and ligand size follows the same trend as that observed for SYK recruitment, in BMDCs and RAW Dectin-1 cells. Therefore to conclude, the effect of ligand size on the activation of Dectin-1 upstream signaling events could be recapitulated in BMDCs and RAW Dectin-1 cells

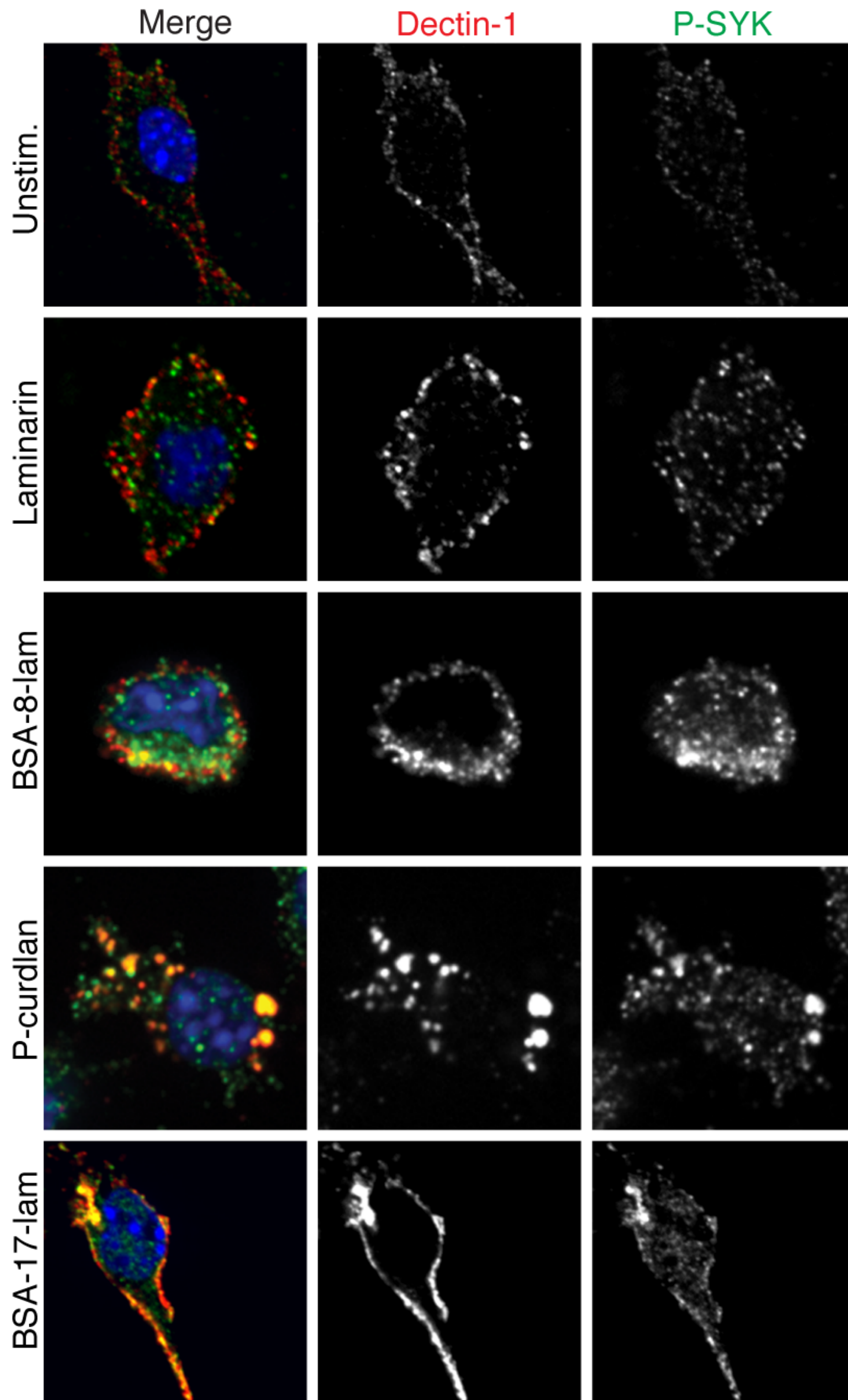


Figure 54: SYK Activation and Recruitment in BMDMs in Response to β-glucans of variable size

Phosphorylation and recruitment of SYK as well as Surface Dectin-1 was observed in response to a variety of

soluble β-glucans of increasing size. Primary BMDMs were serum starved for 5 hours. Cells were then treated at 37°C for 10 mins with 100 μg/mL of ligands (depicted in above figure), or left unstimulated. Cells were then fixed, followed by immunostaining of surface-localized mouse Dectin-1 by incubation with rat anti-mouse Dectin-1 antibodies (R&D) and subsequent labeling with a Cy3-conjugated secondary antibody (in red). Next, the cells were permeabilized, and immunostained for phosphorylated SYK (at residue Y352) before visualization on the confocal microscope. Potent SYK recruitment and colocalization with Dectin-1 puncta at the cell membrane are obvious in cells treated with BSA-17-lam or P-curdlan. Images shown above are representative of three independent experiments.

Finally we wanted to validate that the observed Dectin-1 puncta formed in primary cells colocalize with bound Dectin-1 ligands. We performed a ligand-binding assay on BMDMs and BMDCs as as that described for Figure 35. Cells were treated with AF546-labeled ligands o (pseudocolored in blue) for 5 minutes on ice and then cells were thoroughly washed by PBS to remove unbound ligand. Cells were then fixed and surface Dectin-1 was immunostained (red in colour), followed by visualization on a confocal microscope **(**Figure 35**)**.

Indeed, as illustrated in Figure 35, regions of intense Dectin-1 staining perfectly colocalized with the labeled ligands. Moreover, AF546–BSA-8-lam produced less intense Dectin-1 puncta than the AF546–BSA-17-laminarin with a higher valency of laminarin molecules. Thefore binding of larger ligands could be inducing the formation of high intensity Dectin-1 puncta, which are most probably clusters of Dectin-1. This is in alliance with our results observed for RAW Dectin-1 cells (Figure 43 & Figure 42).

In summary, to conclude this section, examining the activation of selected Dectin-1 upstream signaling events in BMDMs and BMDCs, confirmed that we can recapitulate results from our model cell line of 'RAW Dectin-1' cells, in the more native cell system of primary immune cells endogenously expressing Dectin-1. Moreover results demonstrated in this section, prove that our findings obtained for human Dectin-1 that is heterologously expressed in RAW Dectin-1 macrophages, could be reproduced by the mouse isoform of Dectin-1, which shares more than 65 % sequence homology with human Dectin-1 (Sattler et al., 2012).

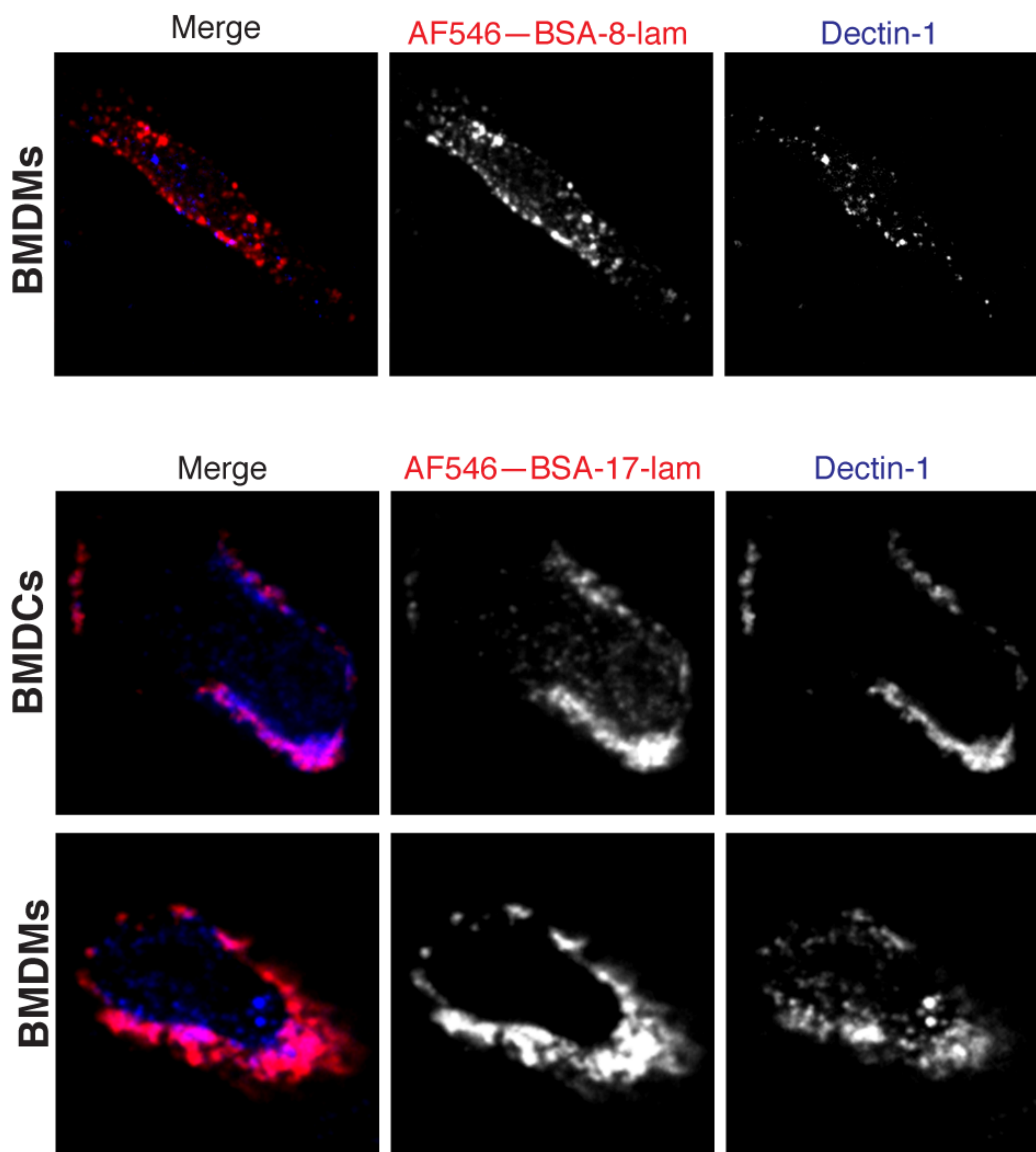


Figure 55: AF546—BSA-laminarin Conjugates Bind to Surface Dectin-1 in BMDCs & BMDMs

Binding of selected AF-546–labeled BSA-laminarin conjugates to BMDMs and BMDCs was examined by immunofluorescence. BMDCs and BMDMs cultured on glass coverslips were treated with ligands AF546–BSA-17-lam, or AF546–BSA-12-laminarin 4°C (on ice for) 5 mins. Ligands were then washed off the cells 5 times with PBS, and then cells were fixed with 4% PFA for 10 minutes at room temp. Rat anti-mouse Dectin-1 primary Ab was then added onto non-permeabilized cells followed by staining with secondary Ab labeled with Cy5 (blue). Coverslips were then by mounted on slides and visualized by confocal microscopy. AF546–BSA-laminarin conjugates were acquired in the Red channel (Cy3) (pseudocolored in red) and Dectin-1 was acquired in the Far red (Cy5) channel (pseudocolored in blue). Images are representative of 3 independent experiments.

3.2.4. The Effect of Ligand Size on Dectin-1–induced Downstream Signaling

In the previous section we examined the effect of soluble Dectin-1 ligands of various size on activation of early Dectin-1 signaling events upstream of the Dectin-1 signaling pathway (section **3.2.3)**. We were then curious to determine if we could see a parallel effect of ligand size on further downstream events of the Dectin-1 signaling pathway

As explained above one of the most crucial, membrane-distal, signaling events activated further downstream of the Dectin-1 signaling pathway, is the activation of the transcription factor NF-κB, which induces the transcription of various cytokines and other key inflammatory mediators **(**see section **1.5.4.1)**. Therefore we sought to investigate the effect of ligand size on the activation key downstream mediator of Dectin-1, NF-κB .

NF-κB is a family of five transcription factors: p65 (also known as RelA), RelB, c-Rel, p50/p105 and p52/p100 (Kingeter and Lin, 2012). These NF-κB protein subunits form homo- or hetero-dimers that are held inactive in the cytosol by the inhibitor protein IκB (inhibitor of κB) (Kingeter and Lin, 2012). Upon Dectin-1 ligation, the canonical NF-κB pathway is triggered by activation of the IKK (inhibitor of κB kinase) complex (Figure 16), which in turn triggers phosphorylation of IκB proteins on conserved serine residues, resulting in their ubiquitination and subsequent degradation, thereby releasing the NF-κB dimers free to be able to translocate into the nucleus and activate gene transcription to induce transcription of cytokines (Dodd and Drickamer, 2001). Before translocating to the nucleus, the p65 subunit of the NF-κB dimer, is phosphorylated downstream of the Dectin-1–induced Raf-1 pathway which leads to its acetylation once present in the nucleus (Gringhuis et al., 2009b). The p65 subunit of NF-κB is the most common subunit of NF-κB dimers involved in NF-κB signaling.

Accordingly, we next sought to examine by an immunofluorescent approach the effect of ligand size on the nuclear translocation of the p65 subunit of the transcription factor NF-κB in RAW Dectin-1 cells, as well as in the primary immune cells, BMDMs and BMDCs.

3.2.4.1. Downstream Signaling Effects of Different-sized Ligands in Dectin-1 RAW Macrophages

We first decided to examine the kinetics of NF-κB translocation in response to ligands of various sizes. As explained in **section 2.7** (Materials and Methods, Chapter 2), RAW Dectin-1 cells were pulse-stimulated with Dectin-1 ligands for 10 minutes at 37°C, then washed thoroughly with

sterile warm PBS to remove excess ligand, followed by another incubation period of 10 or 25 mins at 37°C in plain culture media. This would ensure that the translocation of NF-κB would be due to a single pulse of Dectin-1 stimulation and not due to continuous and repeated pulses of activation, which could result in misleading results. After different incubation times at 37°C, NF-κB translocation was analyzed by cell permeabilization, followed by immunofluorescent staining of the NF-κB p65 subunit, using a mouse anti-p65 (Santa Cruz Biotechnology), with subsequent addition of an anti-mouse Cy3-coupled secondary Ab (red) (Figure 56), or anti-mouse AF488-coupled secondary Ab (green) (Figure 57). Nuclear staining was performed using DAPI (in blue) to be able to locate the cell nuclei.

With all ligand treatments, no nuclear translocation of the p65 NF-κB subunit was observed at the early time point of 10 minutes. However, NF-κB nuclear translocation was observed as early as 20 minutes in cells treated with the larger ligands P-curdlan and ChsA-17-laminarin (chicken-serum albumin protein carrier with 17 laminarin molecules; prepared by the Bundle lab) (Figure 56). Interestingly, at the timepoint of 20 minutes nuclear translocation of p65 was only detected in RAW Dectin-1 cells, treated with P-curdlan and ChsA-17-laminarin, and not in those stimulated with laminarin, a small Dectin-1 ligand (Figure 56). In fact nuclei of cells stimulated by laminarin showed an appearance very similar to that of unstimulated cells. This result is in agreement with the Dectin-1 literature that mentions that laminarin fails to activate induction of Dectin-1 signaling. Also of interest is the stronger nuclear localization of p65 in P-curdlan-treated cells versus BsA-17-laminarin–induced cells, as revealed by the strong colocalization between the DAPI staining (in blue) and the red signal of p65 (Figure 56). This observation is in accordance with the higher molecular weight of P-curdlan versus BSA-17-laminarin. This result also agrees with the well-known capacity of curdlan as a potent Dectin-1 activator, though interestingly our P-curdlan is largely soluble versus the insoluble curdlan extensively used in most Dectin-1 studies.

From the above experiment we were to determine the optimum post-incubation time-point for executing NF-κB further nuclear localization experiments, which is the time-point of 20 minutes (Figure 56). We next decided to move on and examine the effect of a variety of soluble β-glucans, in addition to the effect of a panel of BSA-laminarin conjugates with increasing number of laminarin molecules, on NF-κB nuclear translocation. To confirm that the NF-κB nuclear translocation induced in response to these β-glucans is Dectin-1–specific, we decided to perform

the next nuclear localization experiment in RAW Dectin-1 cells, as well as RAW WT control cells.

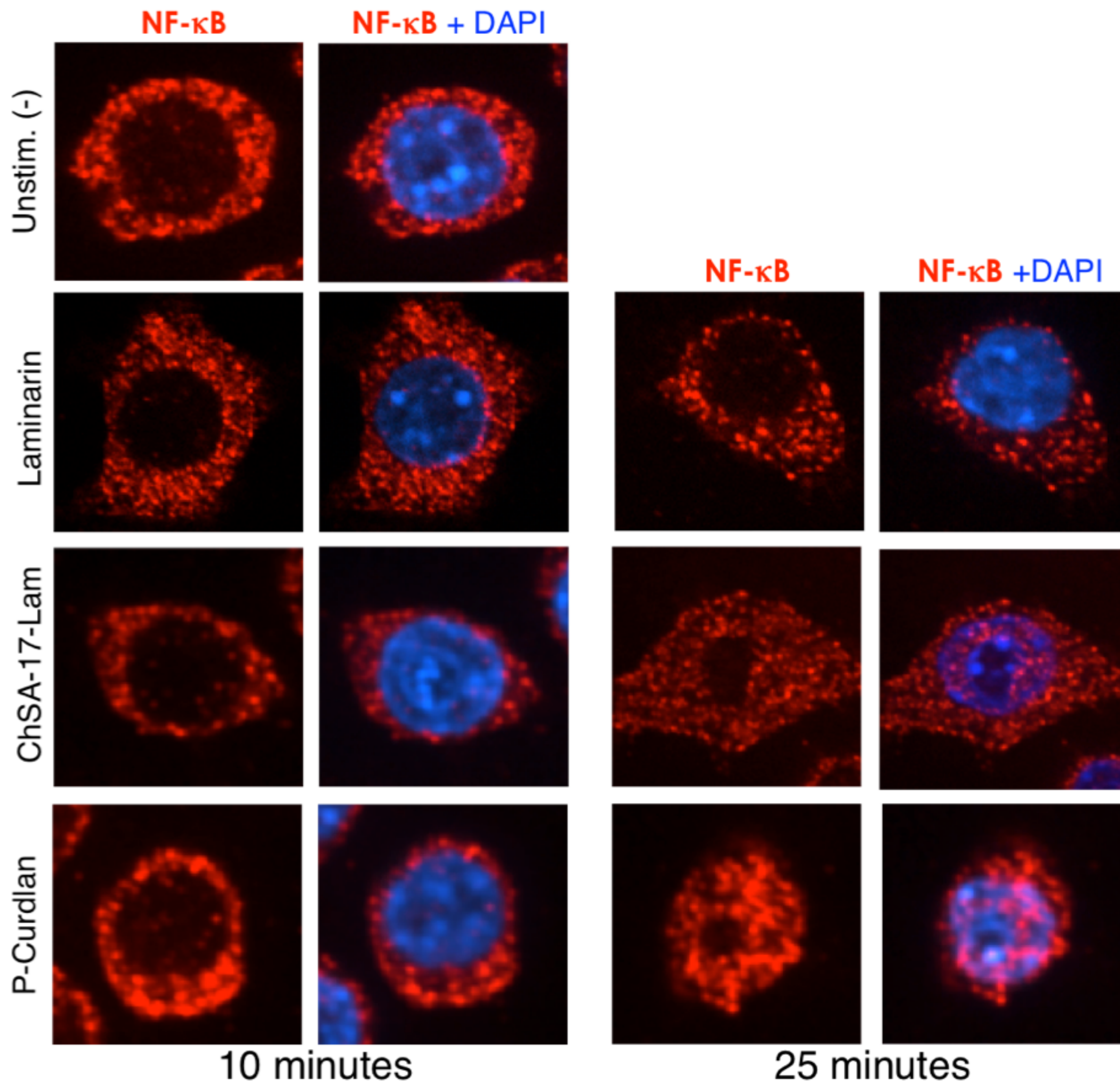


Figure 56: Kinetics of NF-κB Translocation in RAW Dectin-1A Cells in Response to Ligands of Varying Size

RAW Dectin-1 cells were pulse-stimulated at 37°C for 10 minutes with different Dectin-1 ligands of varying sizes, each at a concentration of 100 μg/mL (P-curdlan, ChsA-17-lam, laminarin or not), after which they were rinsed with PBS and incubated for 10 or 20 more minutes at 37°C in culture plain media containing no ligands. The cells were then washed, fixed, permeabilized, immunostained for p65 NF-κB (red) using a mouse anti-p65 (Santa Cruz Biotechnology), with subsequent addition of an anti-mouse Cy3-coupled secondary. Cellular nuclei were labeled with DAPI (blue) to visualize cell nuclei. Images were acquired on the confocal microscope, and are representative of the results from five independent experiments.

We pulse-stimulated both RAW Dectin-1 and RAW WT cells with laminarin, P-curdlan and

BSA-17-laminarin for 10 minutes 37°C, then washed excess ligand off, and cells left to incubate for an additional 20 minutes at 37°C in ligand-free culture medium. This was followed by immunostaining of NF-κB (in green) in permeabilized cells, in addition to DAPI staining of nuclei (in blue) (Figure 57). Only translocation was observed in RAW Dectin-1 cells, and in response to larger-sized ligands, namely BSA-17-lam and P-curdlan, as illustrated in (Figure 57) by the perfect colocalization between NF-κB (in green) and the nucleus (blue). RAW WT cells, lacking Dectin-1 failed to induce nuclear translocation by any of the treated ligands (Figure 57). Therefore, in conclusion NF-κB (p65) nuclear translocation detected in RAW Dectin-1 cells is specific to Dectin-1–mediated signaling.

Since laminarin failed to induce any detectable NF-κB nuclear translocation we were curious to determine if increasing the number of laminarin molecules on the BSA protein carrier would enhance the capacity of laminarin to stimulate NF-κB (p65) translocation (Figure 58B). We thus stimulated RAW Dectin-1 Cells with a panel of BSA-laminarin conjugates with an increasing number of laminarin: 4, 8, 12 and 17 (Figure 58B). Amazingly, all of these BSA-laminarin conjugates were able to induce the nuclear translocation of NF-κB (Figure 58B). Of great interest is that even the BSA-laminarin conjugate with the lowest number of laminarin moleules, namely four, (BSA-4-laminarin) was able to induce nuclear translocation, however stronger nuclear localization was observed with an increase in the number of laminarin molecules in these conjugates (Figure 58B). This indicates that increasing the valency of laminarin molecules exposed on the surface of these conjugates enhances the capacity of the small β-glucan, laminarin, to activate Dectin-1 signaling.

To further validate, the size effect of the soluble β-glucan on its capacity to induce NF-κB signaling we next tested if degrading P-curdlan into smaller fragments, generating the ligand ‘chopped P-curdlan’ would decrease the ability of P-curdlan to induce NF-κB nuclear translocation. Indeed, as seen in (Figure 58A), generating smaller fragments of P-curdlan largely diminished the capacity of P-curdlan to induce NF-κB nuclear localization (Figure 58A). To confirm that P-curdlan induces NF-κB nuclear translocation in a Dectin-1–dependent fashion, RAW Dectin-1 cells were stimulated with P-curdlan in the presence of the SFK (Src Family kinase) inhibitor, PP2 (Figure 58A). As expected, inhibiting SFK activation by PP2, almost completely abolished nuclear translocation stimulated by P-curdlan (Figure 58A). Finally, we wanted to compare the effect of P-curdlan (up to 12,000 glucose residues and MW ≤5000 kDa)

on nuclear translocation of p65, with scleroglucan (>1000 kDa) a soluble, branched β-glucan smaller in size than P-curdlan yet much bigger than laminarin (6–8 kDa) **(**Figure 58**A)**. Indeed, the high molecular weight β-glucan, P-curdlan, induced stronger nuclear localization of NF-κB (p65) than scleroglucan, which is smaller in size than P-curdlan **(**Figure 58**A)**.

In conclusion, the NF-κB (p65) translocation data described above NF-κB signaling is in agreement with our clustering hypothesis stated above in **section 3.1 (**Figure 22**)**, where our findings demonstrate that only larger ligands are capable of inducing Dectin-1 downstream signaling as seen by the marked capacity of larger Dectin-1 ligands to induce NF-κB nuclear tramslocation. Also increasing the valency of a small soluble β-glucan, such as laminarin, exposed on the surface a protein carrier, such as BSA, increases their capacity to activate signaling events further downstream of the Dectin-1 signaling pathway.

Next, to further confirm our key findings from the NF-κB nuclear translocation experiments describe above we were interested to determine if the results that we observed in RAW Dectin-1 cells could be reproduced in the primary immune cells, BMDMs and BMDCs.

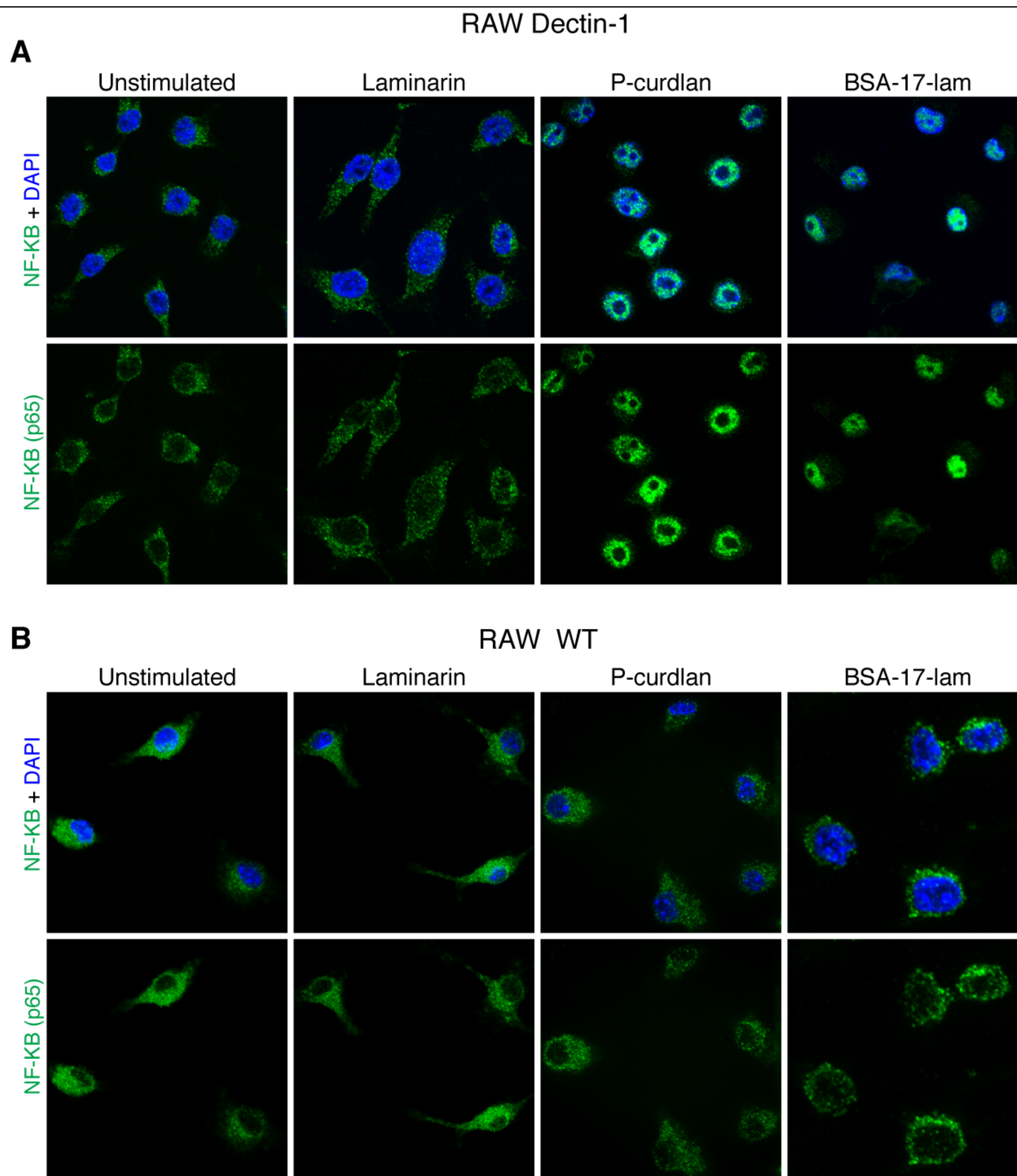


Figure 57: NF-κB Translocation in RAW Dectin-1A versus RAW WT Cells in Response to Ligands of Varying Size

RAW Dectin-1 or RAW WT cells were pulse-stimulated at 37°C for 10 minutes with different Dectin-1 ligands of varying sizes as labeled in figure, each at a concentration of 100 μg/mL (P-curdlan, BSA-17-lam, laminarin or not), after which they were rinsed with PBS and incubated for 10 or 20 more minutes at 37°C in culture plain media containing no ligands. The cells were then washed, fixed, permeabilized, immunostained for p65 NF-κB (green), using a mouse anti-p65 (Santa Cruz Biotechnology), with subsequent addition of an anti-mouse AF488-coupled secondary Ab (green) cell nuclei were labeled with DAPI (blue) to visualize cellular nuclei. Images were visualized on the confocal microscope, and are representative of the results from four independent experiments.

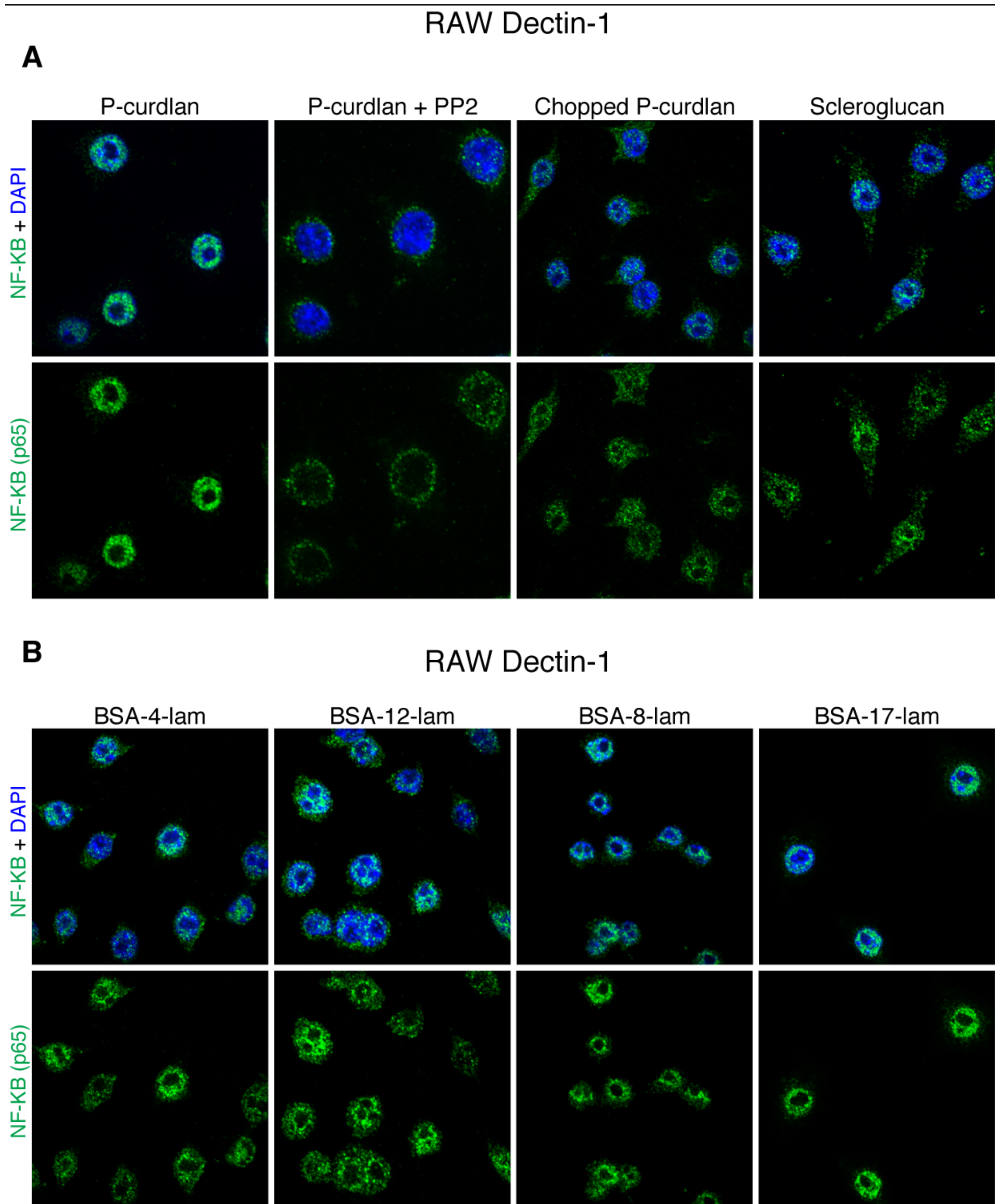


Figure 58: NF-κB Nuclear in RAW Dectin-1 Cells as a Function of Ligand Size

RAW Dectin-1 were pulse-stimulated at 37°C for 10 minutes with ligands of different size indicated in figure, each at a concentration of 100 µg/mL (P-curdlan, BSA-17-lam, laminarin or not). NF-κB translocation to the nucleus was determined upon an additional 20-min incubation of the cells following ligand incubation. Cells were fixed and permeabilized, and NF-κB was labeled using the rabbit anti-p65 Ab (Santa Cruz Biotechnology), followed by an

anti-rabbit AF488 (green) Ab in parallel to DAPI (blue). Images were acquired on the confocal microscope. **(A)** Demonstates the effect of larger-sized ligands P-curdlan and scleroglucan, and chopped P-curdlan. Also shown in top panel is the effect of the SFK PP2 inhibitor on P-curdlan-induced nuclear translocation. For PP2 inhibition, cells were then pretreated with PP2 (at 10 μM), for 30 mins before ligand stimulation. Cells were then stimulated as describe above with P-curdlan in the presence of the pharmacological inhibitors PP2 (at 10 μM). **(B)** NF-κB translocation was monitored in response to a panel of BSA-laminarin conjugates with an increasing number of laminarins. Data are representative of four similar experiments.

3.2.4.2. Ligand Size Effects on Downstream Dectin-1 Signaling in BMDMs and BMDCs

We first decided to examine the effect of selected, soluble β-glucans of varying molecular weight: P-curdlan and laminarin; as well as, a panel of BSA-laminarin with increasing numbers of laminarin (4, 8, and 17). We performed an immunfluorescent NF-κB (p65) translocation experiment exactly similar to the ones described above. Briefly, cells were stimulated with ligands, and then permeabilized cells were immunostained for (NF-κB) in green), and cell nuclei were DAPI-stained (in blue), followed by detection under the confocal microscope (in blue) **(**Figure 59). Similar to results obtained in above section (3.2.4.1) for RAW Dectin-1, no nuclear translocation of NF-κB was observed upon stimulation with strong nuclear colocolaziation of NF-κB was observed in cells stimulated with large ligands, such as P-curdlan and BSA-17-lam. Interestingly, increasing the number of laminarin on the BSA-laminarin conjugate progressively increased the magnitude of p65 nuclear translocation **(**Figure 59**)**.

Therefore the correlation between ligand size and NF-κB nuclear translocation in BMDCs largely follows that detected for RAW Dectin-1 cells.

In parallel to the NF-κB nuclear translocation experiment described above for BMDCs, the same experiment was repeated in BMDMs. Similar to our results from BMDC, P-curdlan and all of the BSA-laminarin conjugates (with 4, 8, 12 and 17 laminarins), were able to induce NF-κB nuclear translocation in BMDMs, as observed by strong colocalization between NF-κB (in green) and cell nuclei (blue) **(**Figure 60). Also similar to the trend observed in BMDCs, increasing the valency of laminarin in the BSA-conjugate increases the potency of nuclear translocation. Again, cells treated with laminarin showed no detectable nuclear localization of NF-κB, which is greatly similar to what has been observed in unstimulated cells **(**Figure 60**)**.

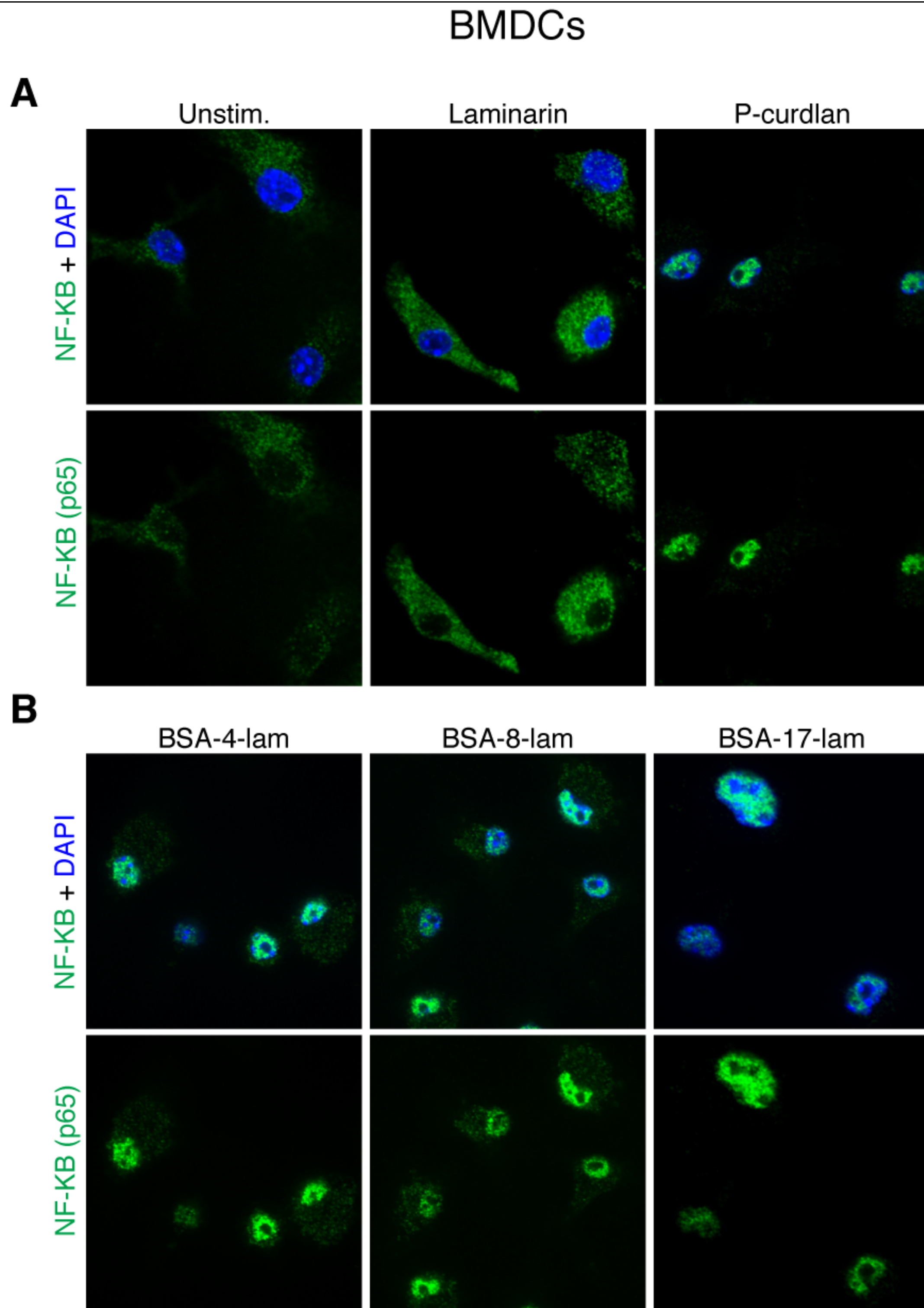


Figure 59: Effect of Dectin-1 Ligands of Variable size on NF-κB Nuclear Translocation in BMDCs

BMDCs were treated with soluble ligands size indicated in figure (P-curdlan and laminarin), as well as with a panel of BSA-laminarin conjugate with increasing numbers of laminarin (4, 7, 8, 12 laminarins). Cells were pulse-stimulated at 37°C for 10 minutes with ligands, each at a concentration of 100 μg/mL, followed by an additional 20-min incubation of the cells 37°C, after which NF-κB translocation to the nucleus was determined. Cells were fixed and permeabilized, and NF-κB was labeled using the rabbit anti-p65 Ab (Santa Cruz Biotechnology), followed by an

anti-rabbit AF488 (green) Ab in parallel to DAPI (blue). Images were acquired on the confocal microscope. Data is representative of 3 similar experiments.

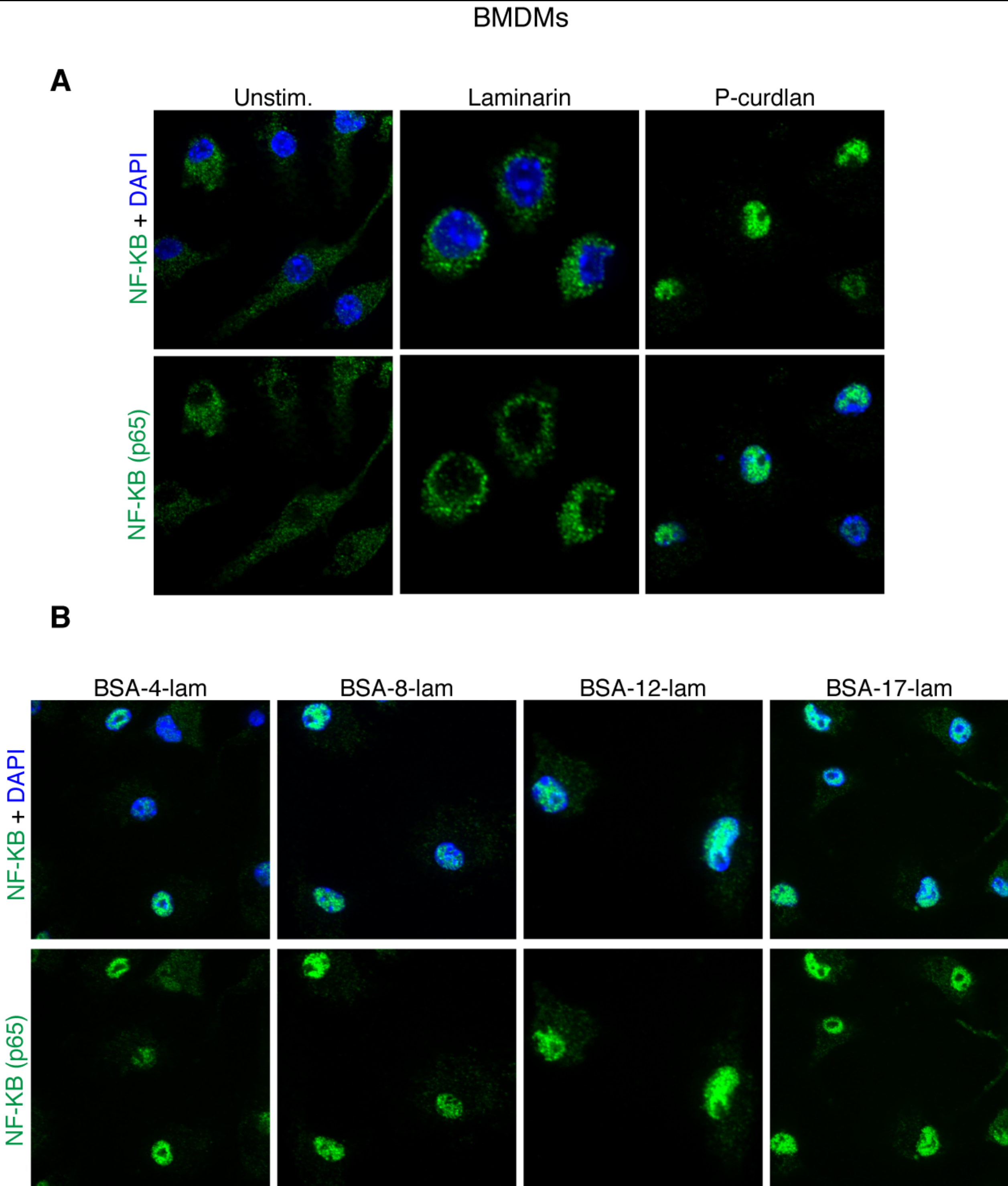


Figure 60: Ligand Size Effects on NF-κB Nuclear Translocation in BMDMs

BMDMs were pulse-stimulated at 37°C for 10 minutes with different Dectin-1 ligands of varying sizes (P-curdlan, BSA-17-lam, laminarin or not), as well as a panel of BSA-laminarin conjugates each at a concentration of 100 μg/mL, after which they were rinsed with PBS and incubated for 20 more minutes at 37°C in culture plain media

containing no ligands. The cells were then washed, fixed, permeabilized, immunostained for p65 NF-κB (green), using a mouse anti-p65 (Santa Cruz Biotechnology), with subsequent addition of an anti-mouse AF488-coupled secondary Ab (green) cell nuclei were labeled with DAPI (blue) to visualize cellular nuclei. Images were visualized on the confocal microscope, and are representative of the results from four independent experiment

Overall, we were able to recapitulate our RAW Dectin-1 results for the effect of ligand size on Dectin-1 activation of NF-κB, in primary innate immune cells, BMDCs and BMDMs (Figure 59, Figure 60, Figure 57, & Figure 58B).

In conclusion so far, larger ligands have a higher capacity of inducing Dectin-1 downstream signaling, such as the key and further downstream event of NF-κB activation followed by nuclear translocation. Moreover, our results in this **section 3.2.4**, demonstrate that the small β-glucan laminarin, in consistence with the literature, fails to induce the translocation of NF-κB to the nucleus as compared with ligands of larger size (P-curdlan and scleroglucan) or in BSA-laminarin conjugates with more than four laminarin molecules. Nevertheless, this result was laminarin quite surprising for us, since as detailed above in **section 3.2.3**, we have uniquely demonstrated that laminarin although a β-glucan of low MW (6–8 kDa), is still capable of activating key upstream signaling events such as SYK and SFK phosphorylation (e.g., as demonstrated by western blots and immunofluorescence (Figure 36, Figure 38, & Figure 42). However, we have intriguingly shown that the magnitude of activation of these upstream signaling events, stimulated in response to laminarin, is much lower than that induced by larger-sized or multivalent Dectin-1 ligands. To date it hasn't been demonstrated that laminarin can induce Dectin-1 signaling at any level of activation, and indeed laminarin is largely considered as a blocker/inhibitor of Dectin-1 signaling. Therefore, we were curious to further understand how a small ligand like laminarin can induce key upstream signaling events, such as SYK and Src phosphorylation yet fails to induce one of the most crucial downstream events of Dectin-1, signaling, namely NF-κB activation. This inquisitively prompted us to closely analyze the Dectin-1 signaling pathway at various levels of activation, upstream and downstream.

The following section demonstrates the investigation that we performed in an attempt to answer this puzzling, yet, intriguing question.

3.2.5. Soluble β-glucans of Variable Size Differentially Activate Dectin-1 Signaling

Our findings from **sections 3.2.3 and 3.2.4**, demonstrate that laminarin, a low molecular weight

β-glucan was able to activate upstream signaling events such as SYK and SFK essential for Dectin-1 activation, yet failed to activate signaling events further downstream of the Dectin-1 signaling pathway, such as nuclear translocation of the transcription factor NF-κB. In contrast, P-curdlan, a high molecular weight soluble β-glucan, and potent agonist of Dectin-1, succesfully activated both of these upstream and downstream signaling events. Therefore we decided to further analyze the Dectin-1 signaling pathway at various upstream and downstream levels, in reponse to stimulation by laminarin, as compared with the much larger β-glucan, P-curdlan. For that purpose, we sought to conduct an immunoblot experiment in which we would investigate the activation of various signaling components of the Dectin-1 signaling pathway. This would enable us to test if these two ligands, of largely variable size, would differentially activate specific components of the Dectin-1 signaling cascade. Additionally, analyzing the magnitude of the resulting signaling events induced by each of these β-glucans, would give further insight into the relationship between ligand size and the capacity of the ligand to induce Dectin-1 signaling.

We then moved on and performed this immunblot experiment, in which we closely examined the ability of P-curdlan and laminarin to fully induce activation of Dectin-1 signaling, starting upstream of the pathway at the level of SFK activation all the way downstream to the level of NF-κB activation (Figure 61). RAW Dectin-1 cells were stimulated with either of these two ligands at various timepoints up to 30 minutes (Figure 61). We then conducted immunoblot analysis of selected components of the Dectin-1 signaling pathway, which are well-known to be activated at various levels (membrane-proximal/upstream or membrane–distal/ downstream) of the signaling cascade. The activity of each of these signaling components was specifically detected by immunblotting of cell lysates via phosph-specific antibodies that recognize the phosphorylated form of the protein at certain residues (Figure 61).

Carefully examining the western blot, the effects of P-curdlan stimulation were then compared to that of laminarin to see if differences in molecular weight would alter the ability to activate Dectin-1 signaling cascades (Figure 61). Indeed, stimulation with both laminarin and P-curdlan induced activation of upstream signaling events, including that of Src family kinases (SFKs), SYK, and even Raf-1 **(see section 1.5.4.2)**. However, P-curdlan triggered higher activation of these events, as detected by the increased intensities of the immunoreactive signal for the phosphoproteins, as compared to stimulation by laminarin (Figure 61). Moreover, laminarin was only able to activate signaling of the upstream components including P-SYK, P-SFK and P-Raf-1,

which is consistent with our previous observations in **sections 3.2.3 and 3.2.4** (Figure 61).

We then continued to analyze activation of signaling events that are even further downstream of Dectin-1 signaling cascade (Figure 61). These later signaling events were related to the activation of both the canonical and non-canonical NF-κB pathways, and included the degradation of IκBα (inhibitor of NF-κB) and the phosphorylations of the canonical NF-κB subunit p65, and the non-canonical NF-κB subunit RelB, as well as p105 (the full-length precursor to the p50 non-canonical NF-κB subunit) (see **sections 1.5.4.1 & 1.5.4.2**) (Figure 61). It is also worth-mentioning that phosphorylation of IKK leads to the phosphorylation and subsequent degradation of IκBα, which releases the p65 NF-κB subunit free within its dimer to become phosphorylated (downstream of Raf-1) and translocate into the nucleus.

Remarkably, P-curdlan was able to trigger higher activation of these events, as detected by the increased intensities of the immunoreactive signal for the phosphoproteins, as compared to laminarin stimulation (Figure 61). However, laminarin failed to activate key components of the NF-κB canonical and non-canonical pathway (Figure 61). Starting at the level of IKK activation/phosphorylation down to the level of activation/phosphorylation of various NF-κB subunits (RelB, p105, and p65), the signal induced by laminarin was completely lost and abolished (Figure 61).

Therefore, in conclusion we determined that P-curdlan, a high molecular weight soluble β-glucan posseses a strong capacity to stimulate Dectin-1 signaling down to the level of activation the transcription factor NF-κB. According to our clustering hypothesis, this effect is likely a result of its extremely large size as a β-glucan (up to 5000 kDa) **(**Figure 22**)**. On the contrary, laminarin, a small soluble β-glucan (6-8 kDa), can only stimulate upstream events in Dectin-1 signaling, but this signal is not carried through to the level of downstream activation of molecules further down in the pathway such as NF-κB.

Perhaps the enhanced activation of downstream signaling events induced by P-curdlan translated to potent stimulation of NF-κB, as P-curdlan strongly activated key components of the signaling machinery required for NF-κB activation, such as activation of IKK, the phosphorylation and degradation of IκBα P-curdlan, as well as phosphorylation of the NF-κB (Figure 61). Moreover, P-curdlan was able to activate the non-canonical NF-κB subunits p105 (the full-length precursor to the p50 subunit) and RelB, which indicates the capacity of P-curdlan a large soluble dcetin-1

ligand to even activate the non-canonical pathway (Figure 61). Laminarin, perhaps, as an effect of its small size, was unable to trigger any of those events, which are events essential to NF-κB activation, confirming its inability to activate NF-κB (Figure 61) (.

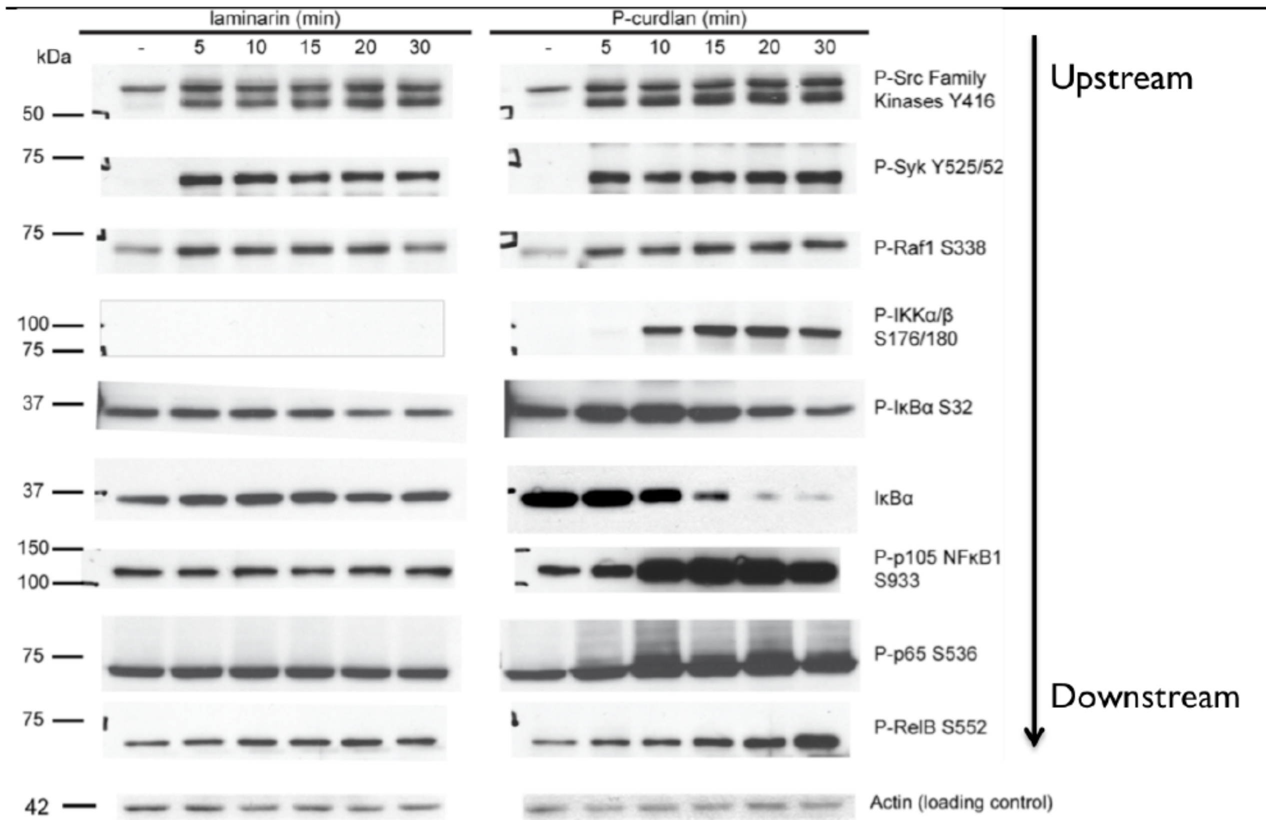


Figure 61: Activation of various Components of the Dectin-1 Signaling Pathway in Response to Laminarin versus P-curdlan

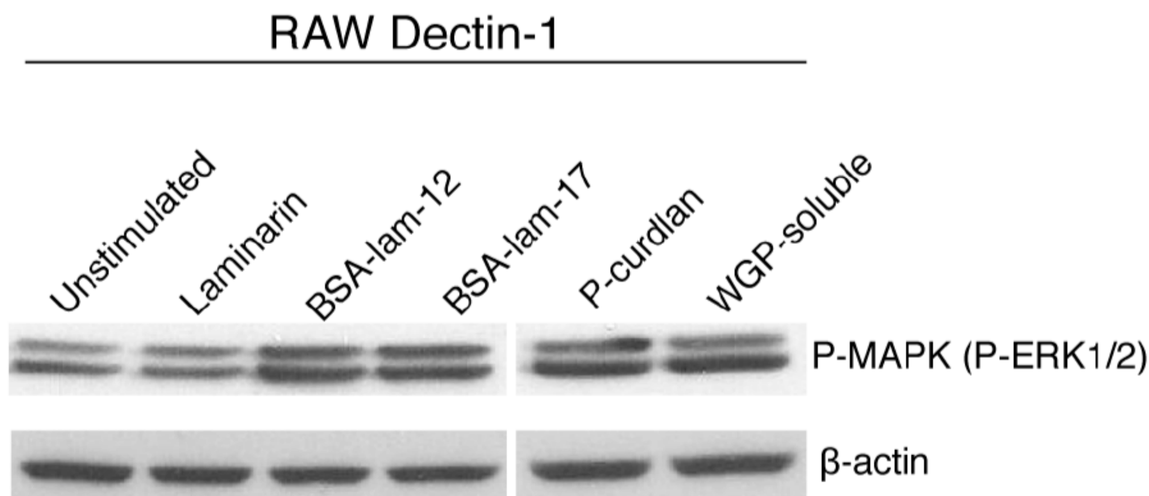
RAW Dectin-1 cells were stimulated continuously in the presence of 100 μg/mL by laminarin (a small Dectin-1 ligand or P-curdlan (a large Dectin-1 ligand) for the indicated times at 37°C. The cells were promptly lysed, processed by SDS-PAGE, and immunoblotted for the presence of phosphorylated Src family kinases, SYK, Raf-1 (on residue S338), the α and β subunits of IKK (on residues S176/S180 or equivalent), IκBα (on residue S32), and the NF-κB subunits p105 (on residue S933), p65, RelB, and finally β-actin as protein loading control. The presence of total IκBα was also detected by immunoblot to assess IκBα degradation, which is a perquisite for NF-κB activation. The lysates for each timepoint were prepared from identical tissue culture plates grown to the same cell density and lysed in the same volume of lysis buffer. Prior to SDS-PAGE, identical lysate volumes and equal amounts of protein (as measured by the BCA protein assay) for each timepoint were loaded onto the polyacrylamide gel. Immunoblot is representative of three independent experiments. The black arrow on the right hand side of the figure indicates the sequence of the Dectin-1 signaling events from upstream to downstream.

We were still puzzled by our results demonstrated in the western blot performed above (Figure 61), which regard the loss of the laminarin signal at a level further downstream of the Dectin-1 signaling pathway. In order to extract further mechanistic insights of how and, exactly when this signal is lost we sought to examine the activation of other well-characterized Dectin-1 signaling components known to be activated downstream of SYK such as ERK1/2 (p42/p44) MAP kinases

(MAPKs), PLCγ2, and PKCδ. We specifically selected these signaling players, as they are not too membrane-proximal (upstream of the pathway), nor too membrane-distal (downstream of the pathway) (Figure 62).

So far in this study, we haven't investigated the effect of laminarin and other ligands of larger size on the activation of the MAP kinases (MAPKs) ERK1/2. Accordingly we were curious to examine by immunoblotting the effect of laminarin on the activation of the MAPKs, ERK1 (p44) and ERK2 (p42), in comparison to larger-sized ligands, including P-curdlan, BSA-17-lam, and BSA-12-lam, and WGPsol (a well-known high MW soluble β-glucan; Biothera) (Figure 62). We conducted an immunoblot experiment, in which RAW Dectin-1 cells were treated for 10 minutes with the ligands mentioned above, or left unstimulated (Figure 62). Amazingly, only the larger Dectin-1 ligands: P-curdlan, BSA-17-lam, and BSA-12-lam, and WGPsol were able to activate Dectin-1 signaling, as detected by higher immunoreactive signals in the western blot for phospho-ERK1/2 in comparison to unstimulated controls unstimulated (Figure 62). On the other hand laminarin didn't produce any activation of the phospho-ERK1/2 and gave a background signal equivalent to that compared to unstimulated controls unstimulated (Figure 62).

As described in section **1.5.4.1**, SYK is a hallmark of Dectin-1 signaling. Accordingly, after showing that, in contrast to laminarin, larger ligands are able to strongly activate the MAPKs ERK1/2, we next wanted to examine in one immunoblot experiment the effect of laminarin on the activation of key signaling effectors activated downstream of SYK including PLCγ2, and PKC, and ERK1/2. We also wanted to examine in parallel, signaling events known to be induced further downstream of Dectin-1–dependent SYK activation, such as degradation of IκBα (inhibitor of NF-κB), and phosphorylation/activation of the canonical NF-κB subunit (p65). As demonstrated in our experiments from **sections 3.2.3 and 3.2.4**, the BSA-laminarin conjugate with 17 laminarins, BSA-17-lam, is a potent water-soluble Dectin-1 ligand capable of strongly inducing various Dectin-1 signaling events, upstream and downstream of the pathway. Therefore, we chose for our next immunoblot experiment to compare the effects of laminarin versus BSA-17-laminarin on activation of the above-mentioned components of the SYK-dependent signaling pathway (Figure 64).



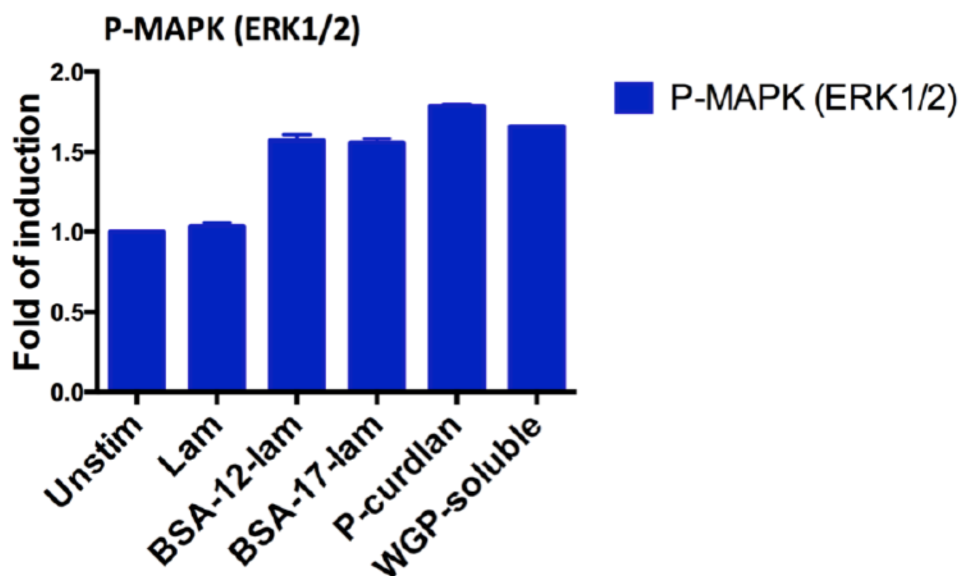


Figure 62: Dectin-1 Activation of the MAPKs, ERK1 (p44) and ERK2 (p42) in Response to Different-Sized Ligands

(A) RAW Dectin-1 cells were serum-starved for 4-6 hours, and then stimulated with media with or without 100 μg/mL of ligand (indicated in above figure) for 10 minutes at 37°C. The cells were promptly lysed, processed by SDS-PAGE, and immunoblotted for the presence of phosphorylated ERK1 and ERK2 (on residues T202 and Y204 or equivalent) or β-actin (as a loading control). Immunoblot is representative of three independent experiments **(A)** Representative blots of phosphorylated ERK1 and ERK2 were shown. **(B)** Densitometry quantification of ERK1 and ERK2 phosphorylation. ImageJ quantification of phosphorylated ERK1 and ERK2 was performed for western blot experiments similar to the one shown in (A) Error bars represent SEM of three independent experiments.

As demonstrated in the western blot in (Figure 64), we stimulated RAW Dectin-1 cells with laminarin or BSA-17-lam for 10 mins and probed by immunobloting for the activation of various

Dectin-1 signaling components activated downstream of SYK. We observed that both laminarin, and BSA-17-laminarin were both capable of activating Src family kinases, Syk, PLCγ2, and PKCδ. However, BSA-17-lam was able to strongly induce much higher levels of phosphorylation/activation of these signaling proteins, as compared to the lower signals detected for laminarin (Figure 64). On the other hand, only BSA-17-lam was able to potently stimulate signaling events further downstream in the SYK-dependent Dectin-1 pathway including ERK1/2(p42/p44) phosphorylation and NF-κB activation (phosphorylation of p65, and degradation of IκBα), whereas, laminarin again failed to induce activation of any these downstream effectors (Figure 64).

These results were intriguing, since a ligand with multiple numbers of the small β-glucan laminarin would fully activate the Dectin-1 signaling pathway all the way down to the level of the transcription factors, wheres laminarin could only activate low levels of phosphorylation of the upstream signaling components SFK, SYK, PLCγ2, and PKCδ (Figure 64). This means that laminarin can only activate upstream events of the SYK-dependent pathway Dectin-1 signaling pathway, just up to the level of PKCδ activation (Figure 64). This result might be because BSA-17-laminarin is a ligand of much larger size and β-glucan valency than laminarin, and therefore it is able to cluster more Dectin-1 as seen by formation of intense Dectin-1 puncta upon binding of BsA-17-laminarin to Dectin-1 (see experiments in section 3.2.3) which in turn possibly facilitates the activation of key upstream mediators such as SFK and SYK above a certain threshold that is sufficient to maintain signaling down to the level of the transcription factors NF-κB. On the other hand, laminarin cannot induce much clustering of Dectin-1, and thus produces limited activation of upstream events at a subthreshold level, which is probably not sufficient to continue the signal further downstream towards the level of NF-κB activation. It is also interesting that laminarin signal is also lost at the level of the MAPKs (ERK1/2), which are known to be activated downstream of PLCγ2 **(see section 1.5.4.1)** (Figure 64). However at this point we don't know how the signal of laminarin stimulation is lost beyond the level of PKCδ (activation and what is reponsible for this 'switching off' of laminarin-induced signaling at the level of MAPK (ERK1/2) and NF-κB activation. It, thus, might seem that activation of the ERK MAP kinases might be essential to transduce the signal further downstream to the level of NF-κB activation.

We were therefore curious to next examine whether inhibition of the MAPK ERK would affect the activation of NF-κB (phosphorylation of p65). Since p65 is phosphorylated downstream of

Raf-1, **(see section 1.5.4.2)**, we used the Raf-1 inhibitor (calbiochem) as a control for p65 inhibition. We then performed an immunoblot experiment where we stimulated RAW Dectin-1 cells with BSA-17-lam, in the absence or presence of the ERK1/2 (p42/p44) MAP kinase inhibitor (calbiochem), or the Raf-1 inhibitor (Figure 63). We chose to examine phosphorylation of p65, as well as, IκBα degradation as NF-κB activation readouts. BSA-17-lam was able to activate these downstream signaling events of NF-κB activation (Figure 63). Interestingly, activation of NF-κB, as seen by phosphorylation of p65, was inhibited by both the Raf-1 and MAPK inhibitors when compared to untreated vehicle controls (Figure 63). However, surprisingly these inhibitors didn't affect IκBα degradation. This means that activation of MAPK is essential for phosphorylation of NF-κB (p65) and not for activation of NF-κB. Also the Raf-1 inhibitor had no effect on ERK activation, indicating that the MAPK ERK is not activated downstream of Raf-1. This is a finding consistent with the literature as MAPKs are thought to be activated downstream of the SYK pathway and not the Raf-1 pathway.

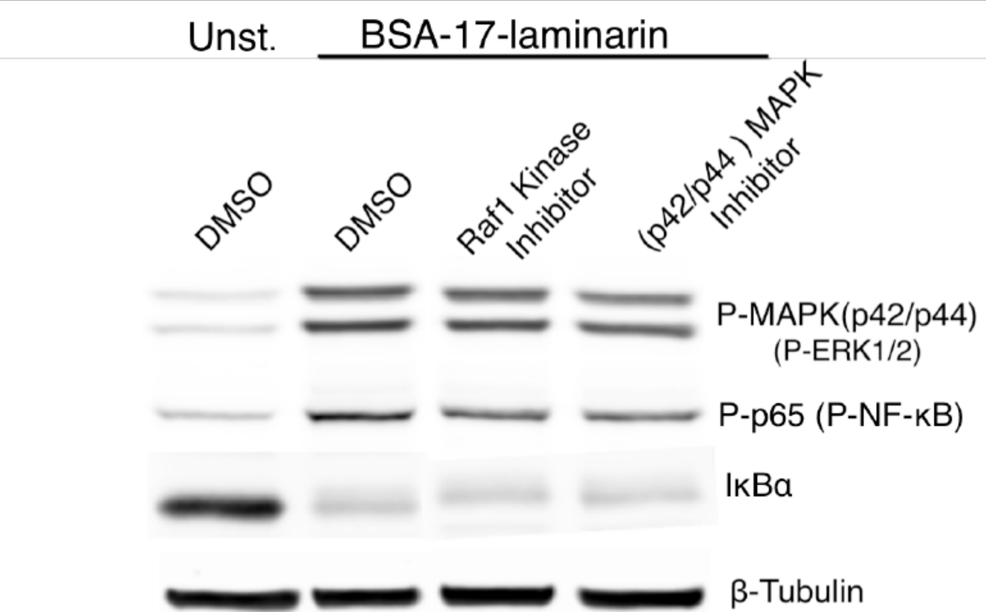


Figure 63: Effect of Inhibitors on MAPK and NF-κB activation

RAW Dectin-1 cells were serum-starved for 4hours and then pre-treated with Raf-1 inhibitor (5 µM; from calbiochem), or ERK1/2 inhibitor (5 µM; from calbiochem), or vehicle control (0.1% DMSO) for 30 minutes at 37°C. They were then stimulated with 100 µg/mL BSA-17-lam in the same solutions for 10 minutes. Cell lysates were then prepared, separated by SDS-PAGE, transferred on nitrocellulose membrane, and blotted with Abs against phospho-65 (Cell signaling), or phosphorylated ERK1 and ERK2 (on residues T202 and Y204 or equivalent), or against total IκBα (to assess IκBα degradation) and β-tubulin (as a loading control). Immunoblots presented are results from two experiments.

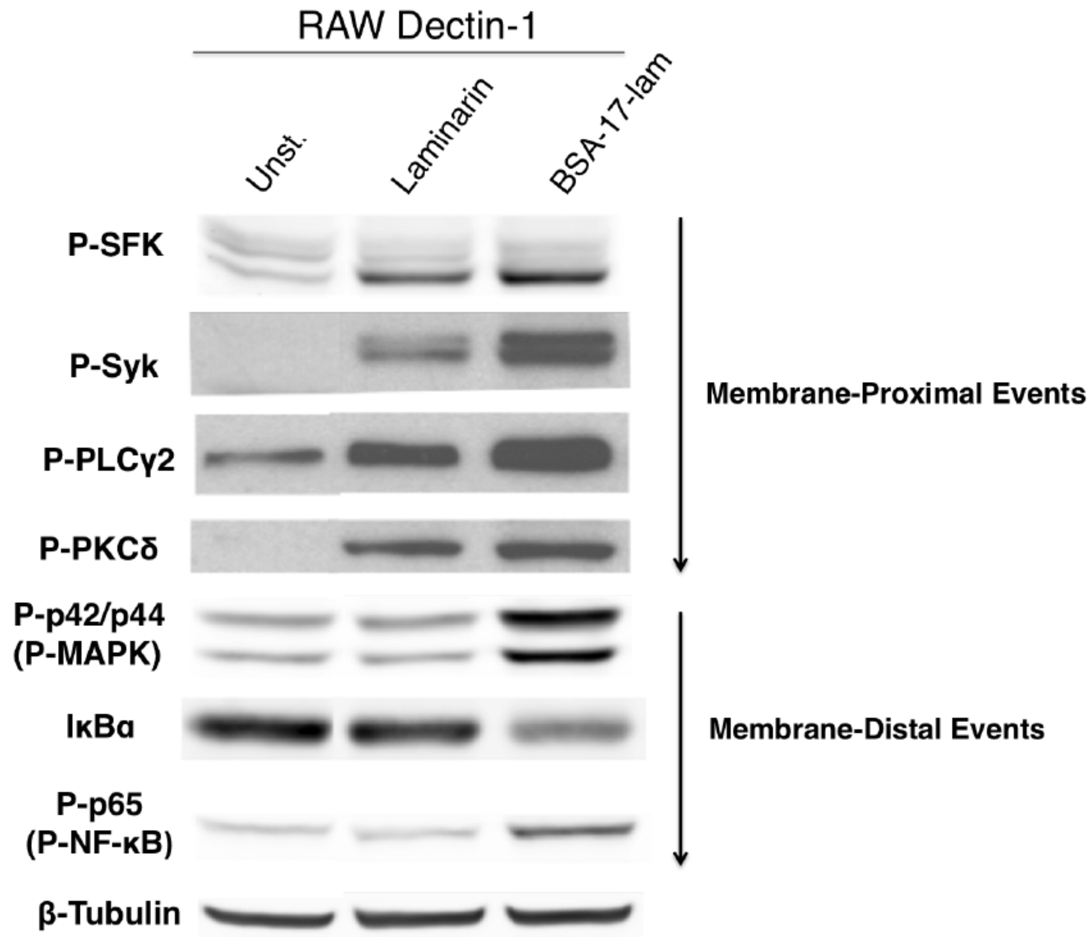


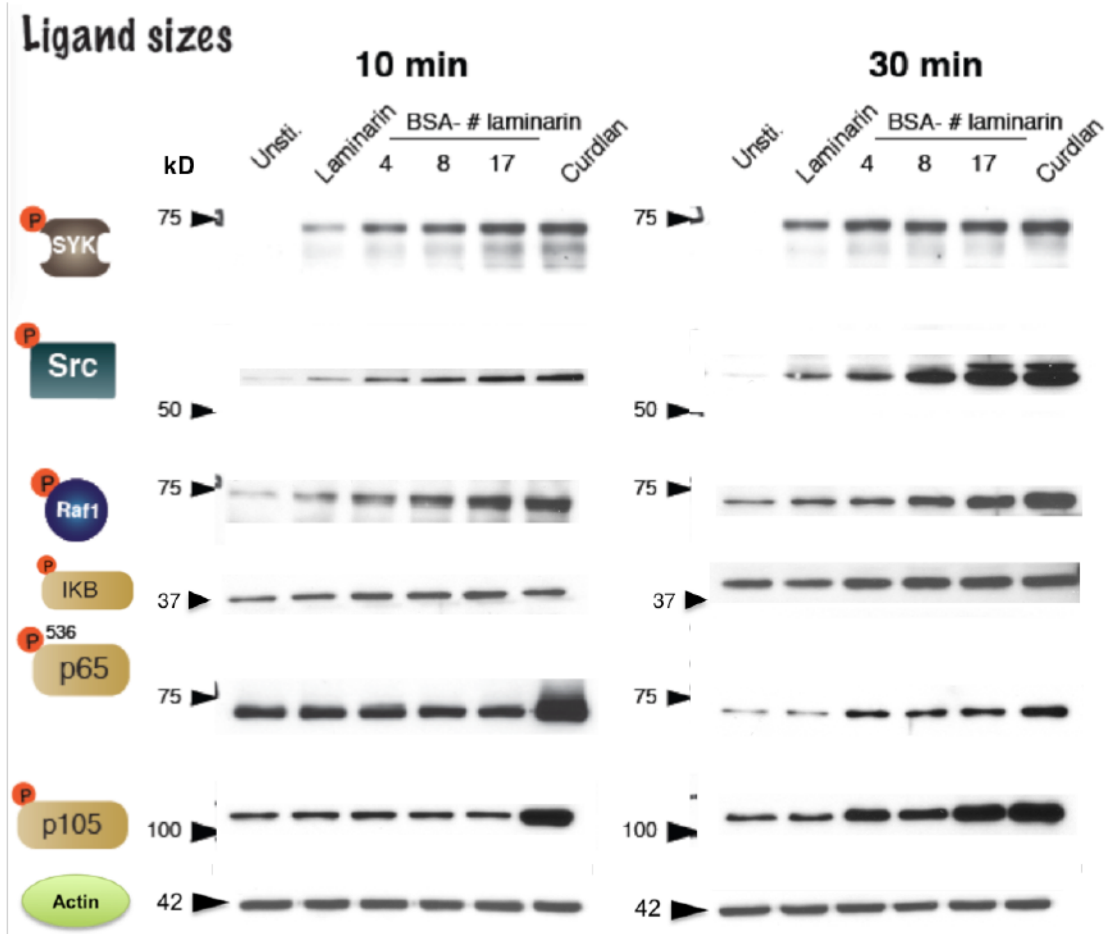
Figure 64: Soluble Ligands of Variable size Differentially Activate Various levels of Dectin-1 Signaling

RAW Dectin-1 cells were serum-starved for 4-6 hours. Cells were then stimulated with serum-free culture media with or without 100 μg/mL of ligand for 10 minutes at 37°C. The cells were promptly lysed, processed by SDS-PAGE, and immunoblotted for the presence of phosphorylated Src family kinases (on residue Y416 or equivalent), phosphorylated SYK (on residues Y525/Y526), phosphorylated PLCγ2 (on residue Y579), phosphorylated PKCδ (on residue Y311), and phosphorylated ERK1 and ERK2 (on residues T202 and Y204 or equivalent) Immunoblots were also performed for the presence of total IκBα (to assess IκBα degradation) and β-tubulin (as a loading control). Immunoblots presented are representative of three independent experiments. The black arrow on the right hand side of the figure indicates sequence of the Dectin-1 signaling events from upstream to downstream.

To date we haven't further investigated how ERK, a MAPK activated downstream of SYK (and not downstream of PKC) could mediate NF-κB activation. We predict that presumably, laminarin could start losing its signal at the level of activation of the MAPK, ERK, which appears to be essential for the subsequent activation the NF-κB activation, thereby preventing the laminarin signal from proceeding further downstream to the level of NF-κB activation.

To summarize our interesting findings from this section, laminarin was only able to activate the upstream components of the SYK-dependent Dectin-1 signaling pathway, down to the level of

PKCδ, after which laminarin seems to lose its signaling capacity **(**Figure 61 & Figure 64**).** This is demonstrated through the loss of the laminarin signal at the level of activation of the signaling machinery required for NF-κB, including the phosphorylation of the IKK kinase complex, as well as degradation of IκBα **(**Figure 61 & Figure 64**).** We also intriguingly discovered that laminarin loses its signal at the level of the activation of the MAPKs, ERK1/2. Therefore ERK might be an essential component for activation of NF-κB. This means that the loss of laminarin signal at the level of MAPK activation could actually be the reason why this signal fails to proceed further downstream for the activation of the canonical p65 subunit, and possibly other non-canonical subunits of NF-κB. However this finding requires further investigation especially at the level of how MAPKs activate NF-κB signaling.


A
Ligand sizes
10 min
30 min
kD
Unsti.
Laminarin
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4
8
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Curdlan
SYK
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IKB
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Actin
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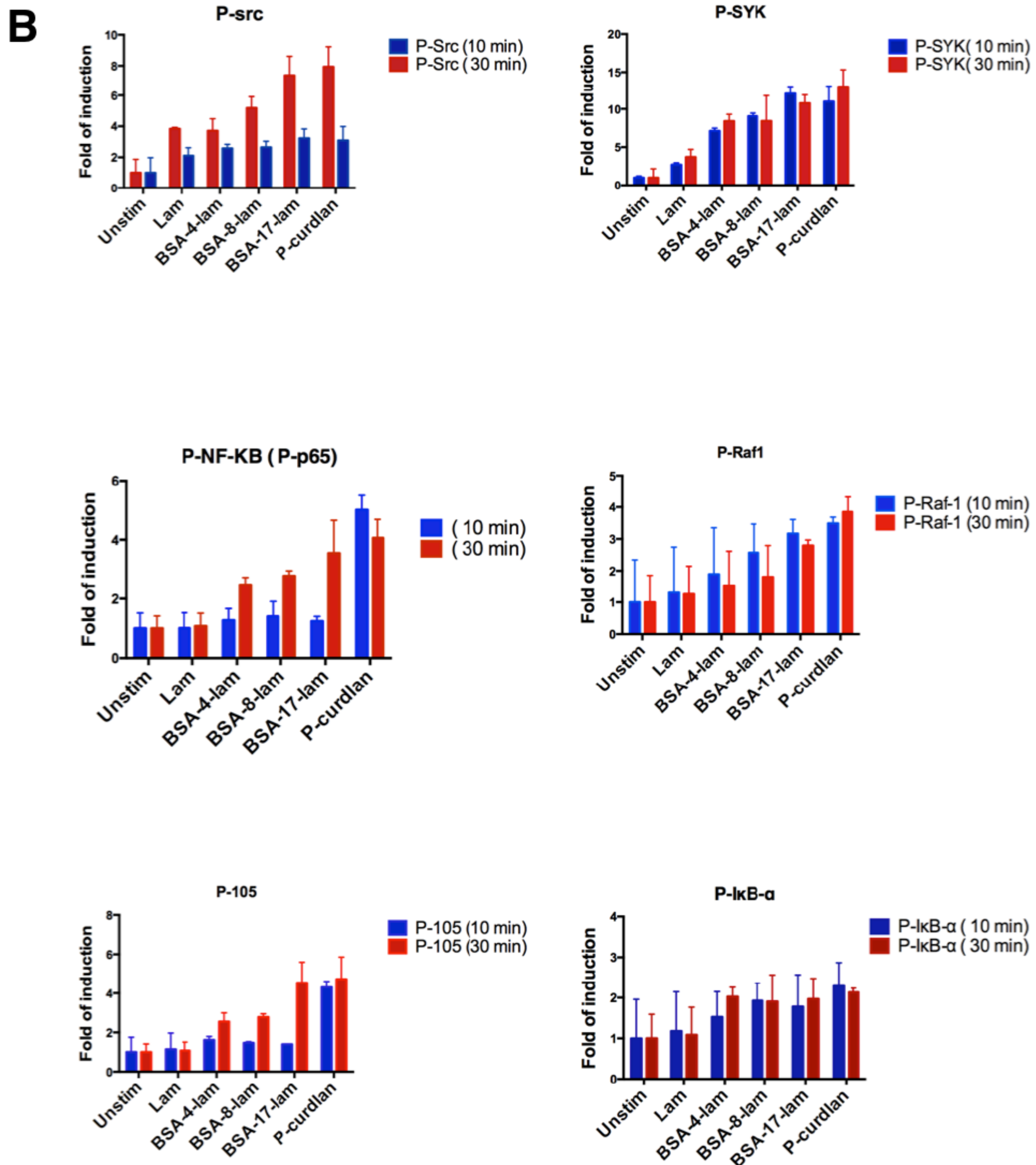


Figure 65: Western Blot Showing Differential Activation of Key Dectin-1 Signaling Players in Response to Ligands of Varying Size

(A) The western blot analysis depicted in Figure 63A summarizes the differential effect of ligands of different size, both qualitatively and quantitatively, on some of the major signaling players of the Dectin-1 signaling pathway including, P-Src, P-SYK, P-Raf-1, P-IκB (inhibitor of NF-κB), P-p65 (canonical subunit of NF-κB), P-p105 (non-canonical subunit of NF-κB. RAW Dectin-1 cells were serum-starved for 4-6 hours. Cells were then stimulated with serum-free culture media with or without 100 μg/mL of ligand for 10 minutes at 37°C. The cells were promptly lysed, processed by SDS-PAGE, and immunoblotted for the presence of phosphorylated protein of the various

signaling readouts, as well as for actin (loading control). Immunoblots presented are representative of three independent experiments. **(A)** Representivitve blots of Key Dectin-1 Signaling Players in Response to Ligands of Varying Size were shown. **(B)** Densitometry quantification of Dectin-1 mediated signaling events. Quantification of P-Src, P-SYK, P-Raf-1, P-IκB, P-p65, P-p105 from 3 independent Western blot experiments was peformed using ImageJ. Error bars represent SEM of three independent experiments.

Furthermore, we still don't understand why the laminarin signal is lost further downstream of the Dectin-1 signaling pathway, although laminarin is capable of activating upstream signaling events. However, quantification of major signaling readouts of the Dectin-1 Signaling pathways by densitometry using ImageJ (Figure 65) demonstrates that laminarin-induced activation of upstream signaling is of much lower magnitude than that activated by larger ligands. This is clear from the enhanced signaling responses triggered by the much larger β-glucan P-curdlan, as well as the BSA-17-laminarin conjugate with multiple laminarin molecules anchored to BSA, as compared with laminarin **(**Figure 61 & Figure 64**)**. This suggests that the size and β-glucan valency of the soluble Dectin-1 ligand affect the capacity for soluble β-glucans to fully activate the Dectin-1 signaling pathway. This may stem from the ability of larger Dectin-1 ligands to induce more Dectin-1 clustering, which gives them a higher capacity of inducing a more sustained Dectin-1 signal that is able to persist till the end of the signaling pathway. Therefore, in conclusion to this section it seems that ligand size differentially affects activation of the Dectin-1 signaling pathway.

Overall, so far in this study we have demonstrated that soluble ligands of larger size have a higher capacity of fully activating the Dectin-1 signaling pathway, all the way from upstream to downstream. We have also observed that larger ligands induce signaling levels of higher magnitude than smaller ones. Therefore the size of the ligand affects Dectin-1 signaling, both quantitatively and qualitatively. Furthermore we have shown that larger ligands strongly bind to high intensity Dectin-1 regions of the plasma membrane or 'puncta' (as visualized by confocal microscopy). I predict that these high intensity Dectin-1 regions of the plasma membrane, or 'puncta', are Dectin-1 clusters.

Therefore, we were next curious to know if ligand size actually affects the clustering/oligomerization state of Dectin-1. This would help us answer the question of whether ligands of larger size have a higher capacity of Dectin-1 activation due to their increased ability to cluster Dectin-1 into higher order structures or oligomers at the plasma membrane, as compared with ligands of smaller size.

3.2.6. Examining the Effect of Ligand Size on Dectin-1 Clustering at the Plasma Membrane

We have shown so far that that larger soluble β-glucans have a higher capacity to quantitatively and qualitatively activate more Dectin-1 signaling events than smaller ligands. Indeed, we have established that the size of the β-glucans positively correlates with the magnitude of the underlying signaling events. This was convenient due to the high solubilty of Dectin-1 ligands developed by the Bundle lab, in addition to the precise differences in size of our soluble ligands. However to date we haven't confirmed if these ligands, when larger in size, actually induce the formation of Dectin-1 clusters on the plasma membrane. Although we have seen by confocal microscopy that larger ligands such as P-curdlan and BSA-17-laminarin induce the formation of high intensity puncta at the plasma membrane, we haven't yet physically examined the formation of these clusters by biochemical techniques nor by high resolution microscopic techniques that would provide more sophisticated details of these high intensity Dectin-1 structures than that offered by conventional confocal microscopy. We and others have proposed that larger ligands could induce clustering of Dectin-1, which in turn could trigger signaling, to date there is no direct evidence showing that Dectin-1 physically forms clusters on the cell membrane in response to binding to β-glucans of high molecular weight. However some studies have shown by an in vitro assay of ligand binding assays to to purified Dectin-1 show that binding of larger ligands induces the aggregation of Dectin-1 into structures of higher molecular weight. Thus, in this section we sought to examine using biochemical approaches and cutting-edge superresolution microscopy, the membrane organization and clustering state of Dectin-1 on the cell surface under different clustering conditions.

3.2.6.1. Chemical Cross-linking of Dectin-1 in Response to ligands of Varying Size

According to our 'Clustering Hypothesis' that we postulated above, we and others have speculated that binding of larger ligands to Dectin-1 induces clustering of the receptor, and that the ligand capacity to cluster the receptor is correlated to the magnitude of signaling elicited by the β-glucan ligand (see section 3.1) (Figure 22). (Adams et al., 2008; Batbayar et al., 2012; Brown, 2006a; Goodridge et al., 2011; Lowe et al., 2001; Qi et al., 2011). Also our observations from confocal images of surface Dectin-1 and fluorescently labeled ligand indicate intense binding and strong colocalization of the ligand with Dectin-1, at areas of the plasma membrane

highly enriched in Dectin-1. Indeed, Dectin-1 upon binding to larger ligands, such as P-curdlan or BSA conjugates with high laminarin (e.g, BSA-17-lam) organizes into high intensity puncta on the plasma membrane that strongly coalesce with the ligand. As mentioned above we believe that these puncta represent Dectin-1 clusters or higher order oligomers/multimer **(see section 3.2.3.1) (**Figure 42 & Figure 45).

Accordingly, to determine if Dectin-1 might cluster after ligand stimulation, we decided to employ a biochemical approach for chemcially cross-linking Dectin-1. In this experiment after the treatment of RAW Dectin-1 cells with soluble β-glucans of different size, cell surface proteins will be chemically cross-linked with a membrane-impermeant cross-linker (Figure 66). Immunblotting of surface Dectin-1 in these cells would alow us to examine by immunoblotting whether Dectin-1 is present as a monomer, dimer or higher-order structure (including oligomers and multimers) on the surface of these cells

To crosslink Dectin-1 on the cell surface we used Sulfo-EGS (Thermo Scientific), a water-soluble, membrane-impermeant cross-linking reagent, which cross-links proteins that fall within a distance of 1.6 nm (16Å) of each other. Oligomerization of the receptor was then investigated by western blotting (Figure 66). Cells were stimulated in the absence or presence of Sulfo-EGS with laminarin, P-curdlan or left unstimulated (Figure 66). After ligand stimulation the cells were lysed, processed by SDS-PAGE, and immunoblotted for Dectin-1. Remarkably a population of Dectin-1 dimers, was apparent in all conditions, as seen by a higher molecular weight thick band of ~80-90 kDa that corresponds approximately to double the molecular weight of monomeric Dectin-1 detected at ~40 kDa (Figure 66). Moreover, as seen in the blot P-curdlan stimulation induced the formation of more dimers than that observed with laminarin, as seen by a highly intense band corresponding to Dectin-1 dimers, which was much thicker and of much higher intensity than that observed for laminarin (Figure 66). It was also intriguing to us to find that even in unstimulated conditions exists as a dimer, and that binding of Dectin-1 to a larger-sized ligand, like P-curdlan, increases the occurrence of this population of dimers increases (Figure 66). Also interestingly, after addition of cross-linker, the presence of higher molecular weight bands of Dectin-1 dimers can be seen together with a corresponding reduction in intensity of the 40-kDa bands corresponding to monomers of Dectin-1 (Figure 66). Notably, cells untreated with the cross-linker didn't reveal the formation of these Dectin-1 dimers, indicating that the chemical cross-linker was successfully cross-linking Dectin-1 at the cell surface. Since all samples exibit

equal amount of protein as demonstrated by actin loading, the corresponding changes in the levels of monomeric and dimeric forms of Dectin-1 are most probably due to interconversion of one form to another.

The observed increase in the formation of Dectin-1 dimers upon ligand binding is in accordance with the suggested model for hemITAM activation of SYK in Dectin-1 signaling and also agrees with the finding that CLEC-2, a closely related CLR of the 'Dectin-1 cluster' Group V CLRS activates SYK through CLEC-2 dimerization (**see sections 1.3.4 & 1.5.4.1,** of *Chapter 1, General Introduction*) (Hughes et al., 2010b; Kerrigan and Brown, 2011b; Severin et al., 2011; Watson et al., 2009). In this model, it is hypothesized that Dectin-1, following ligand binding, may form a dimer structure with SYK **(**Figure 8**)** (Brown, 2006a). This model proposes that dimerization of Dectin-1 occurs via the bridging of two nearby Dectin-1 molecules via the two SH2 domains of SYK (Rogers et al., 2005), where each SH2 domain binds to a single phospho-tyrosine residue within the hemITAM motif of each of the two individual molecules of Dectin-1 **(**Figure 8**,** Figure 16 & Figure 15**)** (Brown, 2006a; Brown et al., 2007; Goodridge et al., 2009b; Osorio and Reis e Sousa, 2011; Rogers et al., 2005). As mentioned above the nature of the interaction of SYK with the hemITAM of Dectin-1 is not clear, especially that that Dectin-1 lacks cysteine residues in its stalk region involved in CLR dimerization.

It is also interesting that Dectin-1 occurs as a dimer in the unstimulated steady state. This means that there is a basal level of pre-exisiting Dectin-1 dimers, that are formed and SYK activation even before ligands bind to Dectin-1 to induce dimerization as proposed by the above model. Again it is hard to understand how these steady-state dimers of Dectin-1 are formed in the absence of ligand binding especially that Dectin-1 lacks cysteine residues essential for CLR dimerization. It is still not known if there are specific residues that could confer the dimerization of this proportion of Dectin-1 receptors that pre-exist as dimers in unstimulated cells, and thus further investigation is required to assess the mechanism of this dimerization one possibility is that a basal level of activated SYK exists in unstimulated condtions, which mediates the bridging of Dectin-1 molecules in to dimers. Indeed we have demonstrated by immunblotting and immunofluorescence experiments (performed in **section** 3.2.3**)** that there is a basal level of SYK phosphorylation/activation as well as recruitment to the plasma membrane in unstimulated cells, which presumably could lead to the formation of Dectin-1 dimers in the steady state. However it needs further investigation to validate if membrane recruitment and activation of SYK are

necessary for the formation of Dectin-1 dimers, and *not vice versa.*

However, despite the detection of dimers in this blot, higher order structures of Dectin-1 oligomers or multimers couldn't be detected. Possibly, the length of the cross-linker, along with the precise molecular conformations needed for the occurrence of crosslinking between neighbouring receptors, may have precluded detection of these events. Furthermore an increase in the formation of Dectin-1 dimers relative to unstimulated control cells, couldn't be observed for laminarin-treated cells, which might be due to inefficient cross-linking of surface Dectin-1 in these cells. Therefore, it is clear that more experiments are required before we can truly conclude that Dectin-1 dimerizes or oligomerizes upon ligand binding. In their least, the findings demonstrate that the proximity of the Dectin-1 receptor to neighbouring Dectin-1 molecules is increased upon ligand stimulation, suggesting that binding of larger-sized lgands may induce clustering of the receptor.

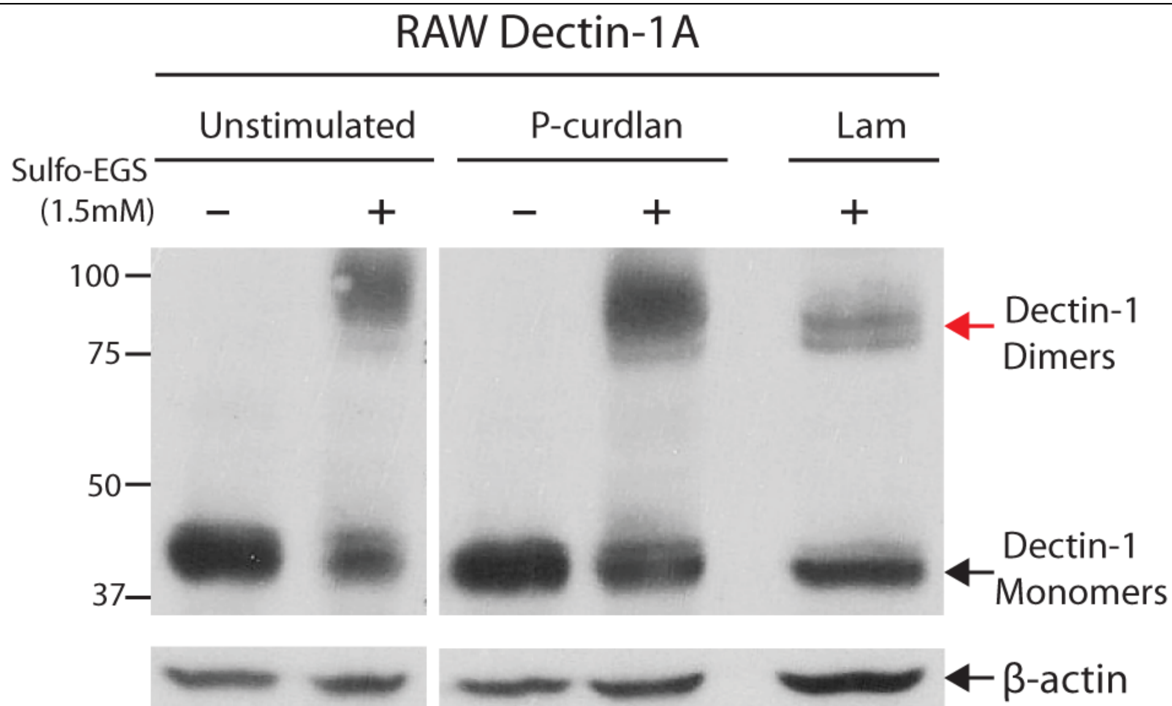


Figure 66: Chemical Cross-linking of Dectin-1 in RAW Cells

Monolayers of RAW Dectin-1 cells at equivalent density were stimulated at 37°C for 10 minutes with 100 μg/mL of laminarin (lam), or P-curdlan or left unstimulated (unstim). The cells were then treated with 1.5 mM sulfo-EGS, or left untreated at 4°C for 2 hours to cross-link surface proteins; the reaction was later quenched with 20 mM Tris. Cell lysates were prepared, proteins were then separated by non-reducing SDS-PAGE (in the absence of β-mercaptoethanol), transferred on nitrocellulose membrane, followed by subsequent immunoblotting for detection of Dectin-1 For immunoblotting, the goat anti–Dectin-1 Ab (R&D Systems) was used at 1:1000, followed by an anti-goat IgG coupled to HRP (1:5000). ECL treatment and film developmen revealed immunoreactive bands suggestive

of receptor monomers (~40 kDa) and dimers formed by cross-linking (~80 kDa), which are indicated by the arrows. Data represents results form two independent experiments.

After biochemically detecting, through chemical cross-linking detecting, an increase in the formation of dimers upon binding of the larger-sized β-glucans, we next decided to further examine the formation of Dectin-1 dimers or higher order oligomers/multimers in reponse to different-sized ligand using 'suprerresolution' microscopic techniques. As decribed in detail, in **section 2.24**, superresolution microscopy is high-resolution single-molecule imaging technique microscopic technique that allows the detection of individual molecules in the cell without being obscured by the ensemble averaging inherent in conventional biochemical methods (Owen et al., 2010). Furthermore, this cutting-edge microscopic technique breaks the diffraction limit providing images with much more enhanced resolution than the diffraction-limited low resolution of images acquired by conventional fluorescency microscopy (including confocal microscopy) (see **section 2.24**) **(**Figure 21). This powerful, state-of-the-art, microscopic technique, together with high-end image analysis of our super-resolution data (performed by Dr. Touret and John Maringa in collaboration with Dr. Jaqaman at UT Southwestern Medical Center, Dallas) provided us with valuable tools essential to extract the single molecule behavior of Dectin-1 at the surface of innate immune cells. This gave us the advantage to accurately analyze at the molecular level, the plasma membrane organization and clustering state of Dectin-1 at the cell surface of RAW macrophages. Results provided by this high resolution imaging approach are expected to provide us with much higher molecular detail and insight about Dectin-1 clustering than we observed using confocal microcopy or conventional ensemble biochemical techniques.

3.2.6.2. Analysis of ligand size effects on Dectin-1 clustering by Superresolution Microscopy using PALM

The previous section indicates that formation of Dectin-1 multimers might be induced by β-glucans. To obtain a more precise and accurate understanding of the molecular events taking place during Dectin-1 signal transduction, we developed single molecule approaches based on PALM (PhotoActivated Localization Microscopy) to determine the localization of the receptors before and after ligand engagement. We quantified the level of clustering by performing a statistical distribution analysis named Ripley's function.

For these PALM experiments, we generated a stable RAW macrophage cell line expressing the photoactivable PAmCherry fused to the N-terminus of Dectin-1. 24 hours prior to the experiment,

cells were plated on squeaky-clean coverslips. Cells were then washed several times with RPMI before being incubated with either no ligand (unstimulated control in HRPMI) or with β-glucan at a concentration of 100 µg/mL for 10 min at 37°C. Fixation was performed on ice after 3 washes with cold PBS using a 3% PFA + 0.2 % Glutaralhedyde solution for 30 min. The coverslips were then washed on image with the next 24 hours on our TIRF microscope. Image sequences were acquired by imaging in the "red" channel (561 nm excitation / 575 ± 20 nm emission) upon constant low photoactivation light (405 nm) excitation. In these conditions, only a few hundred molecules were turned on on each frame allowing accurate single molecule fitting using our Matlab detection program. Following the detections of these single fluorescence spots, we rendered superresolved images using a resolution of 20 nm. This resolution was determined based on the localization accuracy of our imaging and is dereived from the standard error of the Gaussian fitting of the fluorescence features. Figure 67 represents 4 typical images obtain for the rendering of our PALM imaging. Simple visual inspection can convincingly reveal that larger and brighter Dectin-1 structures are formed upon treatment with 100 µg/mL of P-Curdlan compared to the control. But to obtain more precise understanding of the molecular rearrangement taking place during ligand stimulation, we developed more sophisticated analysis based on a statistical analysis of the distribution of the receptors.

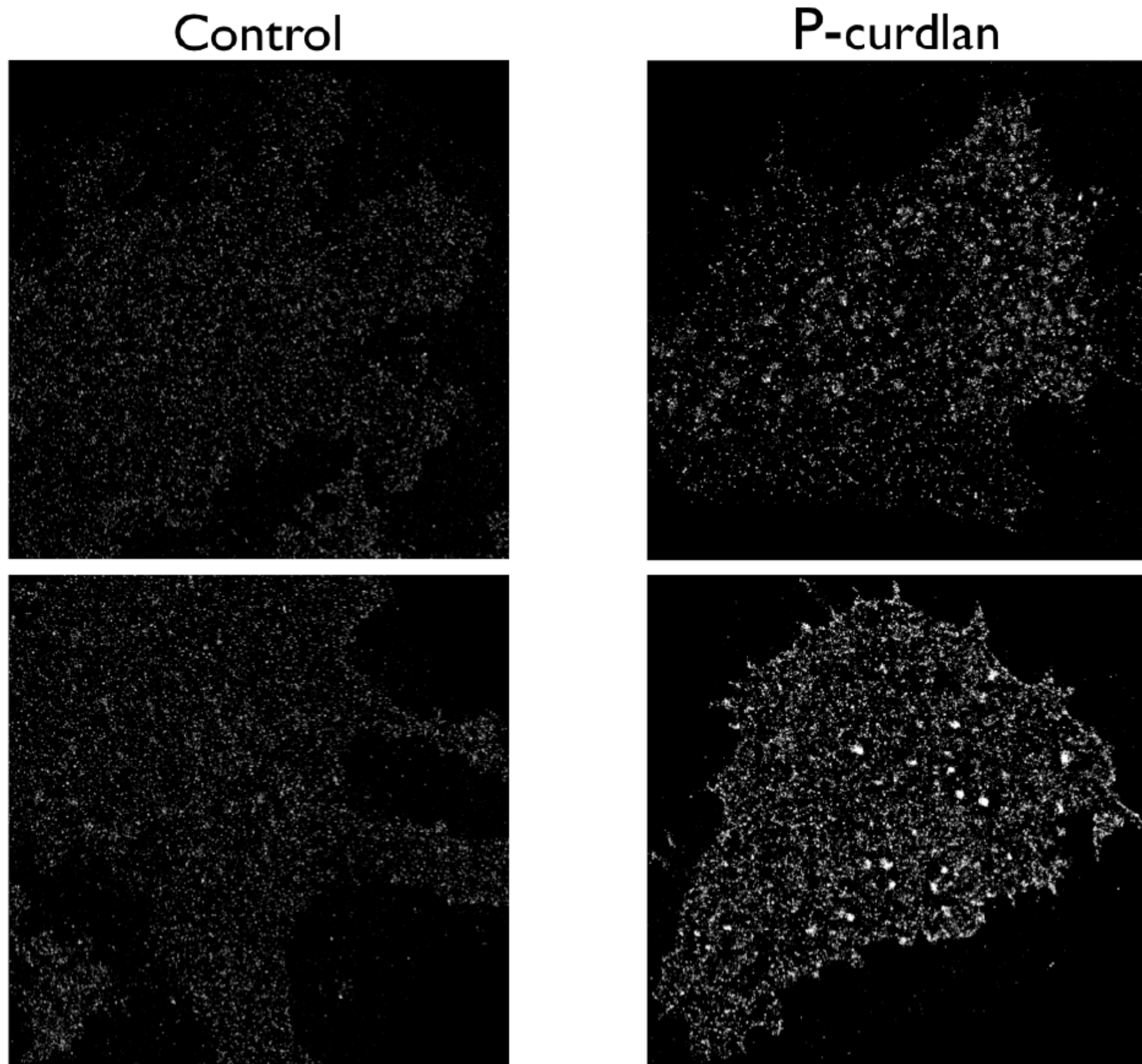


Figure 67: Two Representative PALM Images of PA-mcherry-Dectin-1

Above PALM images of PA-mcherry-Dectin-1 were acquired on a TIRF microscope under control and unstimulated conditions (left column) or after stimulation by P-curdlan for 10 mins at 37°C. Immunofluorescent protocol is as described above in Chapter 2, Materials and Methods. Two representative images are shown for each condition Control and P-curdlan, (Top and bottom panels. The PALM images of PA-mcherry-Dectin-1 visually reveal more Dectin-1 clustering into high intensity puncta than in control conditions.

The spatial distribution analysis software was developed in the laboratory by John Maringa Githaka (a candidate Ph.D. student in the lab) and Dr. Nicolas Touret. The principle is described in Figure 68 (panel A) shows a set of simulated coordinates to help understand the concept. The Ripley's K function is a mathematical tool that compares the number of molecules present in circles of increasing radii around each position to a randomly distributed set of coordinates of

similar density. For a set of coordinates randomly distributed, linearlized Ripleys K analysis, H-function, is about 0 at any radii tested (Figure 68A, blue curve). The second example in red shows a set of simulated clustered points. H-function increases with increasing radii (there are more molecules found in each circles around the molecules than expected if they were randomly distributed).

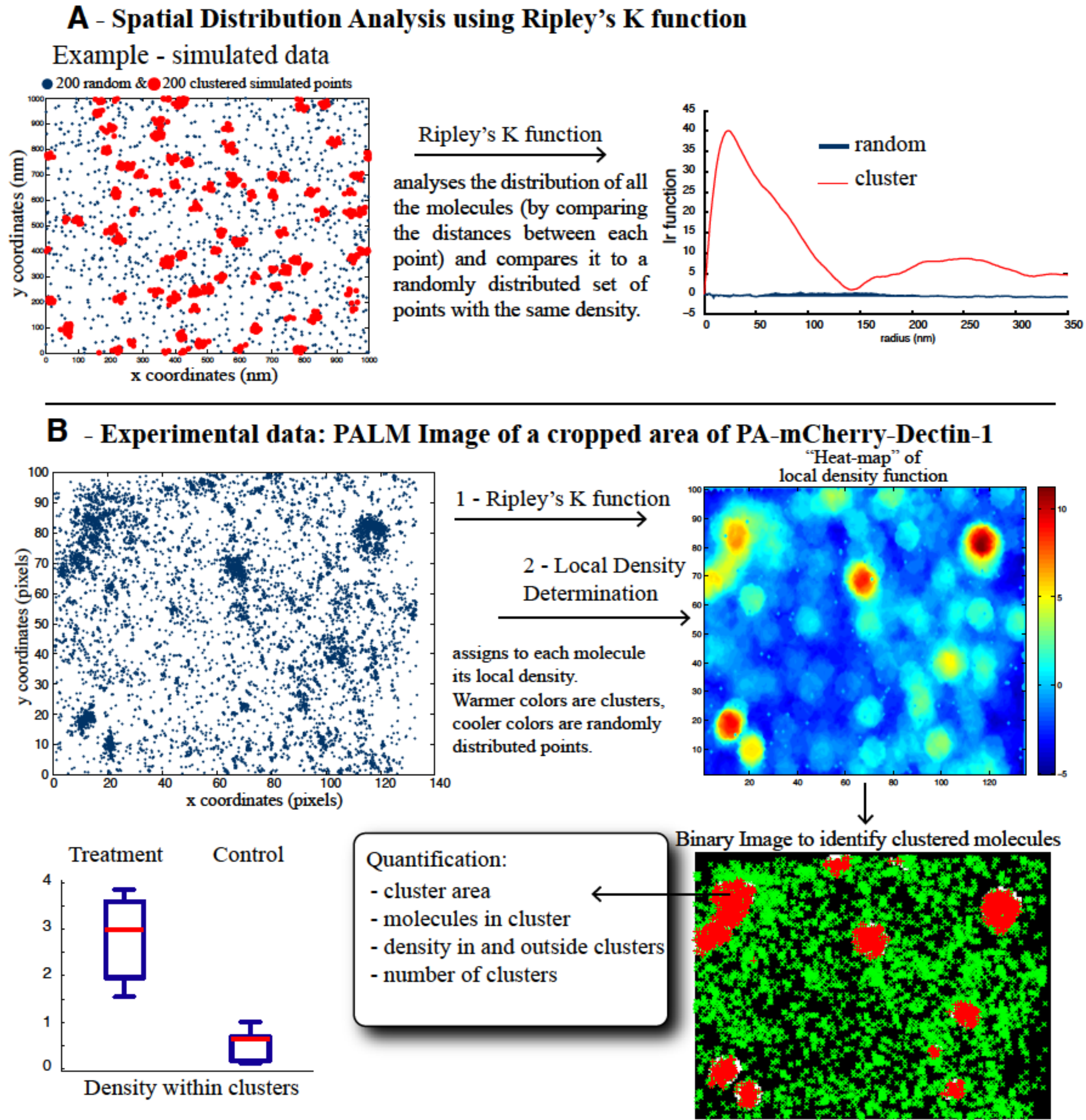


Figure 68: Flow Chart for Clustering Analysis

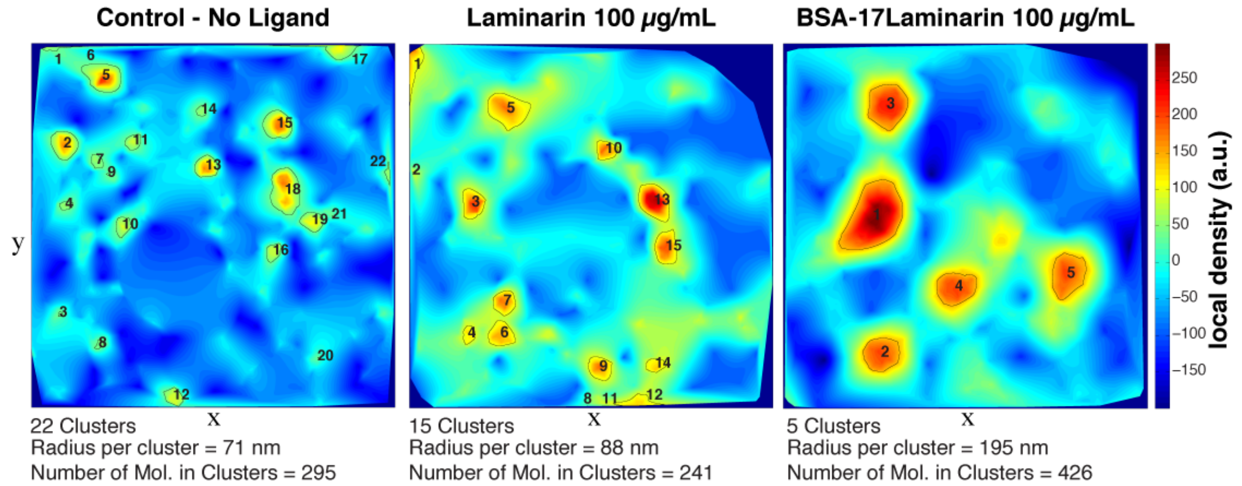
With the coordinates of all the receptors imaged by PALM, a statistical analysis provides a quantitative determination of their clustering. A) Using simulated coordinates, Ripley's K function analysis results in values of local densities (lr) that are near zero for random points (in blue), while clusters (in red) shows a curve that peaks at a radius corresponding to the cluster size. B) Applied to PALM images of CD36 (another membrane receptor studied in the lab), quantitative clustering information can be extracted, such as size, number of clusters, the number and denstiy of receptors within these clusters.

The radius at which maximum of the H-function is obtained correlates with the average size of the clusters. Based on this radius, a local density value can be attributed to each coordinates in the

image and it relates to the density of molecules around each of this receptor. This local density value is then used to produce a “heat-map” where x and y axis are the coordinates in nm and z is represented in pseudo-colors from cold blue (low local density) to warm red (high local density) (Figure 68B). Areas of clustering are then defined by thresholding the local density to that of randomly distributed coordinates). The binary map is produced by converting every pixel that is above the threshold as 1 (the clusters) and everything below as 0 (Figure 68B). Using Matlab function, each clustered region can be isolated and its area, perimeter, location as well as the number of molecules residing in the domain can be extracted. This final segmentation of the clustered area allows the precise measurements of any changes in the distribution of receptors, their density, and the cluster size that could occur upon ligand stimulation.

Figure 69 represents the clustering analysis of PAmCherry-Dectin-1 under control, laminarin and BSA-17 -laminarin treated conditions. Panel A illustrates representative local density “heat-map” of Dectin-1. Under control conditions, many small clusters are dispersed across the cell membrane and contain an average of 25 molecules. Addition of laminarin induces formation of fewer but larger clusters. This phenomena is further exaggerated when the BSA-17 Laminarin was used. Analysis of hundreds of these clusters gave us more robust explanation of the molecular rearragenment undertaken by Dectin-1 (Figure 69B).

At steady-state Dectin-1 is present is smaller clusters of about 66 nm of radius, containing an average of 17 receptors (density in clusters of 1330 molecules per μm^2). The number of cluster per μm^2 is 1.23. This density of cluster per surface area decreases slightly when ligands are added but the difference is not significant. The density of molecules with clusters also decreases slightly from 1370 mol/μm^2 to 1087 for laminarin and 1044 BSA-17Laminarin. This decrease is probably the result of the coalescence of several clusters together. This coalescence is illustrated by the slight decrease in the number of clusters per surface area and by the large increase in cluster radius (from 66 nm to 102 for laminarin and 151 for BSA-17-laminarin) paralled by the increase number molecules present in clusters. These data indicates that ligand binding induces the consolidation of Dectin-1 clusters into larger and platform. When correlated to the capacity of the ligand to stimulate downstream Dectin-1 sigaling molecules, these results suggest that cluster of 66 nm are too small to induce SFK stimulation or SYK activation, but when their size rises above 100 nm, signal transduction is promoted. In conclusion, the increase size of Dectin-1 clusters correlates with stronger activation strength downstream of receptor.



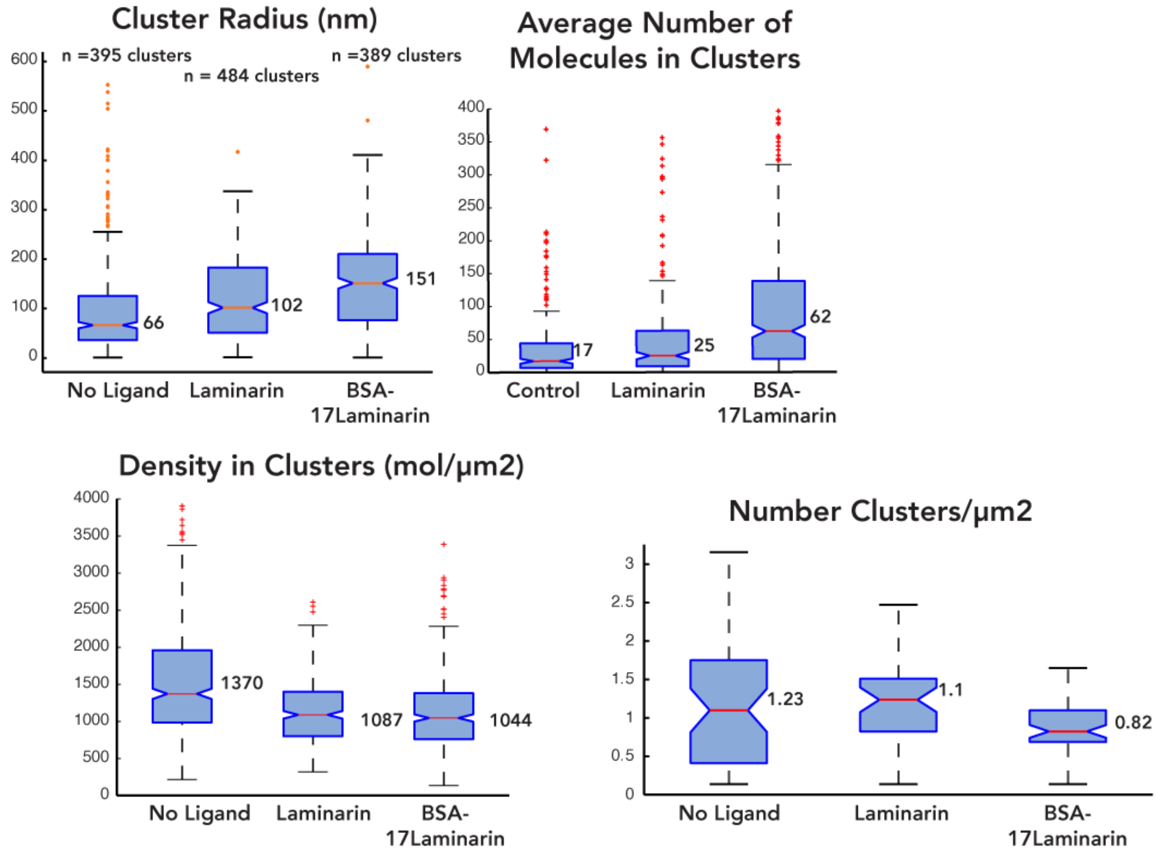


Figure 69: Dectin-1 Clustering Analysis

RAW cells stably expressing PAmCherry-Dectin-1 were either stimulated or not (Control) with 100 μg/mL of laminarin or BSA-17-Laminarin. PALM imaging and single molecule localizations were then analyzed in random

region of interest (R.O.I. of 4 x 4 μm) with our clustering algorithm. Representative heat maps of the local density measured are presented with area of higher density in warmer colors. B) Clustering analysis of thousands of clusters showed that multivalent ligand (BSA-17-Laminarin) induces increase cluster size with higher number of Dectin-1 (151 nm of radius with 62 molecules in average). The comparaison between the density with clusters versus the density of the R.O.I shows a slight increase in the density of molecules in clusters. Together, it suggests that larger ligands induce recruitement of receptors to form larger, but not denser clusters.

In conclusion, larger ligands can induce Dectin-1 Signaling and have a higher capacity of promoting the formation of larger but less dense clusters of Dectin-1. These clusters in turn have a high potential to fully and strongly induce Dectin-1 signaling. The next question that we wanted to address was if the positive effect of Dectin-1 clustering on the activation of Dectin-1 signaling is due to the enhanced capacity of larger-sized ligands size to bring more Dectin-1 molecules together, or because of some molecular determinant of the ligand that could cause a conformational change in Dectin-1.

3.2.6.3. Effect of Antibody Cross-linking of Cell Surface Dectin-1 on Activation of the Receptor

In the previous section **3.2.6.2**, we have proved that soluble ligands of larger size induce Dectin-1 association on into clusters of larger size and higher number of molecules than in unstimulated steady state. As shown in sections 3.2.3, 3.2.4, & 3.2.5**,** larger-sized ligands greatly enhance the signaling capacity of Dectin-1 as manifested by an increase in the magnitude of the underlying signaling events at the upstream and downstream levels. This could be due to the higher capacity of these ligands to induce Dectin-1 clustering. Furthermore the size of the ligand, and presumably the amount of Dectin-1 clustering induced by this ligand, differentialy affects signaling events activated further downstream of the signaling pathway, and determine whther the Dectin-1 pathway is fully activated or not by the β-glucan **(section 3.2.5)**.

Therefore, from what has been described above we have so far in this study established a parallel correlation between ligand size, Dectin-1 clustering and Dectin-1 signaling capacity. However, the positive relationship between Dectin-1 clustering and Dectin-1 activation opened up an intriguing question for us, which is why Dectin-1 clustering by larger ligands induces signaling, and there thought of two possible scenarios for answering this question. The first possibility is that clustering of Dectin-1 because brings more Dectin-1 molecules in proximity together up to a certain level that is sufficient for activating key upstream signaling events such as SFK activation, that in turn trigger the Dectin-1 signaling cascade. The other scenario is that binding of larger ligands to Dectin-1 could induce a conformational change of the receptor or another modification

of the Dectin-1 receptor, which in turn leads to Dectin-1 activation and initiation of its signaling pathway. To answer this question we sought to examine the first possibility, which is that Dectin-1 activation by clustering is due to the gathering of more Dectin-1 together and not because of a conformational change produced in Dectin-1. An excellent approach to address this question is by Ab cross-linking (Ab x-linking) of the Dectin-1 on the suface of cells (Figure 23**,** 3.2.1**)** It has been proved in the Touret lab that the binding of antibodies against Dectin-1, doesn't interfere with the capacity of β-glucan ligands to bind to Dectin-1, i.e., the epitope of Dectin-1 recognized by the Ab is distinct from the ligand binding site (Data not shown). Therefore, the great advantage of Ab x-linking of cell surface Dectin-1 is to induce clustering of Dectin-1 via another location on Dectin-1 that is distinct from the ligand binding site. Accordingly the examined effects of Ab x-linking will not be due to the induction of conformational effects mediated by ligand binding, but only due to the recptor clustering that brings Dectin-1 molecules closer in proximity on the cell surface.

Antibody cross-linking of cell surface Dectin-1 was examined by either Immunofluorescence (IF) or by western blotting experiments using two cell lines exogenously expressing human Dectin-1 on the cell surface, namely RAW Dectin-1 and HeLa Dectin-1 cells. The latter cells are a stable cell line of HeLa cells, which have been developed in the Touret lab by a very similar method to that of RAW Dectin-1 cells. The advantage of HeLa cells is that they are much larger than RAW macrophages and thus easier for visualization of cell surface Dectin-1 and receptor clustering by confocal microscopy. Expression of human Dectin-1 in HeLa Dectin-1 Cells was was confirmed by immunoblotting of these cells in comparison to HeLa wild type (WT) controls (Figure 70). The western blot revealed a high-molecular weight band (at ~40–50 kDA), corresponding to the mature glycosylated form of Dectin-1 expressed on the cell surface, and this band which was not present in HeLa WT control cells (Figure 70).

In general Ab crosslinking of cell surface Dectin-1 was performed on live cells (placed on ice) using bivalent mouse anti-human Dectin-1 antibodies (R&D Systems), which will have a mild cross-linking effect as it only brings two receptors together (bivalent). Subsequent incubation with a polyclonal secondary antibody (that detects multiple epitopes of the primary Ab) will induce formation of larger Dectin-1 clusters. An Isotype IgG control of the same species of the anti-Dectin-1 antibody was used as a negative control for antibody cross-linking, and also to make sure that the observed effects were specific to only Dectin-1 and not due to non-specific

cross-linking of other receptors such as the Fc receptor (FcR). To avoid cross-linking of the FcR we used an FcR blocker before adding the primary antibody onto the cells.

First, we conducted a western blot experiment to determine the effect of Ab x-linking in HeLa Dectin-1 cells. Cells were seeded in a 6 well plate 24 hours before the experiment and then were serum-starved for 4-6 hours to minimize background phosphorylation. At the beginning of the Ab x-linking experiment cells were first blocked with an Fc receptor for 5-10 minutes on ice, to avoid non-specific cross-linking of the Fc receptor by primary Ab. For Ab x-linking of cell surface Dectin-1, cells were incubated for 10 minutes on ice with the primary Ab against human Dectin-1 (mouse anti-human Dectin-1) or with the corresponding isotype control (mouse IgG2b, used as a negative control for cross-linking) for 10 minutes. Cells were then placed on ice to inhibit further cross-linking and washed with ice cold PBS followed by incubation with goat anti-mouse IgG (secondary Ab) for 5 mins at 37°C. Following cross-linking, cells were washed and then standard preparation of lysates was performed (see *Chapter 2, Materials and Methods*) and we chose to probe for SYK phosphorylation as a characteristic read-out of Dectin-1 signaling. We therefore, immunoblotted for phospho-SYK (P-SYK) using two different anti-P-SYK Abs, one detecting SYK phosphorylation at Y525/Y526 and the other detecting P-SYK at Y352. Both are activatory phosphorylations of SYK (Figure 71).

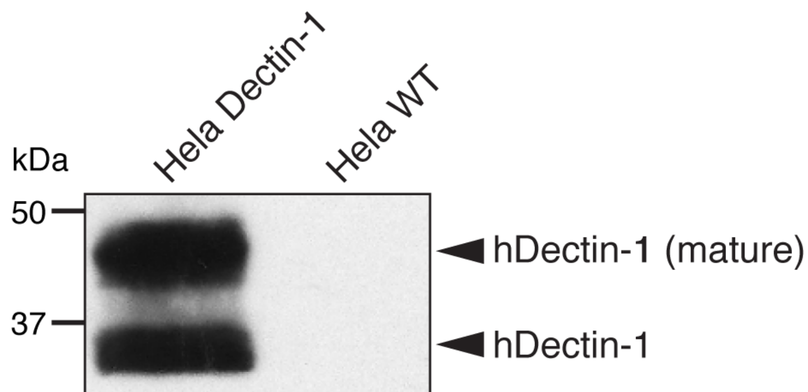


Figure 70: Exogenous Expression of Human Dectin-1 in HeLa Dectin-1 cells

A stable cell line of HeLa Cells exogenously expressing Dectin-1 was developed in the Touret Lab, and were named 'HeLa Dectin-1'. Western blotting of whole-cell lysate prepared from HeLa Dectin-1 (HeLa Dectin-1–expressing cells) and HeLa WT was peformed, by immunoblotting for human Dectin-1 using, the goat anti–Dectin-1 Ab (R&D Systems) (used at 1:1000), followed by an anti-goat IgG coupled to HRP (1:5000). In the HeLa Dectin-1 lane, two bands appeared upon ECL treatment and film development. The upper and large bands correspond to the complex glycosylated form of the receptor, whereas the smaller one (30 kDa) corresponds to the core glycosylated (determined by PNGase and EndoH treatment; data not shown. No expression of Dectin-1 in HeLa WT cells. Experiment was repeated thrice giving the same result.

As illustrated in (Figure 71) western bot analysis of cell lysates remarkably demonstrated that

SYK was only phosphorylated under Ab cross-linking conditions (Figure 71). While pronounced levels of P-SYK were observed in response to Ab x-linking, no phosphorylation of SYK was detected in the isotype negative control or unstimulated conditions, which demonstrated the Dectin-1 specificity of the signaling response observed (Figure 71).

This preliminary result indicated that Ab x-linking of cell surface Dectin-1 specifically activates SYK phosphorylation in HeLa Dectin-1 cells exogenously expressing human Dectin-1.

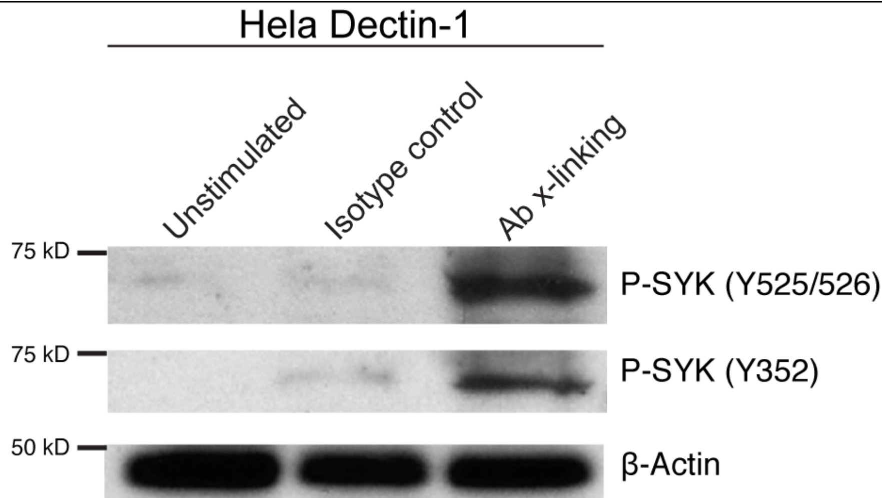


Figure 71: Western Blot Analysis of SYK Phosphorylation in Response to Ab cross-linking of Surface Dectin-1 HeLa Dectin-1 Cells

Serum starved (4-6 hrs) HeLa Dectin-1 cells were trated with FcR blocker for 5 mins on ice. This was followed by incubation at 4°C for 10 min with mouse anti-human Dectin-1 antibodies at 1:1000 (Ab x-linking/cross-linking) or mouse IgG2b isotype control (1:1000), or left unstimulated after which they were removed with several PBS washes, and then replaced with culture media containing goat anti-mouse secondary Ab (1:500) at 37°C for 5 minutes. Cell were then placed on ice and washed 5X with ice cold PBS, after which cell lysates were prepared and separated by reducing SDS-PAGE and western blotted for P-SYK, using rabbit anti-P-SYK (Y525/Y526) or rabbit anti-P-SYK (Y352). We also probed for β-tubulin as a protein loading control. Data is representative of three identical experiments.

Next we wanted to confirm this exciting result in our model of RAW Dectin-1 cells as compared to RAW WT cells (Figure 72). The same western blot experiment was performed as described above, and remarkably we obtained results similar to that observed above for HeLa Dectin-1 cells. Ab x-linking of Dectin-1 in RAW Dectin-1 cells resulted in enhanced phosphorylation of SYK at Y525/Y526, as compared with isotype controls or unstimulated conditions (Figure 72). This indicates that the observed signaling response is specific to Ab x-linking of Dectin-1 and not due to cross-linking of other receptors on the surface of these cells (Figure 72). Remarkably, Ab x-linking didn't activate SYK phosphorylation in RAW WT, further confirming that the Ab x-

linking effects observed in RAW Dectin-1 cells are due to cross-linking of surface Dectin-1 in these cells (Figure 72).

To conclude so far, Ab x-linking induces enhanced SYK phosphorylation in HeLa Dectin-1 and RAW Dectin-1 cells as compared to isotype controls, unstimulated controls as well as RAW WT controls.

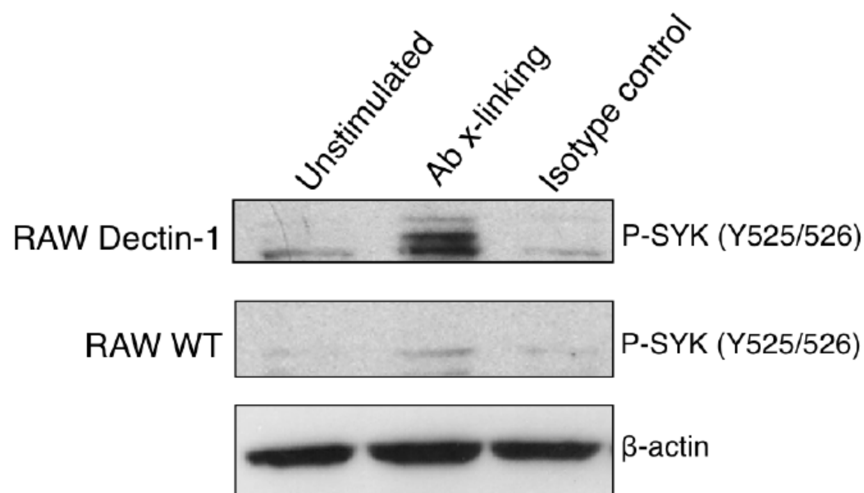


Figure 72: Western Blot Analysis of the Effect of Ab cross-linking on SYK Phosphorylation in RAW Dectin-1 Cells versus RAW WT cells

RAW Dectin-1 or RAW WT cells were serum starved (4-6 hrs) and then treated with Fc receptor (FcR) blocker for 5 mins on ice. This was followed by incubation at 4°C for 10 min with mouse anti-human Dectin-1 antibodies at 1:1000 (Ab x-linking/cross-linking) or mouse IgG2b isotype control (1:1000), or left unstimulated. Cells which they were removed with several PBS washes, and then replaced with culture media containing goat anti-mouse secondary Ab (1:500) at 37°C for 5 minutes. Cell were then placed on ice and washed 5X with ice cold PBS, after which cell lysates were prepared and separated by reducing SDS-PAGE and western blotted for P-SYK, using rabbit anti-PSYK (Y525/Y526) or rabbit anti-P-SYK (Y352). We also probed for β-tubulin as a protein loading control. Data is representative of three independent experiments.

After our Ab x-linking results from western blotting, we were next interested in examining by confocal microscopy the capacity of Ab x-linking to induce the formation of high intensity puncta Dectin-1, as well as SYK phosphorylation and recruitment to the plasma membrane .We thus, performed an Ab-xlinking experiment on HeLa Dectin-1 cells followed by dual immunofluorescence of Dectin-1 (immunostained in red) and phospho-SYK (Y352) stained in green.

For Ab x-linking cells HeLa Dectin-1 and HeLa WT cells were treated with the primary Ab mouse anti-human Dectin-1 (prepared in ice cold PBS) for 10 minutes on ice (Figure 73). This was followed by washing 5x with cold PBS, and then the addition of donkey anti-mouse Cy3-

labeled secondary Ab (red) for 10 mins at 37°C, and then cells were put on ice and washed 5x with cold PBS (Figure 73). This was followed by fixation with 4% PFA, cell permeabilization, blocking followed. Next cells were immunostainined for phospho-SYK using rabbit ant-P-SYK (Y352) and an anti-rabbit AF488-labeled secondary Ab (in green). Cells were then acquired on a confocal microscope (Figure 73).

As illustrated in (Figure 73), HeLa Dectin-1 cells, form high intensity Dectin-1 puncta at the membrane in response to Ab x-linking. Moreover these cells induced much higher levels of SYK phosphorylation (green signal) as compared with HeLa WT controls, which was accompanied with enhanced recruitment of SYK to regions of the plasma membrane highly enriched in Dectin-1 forming apparent P-SYK punctate structures (Figure 73). Amazingly, Dectin-1 and SYK puncta perfectly colocalized which is a similar result to that obtained by stimulation of cells with larger ligands (as seen in zoom in of Figure 73). SYK puncta were not observed in HeLa WT controls and the P-SYK signal was generally much weaker than in HeLa Dectin-1, indicating the specificity of the response to Ab x-linking of cell surface Dectin-1 (Figure 73).

We next wanted to validate this result in RAW Dectin-1 cells by analyzing the effect of Ab x-linking on SYK phosphorylation/membrane recruitment, and the formation Dectin-1 puncta by immunofluorescence. Therefore an identical immunofluorescence (IF) Ab x-linking experiment was performed on RAW Dectin-1 cells, as described above for HeLa Dectin-1 cells (Figure 74). We obtained an exactly similar result for SYK recruitment and phosphorylation as that observed for HeLa Dectin-1 cells. In summary, Ab x-linking resulted in enhanced recruitment of P-SYK (green) to the plasma membrane which strongly coalesced with highly intense Dectin-1 puncta formed at the membrane (red) (Figure 74).

These (IF) Ab x-linking experiments demonstrate the capacity of Ab x-linking of cell surface Dectin-1 to induce pronounced SYK phosphorylation and formation of P-SYK punctate structures that recruit to regions of the plasma membrane high in Dectin-1 intensity (Dectin-1 puncta) (Figure 73 & Figure 75).

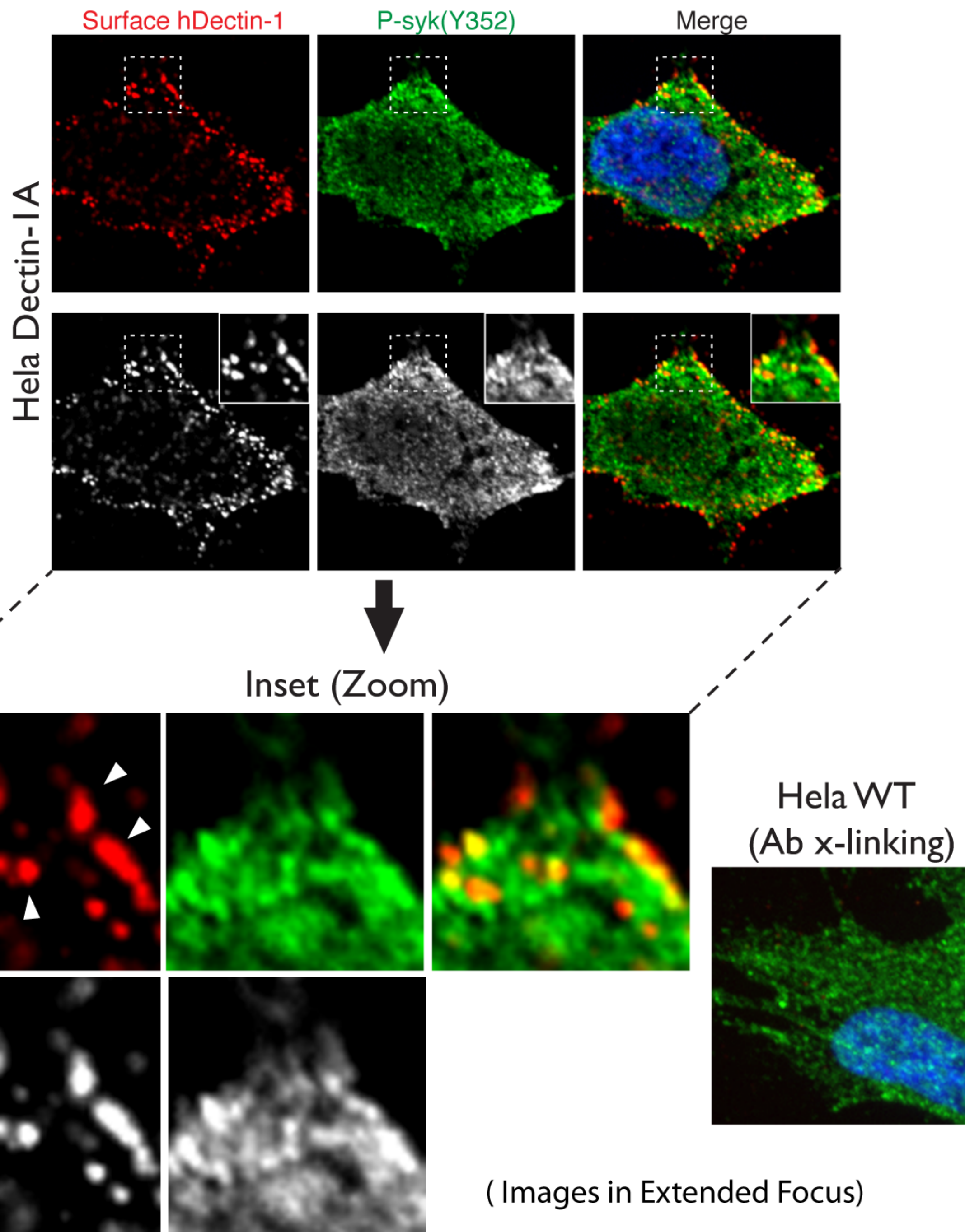


Figure 73: Effect of Ab Cross-linking in HeLa Cells on SYK Recruitment and Formation of Dectin-1 Puncta

Ab cross-linking (Ab cross-linking) cells was performed in HeLa Dectin-1 cells, examine the effect of clustering Dectin-1 on SYK phosphorylation. Cells were serum-starved (4-6 hours) after which cells treated with the primary Ab mouse anti-human Dectin-1 (prepared in ice cold PBS) for 10 minutes on ice or with mouse IgG2b isotype control This was followed by washing 5x with cold PBS, and then the addition of donkey anti-mouse Cy3-labeled secondary Ab (red) for 10 mins at 37°C, and then cells were put on ice and washed 5x with cold PBS. This was

followed by fixation of cells with 4% PFA, permeabilization (0.1% TX-100) and blocking followed by imunostaining of phospho-SYK using rabbit anti-P-SYK (Y352) and an anti-rabbit AF488-labeled secondary Ab (in green). Apparent overlap between Dectin-1 puncta/clusters and phospho-SYK reflects the recruitment of signaling molecules at the site of Dectin-1 clusters. The inset (dashed white square) of the top image has been zoomed to reveal more molecular details. White arrows in zoomed images of panel 1, indicate the colocalization between Dectin-1 puncta (clusters) and phospho-SYK (green) induced by Ab x-linking, and reflects the recruitment of signaling molecules at the site of Dectin-1 clusters. Also depicted on the left hand side in figure is an image of a RAW WT cell showing no SYK recruitment to the membrane. Data is representative of 4 independent experiments Data represents results from 3 similar experiments.

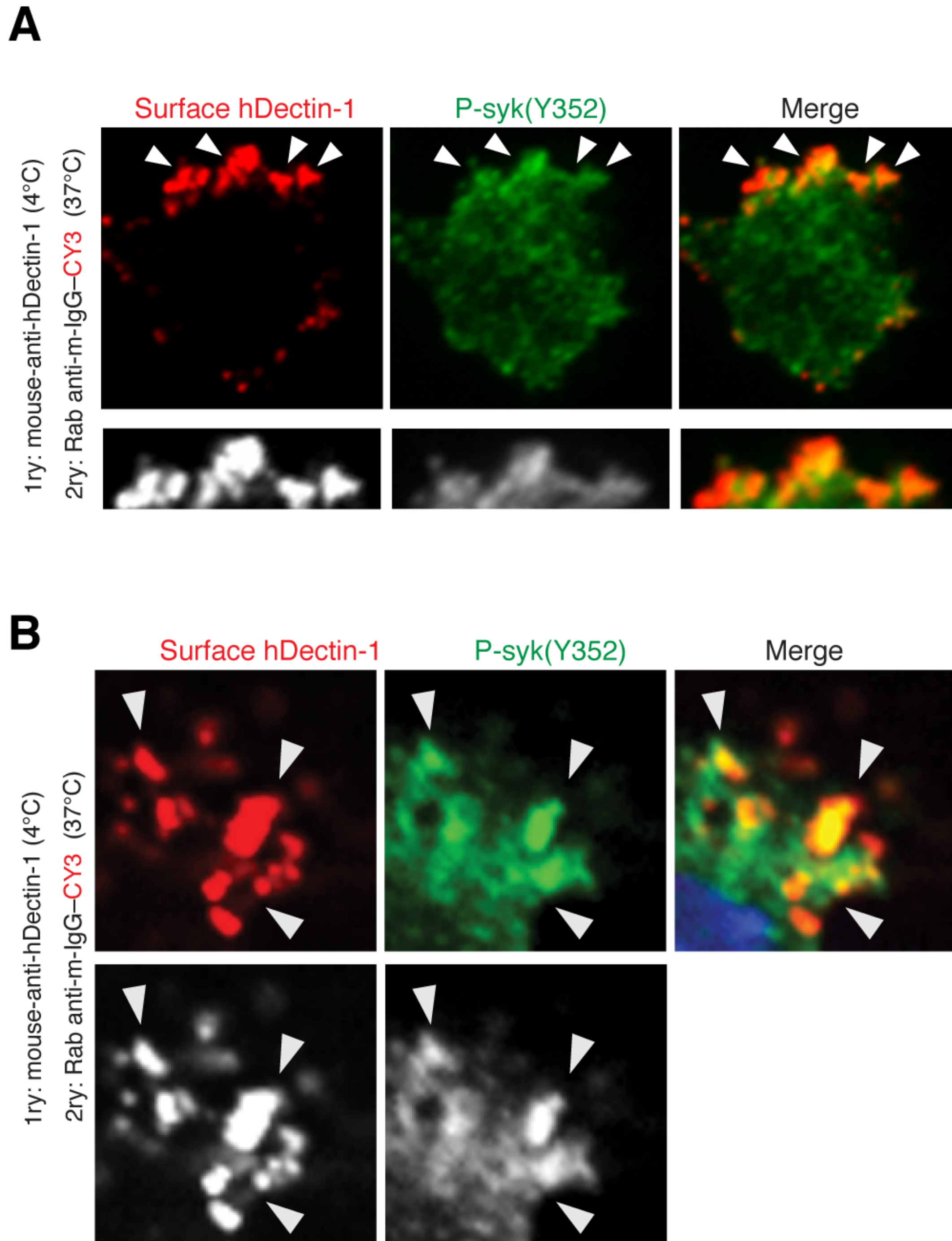


Figure 74: Dectin-1 Puncta Formation and SYK Recruitment in Response to Ab cross-linking in RAW Dectin-1 Cells

Ab cross-linking (Ab x-linking) of cells was performed in RAW Dectin-1 Cells to visualize the effect of clustering Dectin-1 on SYK phosphorylation. Cells were serum-starved for 4-6 hours and treated with the primary Ab mouse anti-human Dectin-1 (prepared in ice cold PBS) for 10 minutes on ice or with mouse IgG2b isotype control This was followed by washing 5x with cold PBS, and then the addition of donkey anti-mouse Cy3-labeled secondary Ab (red) for 10 mins at 37°C, and then cells were put on ice, washed with 5x cold PBS. This was followed by fixation of cells

with 4% PFA, permeabilization, blocking, and then imunostaining of phospho-SYK using rabbit anti-P-SYK (Y352) and an anti-rabbit AF488-labeled secondary Ab (in green). Apparent overlap between Dectin-1 puncta/clusters and phospho-SYK reflects the recruitment of signaling molecules at the site of Dectin-1 clusters. Data represents results from 3 independent experiments. **A** and **B,** represent images for two selected cells. White arrows shown in images of cells in (A) and (B) indicate the strong colocalization between Dectin-1 puncta (clusters) and phospho-SYK in images from panel one induced by Ab x-linking, and reflects the recruitment of signaling molecules at the site of Dectin-1 clusters. Data is representative of 4 independent experiments.

Dectin-1 signaling involves many tyrosine phosphorylation events mediated by tyrosine kinases such as SYK and SFKs, and even by dual-specificity MAPKs. Indeed, we have observed that Dectin-1 upon ligation with a larger-sized ligand such as P-curdlan, induces a marked amount of global tyrosine phosphorylation in these cells as compared to unstimulated controls (Figure 75). Therefore, using the same phosphotyrosine antibody (mouse anti-phosphotyrosine, clone 4G10, from Millipore) used in the western blot of Figure 75 to detect global tyrosine phosphorylation we performed an immunofluorescent experiment in HeLa Dectin-1 cells to visualize the effect of Ab x-linking on tyrosine phosphorylation in these cells (Figure 76B).

A similar immunofluorescent experiment was performed as decribed above, where Ab x-linking of cell surface Dectin-1 was conducted in 'HeLa Dectin-1' cells (Figure 76B). Briefly, cells were treated with primary Ab (Mouse anti-human Dectin-1 at 1:1000) or with mouse IgG2b isotype control (1:1000) for 10 minutes on ice, followed by thorough washes with PBS and incubation with secondary Ab (Rabbit anti-mouse Cy3 coupled antibody, used at 1:500) (red signal) for 10 mins at 37°C (Figure 76B). Cells were then placed on ice and washed 5x with cold PBS, fixed with 4% PFA, permeabilized and blocked, followed by immunostaining of global phospho-tyrosine with a FITC-coupled anti-phosphotyrosine antibody, clone 4G10 (Millipore) (green signal). Cells were then visualized by confocal microscopy (Figure 76B).

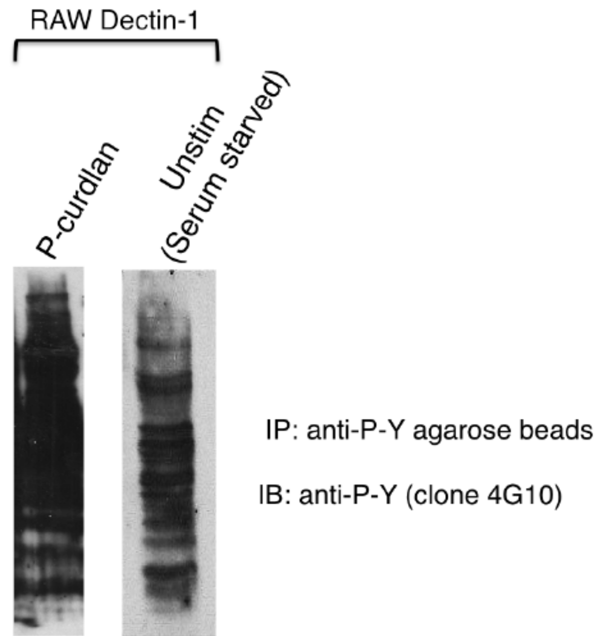


Figure 75: Global Tyrosine Phosphorylation Upon Dectin Stimulation by a Large-sized Dectin-1 Ligand (P-curdlan)

RAW Dectin-1 Cells were probed for global tyrosine phosphorylation in response to the large ligand P-curdlan.Cells were harvested, washed with PBS and treated with 100μg/ml of P-curdlan for 10 minutes or left unstimulated, and then lysed in special phospho-lysis buffer containing phosphatase inhibitor cocktail (Roche) for 20 minutes at 4°C. Post-nuclear lysates were precleared to remove non-specific binding of proteins to agarose beads. Preclearing was performed by incubation of cell lysates with agarose beads for 2 hours with gentle mixing at 4°C on a rotator for 4 hours. Beads were then pelleted by centrifugation at 8,000 rpm. Precleared lysates ere incubated with anti-phosphotyrosine Ab (clone 4G10) conjugated to agarose beads with gentle mixing at 4°C on a rotator for 4 hours. Beads were pelleted and washed three times with lysis buffer before resuspension of eluted proteins in 2x sample buffer and boiling for 3 minutes. Samples were then separated by SDS-PAGE, transferred on nitrocellulose membrane, and blotted with mouse anti-phosphotyrosine Abs (Millipore) to detect tyrosine phosphorylated proteins Western Blot is representative of two independent experiments. Blot reveals intense tyrosine phosphorylation upon treatment of cells with a Dectin-1 ligand of high molecular weight (P-curdlan) as compared to unstimulated cells.

As seen in (Figure 76B), Ab cross-linking of surface Dectin-1 in HeLa Dectin-1 Cells produced high levels of tyrosine phosphorylation (green) as demonstrated by an intense green signal in the confocal images of these cells. Amazingly, this intense green signal, which represents phospho-tyrosine, strongly colocalized with high intensity Dectin-1 puncta (red) formed at the plasm membrane (Figure 76B). Therefore Ab x-linking of cell surface Dectin-1 induces high levels of tyrosine phosphorylation, which is reminiscent of the effect observed upon stimulation of Dectin-1 with a larger-sized ligand such as P-curdlan (Figure 75). Excited by this result, we were curious to know if SYK phosphorylation observed in HeLa Dectin-1 and RAW Dectin-1 cells in response to Ab x-linking was higher than that induced by a smaller ligand such as laminarin. To investigate this question we next performed a western blot Ab x-linking experiment on HeLa Dectin-1 cells (Figure 76A). Cells were either induced by Ab x-linking of Dectin-1, or treated

with isotype negative clustering control or with laminarin. We then monitored SYK phosphorylation by immunoblotting cell lysates with a phospho-specific antibody against P-SYK (anti-P-SYK for residues Y525/Y526, cell signaling), as well as an antibody against total SYK (for detection of total SYK, phosphorylated and unphosphorylated protein) (Figure 76A). Amazingly, SYK phosphorylation was only induced in cells stimulated by Ab x-linking. These cells produced an immensely high P-SYK signal compared to cells treated with laminarin or isotype control. In contrast the total level of SYK was the same in all conditions, indicating that the differential levels of SYK phosphorylation observed are related to the various conditions used, and not to unequal loading of protein. Since laminarin has been demonstrated by us in section 3.2.6.2**)**, to possess a very limited capacity of clustering the Dectin-1 receptor into large aggregates, the above result indicates that the the pronounced signaling effects produced by Ab-x-linking are most likely due to the gathering of Dectin-1 receptors into large-sized clusters **(**see Figure 69**)**.

Finally, to confirm our results from this section we performed an IF Ab x-linking experiment on RAW Dectin-1 cells using more controls. One of these controls included a condition where we treated cells as usual on ice (at 4°C) for 10 mins, and instead of the usual incubation with secondary Ab at 37°C for 5 minutes, cells were placed on ice and treated with the cold secondary Ab (prepared in ice cold solutions) at at 4°C (Figure 77). Treating cells with secondary Ab at low temperatures is expected to reduce the ability of the secondary Ab to cluster/cross-link cell surface Dectin-1 molecules due to the decrease in the mobility of the Dectin-1 receptor at low temperatures, which in turn reduces the ability of the secondary Ab to cross-link the anti-Dectin-1 primary Abs. Another control condition that we employed for this Ab x-linking experiment, was just treating cells with primary Ab as usual for 5 mins on ice (at 4°C), without further addition of secondary Ab (Figure 77). This is predicted to produce a very mild cross-linking effect of Dectin-1 receptors on the cell surface. We also included in the experiment our regular isotype control, which is a negative control for cross-linking. Finally we were interested in examining the effect of the PP2 SFK inhibitor on SYK phosphorylation, membrane recruitment and Dectin-1 puncta formation induced by Ab x-linking as shown in (Figure 74). This would demonstrate if SFK phosphorylation of the Dectin-1 hemITAM is essential for the formation of Dectin-1 clusters or Puncta, as we have demonstrated above for the effect negative of PP2 on the formation of Dectin-1 puncta and SYK recuitment (see section 3.2.3.1**,** Figure 51**)**.

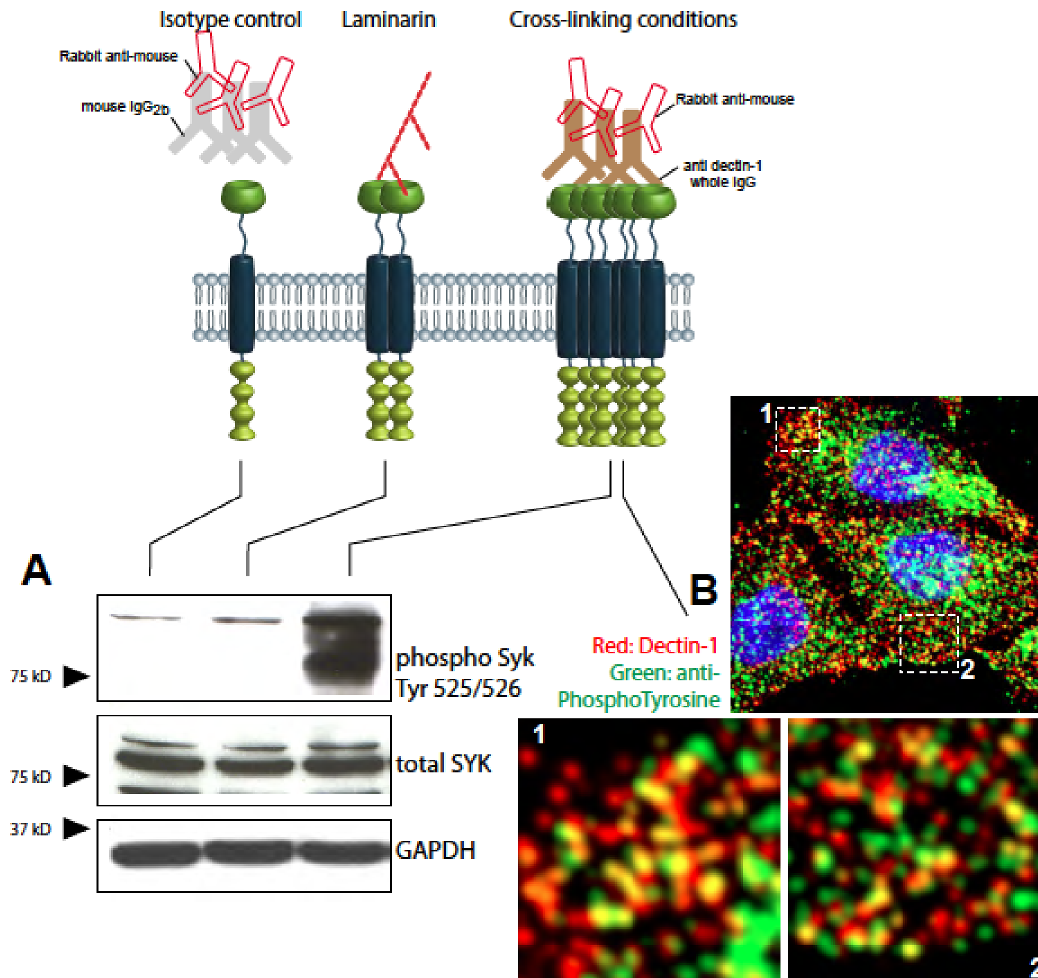


Figure 76: Dectin-1 Cross-linking Induces Tyrosine Phosphorylation and SYK Activation in HeLa Dectin-1 Cells

The activation/phosphorylation of SYK and tyrosine phosphorylation in response to Dectin-1 stimulation was tested by immunoblotting (A) and immunofluorescence (B). Following the treatment of HeLa Dectin-1 cells with the various conditions described in the upper schematic (see text) **(A)** Using a mouse isotype control IgG2b antibody and secondary rabbit anti-mouse antibody or adding 100 μg/ml of laminarin did not induce SYK activation (phosphorylation). When Dectin-1 was clustered by cross-linking using mouse anti-human Dectin-1 (10 mins at 4°C) followed by rabbit anti-mouse antibodies (for 10 mins at 37°C). SYK, was found to be phosphorylated on tyrosine 525/526, which reflects its activation. We also probed for total SYK and GAPDH (as protein loading control) **(B)** Following cross-linking, fixation and immunolabeling with a rabbit anti-mouse Cy3 coupled antibody, HeLa Dectin-1 cells were permeabilized with 0.1% TX-100 and stained with a FITC-coupled anti-phosphotyrosine antibody (clone 4G10, Millipore). Cells were imaged by confocal microscopy. Insets (1) and (2) represent zoomed areas of the cells (1) and (2) from theimage above. These insets demonstrate the remarkable overlap between Dectin-1 clusters and phospho-tyrosine residues is obvious and it reflects the recruitment of signaling molecules at the site of Dectin-1 clusters. Data is representative of 3 independent experiments.

After treating RAW Dectin-1 cells with these various clustering, non-clustering, and inhibitor conditions, standard IF protocol was performed, as described above. After cell treatment, cells were fixed and immunostained for surface Dectin-1 using a Donkey anti-mouse Ab coupled to Cy3 (in red). Cells were then permeabilized to detect phospho-SYK at using a rabbit anti-P-SYK

(Y352) and anti-rabbit secondary Ab coupled to AF488 (green) (Figure 77). As illustrated in Figure 77, regular Ab x-linking of RAW Dectin-1 cells using primary Ab (mouse anti-human Dectin-1, 1:100) at 4°C followed by incubation with secondary Ab (Donkey anti-mouse IgG used at 1:500) at 37°C induces the formation of high intensity puncta of Dectin-1 and SYK puncta l at the plasma membrane, which demonstrate perfect colocalization in merged confocal images. As expected, isotype control conditions failed to induce such effects in RAW Dectin-1 cells. Indeed there was a very low level of P-SYK, which was mostly cytosolic and showed no visible recruitment to the plasma membrane (Figure 77). As previously shown by us in Figure 26, the isotype IgG control wasn't able to recognize Dectin-1 expressed on the surface of these cells indicating the specificity of the Dectin-1 Ab for binding to, and cross-linking, surface Dectin-1 in these cells (Figure 77). Intrigingly, treatment of cells with only primary antibody (at 4°C) without subsequent addition of secondary Ab, didn't produce any of the effects observed upon induction of cell surface Dectin-1 clustering by full Ab x-linking (Figure 77). This has a very important implication, as it means that binding of just the primary Ab to a site on Dectin-1, distinct from the ligand binding site, and known not to induce any conformational change in Dectin-1, doesn't induce Dectin-1 signaling. Only when secondary Ab was added at 37°C leading to the cross-linking and clustering of the receptor, Dectin-1 was able to activate signaling (Figure 77). To further validate this result, the non-clustering control condition using both primary and secondary Ab at 4°C didn't induce any visible SYK recruitment/activation and Dectin-1 formation as that detected with regular Ab cross-linking condition (primary Abs at 4°C, and secondary Ab at 37°C) (Figure 77). This non-signaling effect observed in thse cells is most likely due to the mild magnitude and nature of Dectin-1 cross-linking induced by this condition, which seems to be unsufficient for inducing Dectin-1 signaling.

Finally, we investigated the effect of PP2 on SYK on the above-described signaling effects observed in response to Ab x-linking (Figure 77). Excitingly, treatment of RAW Dectin-1cells with the SFK inhibitor PP2 during the Ab x-linking of surface Dectin-1, inhibited the recruitment and activation of SYK, as demonstrated by reduced levels of SYK phosphorylation and the mostly cytosolic appearance of P-SYK in these cells (Figure 77). Furthermore PP2 inhibited the formation of high intensity Dectin-1 puncta on the cell membrane of the cells (Figure 77). This result is reminiscent of the inhibitory effect of PP2 on Dectin-1 and SYK puncta formation at the cell membrane, in cells treated with larger ligands, such as P-curdlan and BSA-17-lam in

presence of PP2 (Figure 49). This implicates that SFK activation could be required for the formation of Dectin-1 high intensity puncta at the cell membrane or in other words, Dectin-1 clusters. This effect of PP2 on Dectin-1 clustering is interesting as it implies that the phosphorylation of the hem ITAM motif is essential for the formation of Dectin-1 clusters, which in turn might be due to the recruitment of SYK to these the phosphorylated hemITAMs on two individual Dectin-1 molecules thereby bridging these two molecules into a stable Dectin-1 dimer.

Overall we have shown in this section that Ab-crosslinking of the receptor, is able to induce Dectin-1 clustering (as seen by the formation of intense Dectin-1 puncta at the cell membrane), which in turn activate key upstream signaling events required for Dectin-1activation mainly SYK phosphorylation and recruitment to the plasma membrane. This provided insight into the mechanism by which the binding of Dectin-1 to to large-sized ligands induces signaling, which is most probably due to the clustering of the receptor by the ligand, and not due to ligand induction of conformational changes in the Dectin-1 receptor.

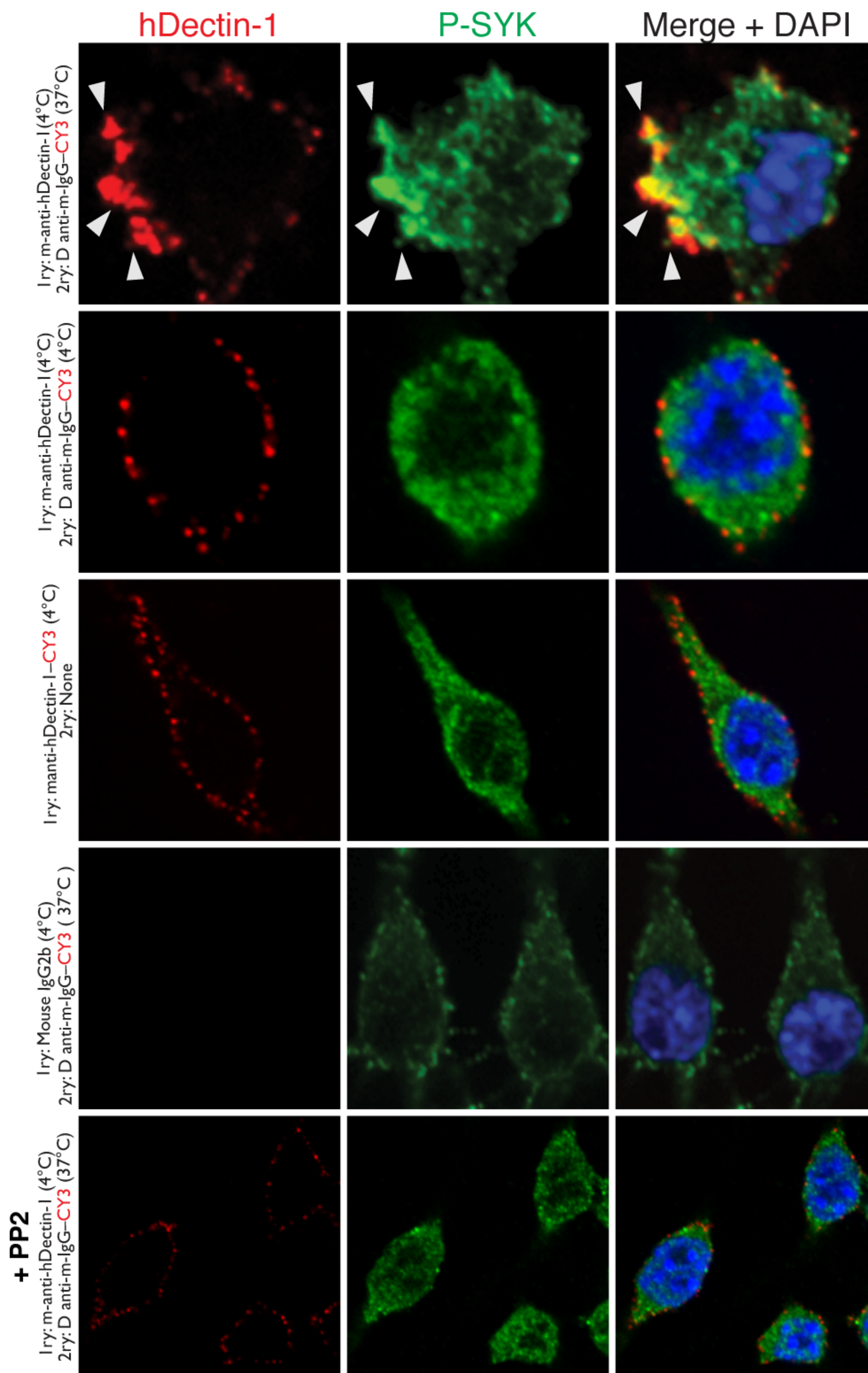

RAW Dectin-1
hDectin-1
P-SYK
Merge + DAPI
1ry: m-anti-hDectin-1(4°C)
2ry: D anti-m-IgG–CY3 (37°C)
1ry: m-anti-hDectin-1(4°C)
2ry: D anti-m-IgG–CY3 (4°C)
1ry: manti-hDectin-1–CY3 (4°C)
2ry: None
1ry: Mouse IgG2b (4°C)
2ry: D anti-m-IgG–CY3 (37°C)
+ PP2
1ry: m-anti-hDectin-1 (4°C)
2ry: D anti-m-IgG–CY3 (37°C)

Figure 77: Monitoring Dectin-1 Puncta formation and SYK Recruitment in Response to various Ab cross-linking conditions and SFK inhibitor

Ab cross-linking (Ab x-linking) of RAW Dectin-1 cell was employed under different clustering and non-clustering conditions as depicted in figure and described in text. For the last panel of images cells were pretreated with 10µM PP2 for 30 mins and then cells were washed, then Ab x-linking was performed as usual but in the presence of PP2. After each of these cross-linking, inhibitor-treated, and non-cross-linking control conditions cells were fixed and immunolabeled for surface Dectin-1 with a Donkey (D) anti-mouse Ab coupled to Cy3 (red). Cells were then permeabilized with 0.1% TX-100 and stained for P-SYK using a rabbit anti-P-SYK (Y352) Ab and anti-rabbit secondary Ab coupled to AF488 (green). White arrows in images of panel 1, indicate the strong colocalization between Dectin-1 puncta (clusters) and phospho-SYK in images from panel one induced by Ab x-linking, and reflects the recruitment of signaling molecules at the site of Dectin-1 clusters. Data is representative of 4 independent experiments.

3.3. Discussion

Many studies have demonstrated the immunomodulatory effects of polysaccharides, including β-glucan, isolated from mushrooms, fungi, yeast, algae, lichens, and plants (Novak and Vetvicka, 2008). Recently, the molecular mechanism of fungal pathogen recognition by innate immune cells, such as macrophages and DCs, has become clearer with the identification of Dectin-1 as the major PRRs for β-glucans, which reside in the fungal cell wall To date, despite the amount of studies/research on Dectin-1 signaling, it is still unclear how β-glucans are able to activate Dectin-1 to initiate immune responses. Accordingly the main purpose of this study was to elucidate the molecular mechanisms responsible for Dectin-1 activation by β-glucans.

There is a great controversy in the Dectin-1 field regarding the capacity of β-glucans to activate Dectin-1 signaling. Soluble β-glucans are deemed by some as biologically inactive ligands, incapable of activating Dectin-1, and in some of these studies it has been claimed that only particulate insoluble β-glucans are able to induce Dectin-1 signaling (Brown, 2003, 2006c; Goodridge et al., 2011). However, it is noteworthy that most of the data of Dectin-1 signaling has been generated using live fungi and particulate β-glucans that are largely insoluble. Indeed, a great caveat in interpreting Dectin-1 data is that the performed studies didn't employ highly purified, water-soluble β-glucans to stimulate Dectin-1, whereas few studies have examined the mechanism of purified, soluble β-glucans, used as a biological response modifiers or immunomodulatory supplements to augment the host immune response (Tsoni and Brown, 2008b). In this study we were fortunate to have had the advantage of overcoming all of these problems faced by others in studying the mechanisms of Dectin-1 activation and signaling by β-glucans, where we had access to highly soluble and 100% pure β-glucans through our collaboration with the group of Dr. Bundle (Department of Chemistry, U of A), of great expertise

in carbohydrate chemistry. Therefore, we were interested in this study to understand whether and how soluble β-glucans can activate Dectin-1 signaling.

Previous reports have indicated that the physicochemical properties of β-glucans such as primary structure, polymer size, solution conformation, molecular weight, surface charge and side-chain branching may be important determinants for the recognition and interaction with Dectin-1, which is considered the main receptor for β-glucans (Barsanti et al., 2011; Brown et al., 2002a; Tsoni and Brown, 2008b). These parameters, may play a role in determining whether and with what affinity β-(1,3)-glucans bind to Dectin-1 to induce signaling and modulate immune function (Mueller et al., 2000). Interestingly, it has been widely recognized in the β-glucan literature that the small soluble β-glucan, laminarin is unable to activate Dectin-1 signaling, whereas higher molecular weight β-glucans, including particulate β-glucans, have a much higher potential to induce Dectin-1 signaling. An interesting comparative study by Rice *et al.* (2004) (Rice et al., 2005), compared the pharmacokinetics of three well-characterized soluble β-(1,3)-glucans (laminarin, sceleroglucan and glucan-phosphate) of different molecular sizes, branching frequency, and solution conformations (Rice et al., 2005). All three β-(1,3)-glucans exhibited similar pharmacokinetics although their physicochemical properties varied considerably. Interestingly, these three different-sized β-(1,3)-glucans (laminarin, sceleroglucan and glucan-phosphate) have been demonstrated by numerous studies to display different bioactivity. Despite that, these three glucans were shown to bind to Dectin-1, laminarin doesn't stimulate innate immunity, whereas sceleroglucan and glucan-phosphate enhance immune function (Mueller et al., 2000; Williams et al., 1999). This data suggests that size of the β-glucans largely affects the bioactivitiy of the glucan. Therefore it has been proposed by us and others that large ligands might have a higher capacity to induce the clustering of the Dectin-1 receptor, which could in turn enhance the potential of Dectin-1 to trigger signaling. However, the precise relationship between the size of the β-glucan and Dectin-1–mediated induction of signaling and subsequent immune responses has not been defined. This was due in part to the lack of well-characterized β-glucans with varying molecular weights and conformational structures. Therefore taking advantage of our excellent system of different-sized highly soluble β-glucans as well as the BSA-laminarin conjugates with a precise increase in numbers of laminarin in addition to the appropriate cell lines expressing Dectin-1 (described above in **section 3.2.2**, mostly provided by Dr. Bundle's group), we were able to successfully establish a parallel correlation between the size

of the soluble ligand, its capacity to cluster Dectin-1, and its potential to induce Dectin-1 signaling.

Along the line of ligand-size/Dectin-1 relationship, we have most importantly demonstrated in this study that laminarin, a small soluble β-glucan (6-8 kDa) widely used as a Dectin-1 blocker for the purpose of inhibiting Dectin-1 signaling, can actually stimulate Dectin-1 upstream components of the signaling pathway including activation of the key effectors of Dectin signaling: Src family kinases (SFKs), SYK, PLCγ2, and PKCδ. It hasn't been previously demonstrated that laminarin can activate any of these key membrane-proximal signaling events. Most of these studies were just focused on examining the effect of laminarin in inducing downstream events of Dectin-1 signaling such as NF-κB activation and cytokine production, which are events that are not activated by laminarin. Amazingly, laminarin could even induce key signaling effectors that are known to be activated downstream of SYK, such as PKCδ and PLC-γ2, and indeed the laminarin signal continues activation all the way down to the level of PKCδ activation, after which it loses its signal, especially at the level of the activation of the IKK kinase and other components of the signaling machinery required for the activation of NF-κB. Interestingly, laminarin despite activating these critical upstream signaling events, induces them at a significantlylower levels than larger-sized ligand such as P-curdlan and BSA-17-laminarin Therefore we have re-identified laminarin in this study not as an absolute blocker of Dectin-1 signaling, but as a small soluble β-glucan with an ability to induce upstream events in Dectin-1 signaling, but failing to carry this signal through to downstream molecules of the Dectin-1 signaling pathway such as the ERK MAP kinases and NF-κB. These downstream effectors are indispensable to the induction of cytokines, chemokines, costimulatory molecules and other key proinflammatory mediators essential for the induction of immune responses, specifically important for antifungal immunity. Also, we found out that the laminarin signal is lost at the level of ERK MAPK activation, and interestingly, we demonstrated that ERK activation is upstream of p65 NF-κB phosphorylation. Therfore the loss of the capacity of laminarin to activate the Dectin-1 signaling pathway at the level of the ERK MAPK, could be the reason why laminarin fails to activate Dectin-1signaling all the way down to the level of NF-κB activation Accordingly, we have re-identified lamnarin as a soluble β-glucan with no immunomodulatory properties, yet with the ability of inducing limited levels of upstream Dectin-1 signaling that is not sufficient to fully activate the signaling pathway all the way downstream. This key finding is the first mechanistic

insight into why laminarin as a small soluble β-glucan is not a biological activator of immunodulatory responses in comparison to other high molecular weight β- glucan. On the other hand, we have identified P-curdlan as a high molecular weight β-glucan (≤ 5000 kDa), which despite being highly water-soluble, demonstrated a strong capacity to stimulate Dectin-1 signaling down to the level of the transcription factor NF-κB. Moreover, we have identified the BSA-laminarin conjugate, with 17 laminarin molecules anchored to the protein carrier BSA (BSA-17-laminarin), as a soluble Dectin-1 ligand and potent activator with the capacity to fully activate all components of the Dectin-1 signaling pathway. BSA-laminarin conjugates with less number of laminarin molecules, were also able to activate Dectin-1 signaling events all the way down to the level of NF-κB activation, yet a progressive increase in the magnitude of signaling was observed with an increase in the valency of the number of laminarins conjugated to the protein carrier. *Therefore, the differential capacity of these ligands to activate Dectin-1 both quantitatively (magnitude of signaling response) and qualitatively (activation of certain components of the signaling pathway) is most likely a result of its size as a β-glucan.*

We have also demonstrated in this study that the reason, larger ligands have higher capacity for activating Dectin-1 signaling stems from their ability to induce clustering of Dectin-1 into higher order structures, which is in accordance with our clustering hypothesis (Figure 22). We were able to establish a correlation between the size of the ligand and the magnitude of the underlying signaling events. We have indeed shown that larger ligands can stimulate the early signaling events of Src family kinase (SFK) and SYK activation, and that these ligands could indeed activate more components of the Dectin-1 signaling pathway (section 3.2.3). In parallel we observed that our soluble β-glucans were able to bind to Dectin-1, and that the larger ligands were able to induce the formation of high intensity Dectin-1 puncta at the plasma membrane to which SFKs and SYK were recruited. We speculated that these highly intense puncta corresponded to Dectin-1 clusters formed by the large-sized β- glucans. By closer analysis of these clusters using the suprresolution 'single molecule' imaging technique of PALM, which facilitates the detection of single molecule events with high resolution, we were able to definitively prove that these high intensity Dectin-1 puncta were indeed higher order clusters of Dectin-1 **(see section 3.2.6.2)**. The larger-sized ligands P-curdlan and BSA-17-lam were able to induce clusters of larger size than laminarin. We also demonstrated that at steady-state, there are pre-existing clusters of Dectin of small size. These clusters coalesce together upon binding of

larger-sized ligands. Interestingly, although the cluster radius and the number of Dectin-1 receptors within the cluster increase with increasing ligand size, the density of Dectin-1 molecules within the cluster does not. This could imply that key signaling partners or membrane proteins / receptors are recruited to, or diffuse into, these clusters thereby inducing molecular events that could potentially trigger Dectin-1 signaling. For instance, clustering of Dectin-1 could mediate the recruitment of critical signaling partners such as members of the Src family kinases (SFKs), which are key to the activation of Dectin-1. Moreover it is possible that clustering mediates the interaction of Dectin-1 with other membrane proteins such as tetraspanins (e.g. CD63 and CD37), or the collaboration of Dectin-1 with other pattern recognition receptors (PRRs). Indeed Dectin-1 is known to interact with PRRs including TLRs (TLR2/TLR4), and CLRs, e.g., galectin, Dectin-2, or Mincle **(see section 1.5.4.4)**. These first level of Dectin-1 collaborations might be essential for activation of Dectin-1 to turn induction of antifungal immune responses. Also tetraspanins are scaffolding proteins that could possibly diffuse into Dectin-1 clusters formed upon ligand binding for the purpose of stabilizing the receptor in these clusters, which in turn could promote Dectin-1 activation. Indeed the tetraspanin CD37, has been shown to physically interact and collaborate with Dectin-1 for the production of cytokines. Furthermore, cytoskeletal proteins such as actin and microtubules could diffuse into these newly formed Dectin-1 clusters further stabilizing the clusters to induce signaling from Dectin-1

On the other hand, another advantage of receptor clustering induced by binding to ligands of larger size, is that, essential receptors and kinases are segregated from phosphatases, such as CD148 and CD45 leading to a net increase in phosphorylation and activation of the Dectin-1 receptor. Indeed a recent study by Goodridge *et al.* (2011), proposed a model mechanism for Dectin-1 activation by particulate ligand (Figure 16). In this model the CD45 and CD148 tyrosine phosphatases are excluded from the clustered sites of Dectin-1 induced by particulate β-glucan, thereby enabling downstream signaling through the hemITAM of Dectin-1 by allowing the activation of SFKs. However in contrast to our findings, they proposed in this study that this Dectin-1 clustering and activation is only induced by binding to particulate insoluble β-glucan and not by soluble β-glucan. Interestingly, we have demonstated in this study that soluble β-glucans are able to induce Dectin-1 activation, but only when they are large enough to mediate Dectin-1 aggregation into large-size clusters. This is a novel finding, and we are the first to establish this ligand-size/Dectin-1clustering/signaling capacity positive correlation. We believe

that large soluble ligands induce clustering of Dectin-1 and in fashion similar to the Goodridge model of Dectin1 activation, this clustering leads to the segregation of CD45 and CD148 phosphatases for the Dectin-1 cluster, thereby facilitating the activation of SFKs which phosphorylate the hemITAM motif and subsequently Dectin-1 activation.

Receptor clustering is an emerging concept in the field of signal transduction, and has been shown to be essential for activation of several immune receptors such as the FcR, BCR and TCR Furthermore, CLRs such as DC-SIGN, CLEC-9A and CLEC-2 have been shown to require receptor clustering for activation of signaling. In fact, CLEC-2, a closely related member of the 'Dectin-1 NKCL cluster' or 'group V' CLRs, which posseses a hemITAM motif like Dectin-1. In contrast to ITAM receptors, which require dually phosphorylated tyrosines for SYK recruitment, hemITAM receptors, phosphorylation of only the membrane proximal tyrosine is sufficient for SYK association with Dectin-1 and CLEC-2, even though both SH2 domains of SYK are still required. Dectin-1 was the first receptor shown to signal via this pathway which although is quite similar to ITAM signaling, is unique in its dependence on only a single tyrosine present within the hemTAM motif. It is still unclear how SYK could be recruited to the monophosphorylated hemITAM of Dectin-1, especially that it requires both SH2 domains for the interaction with classical ITAM receptors. Interestingly, CLEC-2 has been shown to be active as a dimer and to activate SYK by receptor dimerization. Therefore the current model for SYK activation by hemITAM-containing receptors, such as CLEC-2 and Dectin-1 is that SYK could bind to two single phosphorylated tyrosines on adjacent clustered Dectin-1 receptors. This would lead to recruitment of SYK to single phospho-tyrosines on the hemITAMs by bridging two monophosphorylated Dectin-1 receptors. Indeed, consistent with this model, our chemical cross-linking data revealed that Dectin-1 exists as dimers in the steady-state and upon binding of Dectin-1 to the larger-sized ligands, this basal level of Dectin-1 dimers significantly increases. In alliance with this result, clustering analysis of our superresolution data demonstrated that Dectin-1 pre-exists as smaller sized clusters that increase in size upon binding to larger ligands due to fusion of these small pre-existing clusters. Therefore it seems that Dectin-1 binding to larger ligands mediates clustering of Dectin-1 or enhances dimerization of the receptor, leading to an increase in the proportion of the population of Dectin-1 dimers, possibly present in clusters. In accordance with the current model described above for activation of SYK in hemITAM signaling from Dectin-1, the formation of a larger platform of Dectin-1 dimers lead promote the activation

of SYK. SYK is a key upstream mediator involved in the initiation of early Dectin-1 signaling events that further trigger several signaling cascades that are crucial to the Dectin-1 signaling pathway.

Interestingly, inhibition of SFKs led to the decrease in the formation of Dectin-1 puncta and SYK recruitment to the plasma membrane in response to stimulation of cells by larger ligands or by Ab-x-linking. This inhibition led to an immunostaining pattern of SYK and Dectin-1 in confocal images similar to that observed in cells treated with a smaller ligand such as laminarin. This indicates that SFK activation is essential for Dectin-1 clustering and SYK recruitment. Inhibition of SYK recruitment is understandable as phosphorylation of the hemITAM motif of Dectin-1 is required for SYK recruitment. However, it is puzzling that SFK phosphorylation affects clustering of Dectin-1. However clustering by larger ligands, or by particulate ligands as proposed by Goodridge *et al.* (2011) could initially induce Dectin-1 clustering, which leads to exclusion of the receptor tyrosine phosphatases, CD45 and CD148, thereby inducing the activation of SFKs. The latter, in turn induce the tyrosine phosphorylation of the hemITAM motif of Dectin-1, leading to recruitment and activation of SYK by binding via its dual SH2 domains to two monophosphorylated Dectin-1 receptor. Therefore receptor clustering by larger ligands could bring neighbouring Dectin-1 molecules in close proximity, which facilitates the bridging between closely-spaced Dectin-1 receptor dimers via interaction with the two SH2 domains of SYK, which further stabilizes Dectin-1 clusters. Also consistent with this hypothesis is that basal level of SYK recruitment and activation in unstimulated cells reflects the low proportion of Dectin-1 dimers present in the steady state. Nevertheless, further investigation is required to verify this hypothesis and examining the interaction between Dectin-1 and SYK, by a technique such as SPR (surface plasmon resonance) could provide evidence for this model of formation of Dectin-1 dimers.

Finally, as demonstrated and discussed in the results**,** we ruled out, via Ab x-linking, the possibility that ligand binding induces a conformational change of the receptor that activates Dectin-1. Only when cell surface Dectin-1 receptors are brought in close proximity and clustered by Ab x-linking, do they activate Dectin-1. When primary Ab was bound live to cell surface Dectin-1, no activation of Dectin-1 was observed.

To conclude, in this study we have validated our Dectin-1 clustering hypothesis **(**Figure 22**),** by

successfully establishing a correlation between ligand size, Dectin-1 clustering, and Dectin-1 activation. We speculate that in this model, clustering of Dectin-1 by larger-sized soluble ligands (e.g. P-curdlan) could induce the activation of the key early signaling players including SFKs and SYK phosphorylation through their recruitment to Dectin-1 clusters. Above the threshold of more than 50 receptors within clusters larger than 100 nm in radii, Dectin-1 signaling pathway appears to be fully activated, and leds to the activation of key transcriptions factors, mainly NF-κB. This in turn enables Dectin-1 to induce immune responses essential for antifungal immunity. In contrast, smaller soluble ligands such as laminarin only activate the key Dectin-1 upstream signaling events at subthreshold levels which are not sufficient to fulfill signaling till the end of the pathway. See final model (*Discussion, Chapter 5,* Figure 92**)**

Despite all the mechanistic insights provided by this study, supplementary investigations are needed to further validate our model of Dectin-1 activation by clustering. We need to better understand, at the molecular level, how exactly Dectin-1 clustering mediates the receptor signal transduction. The answer to this future question most likely needs to be address determining all the components of the Dectin-1 clusters and by characterizinz the dynamic rearrangements of these molecules during β-glucan stimulation. Without strong expertise in single molecule microscopic technique and superresolution imaging, we will undertake diffusion analysis of Dectin-1 (described in *Chapter 5*). Further molecular insight into the mechanism of Dectin-1 activation by clustering could be obtain by investifgating how varying size ligands could differentially activate more components of the signaling pathway that haven't been tested yet.

Most importantly, in this study we have conclusively demonstrated, in contrary to what has been reported mostly in the literature, that soluble β-glucans are indeed able to activate Dectin-1 but only when they are large size. We demonstrated that laminarin a small β-glucan is incapable of activating Dectin-1 yet increasing the number of laminarin molecules exposed on a protein carrier enhances the Dectin signaling capacity. In fact the BSA-4-lam was able to activate Dectin-1 signaling all the way downstream to the level of NF-κB activation, this indicates that a minimal valency of four laminarin molecules is sufficient to fully activate the Dectin-1 signaling pathway, which in turn is presumably sufficient for activating immune responses. This intriguing finding validated our model of Dectin-1 activation by clustering, and prompted us in the next chapter *(Chapter 4)*, to apply our clustering hypothesis for the generation of an efficient anti-fungal vaccine against *C. albicans.*

Chapter 4. VACCINE TARGETING TO THE C-TYPE LECTIN RECEPTOR DECTIN-1 CAN BENEFIT IMMUNOMODULATION AND AUGMENT THE IMMUNE RESPONSE MEDIATED BY A TETANUS-TOXOID VACCINE CONJUGATE AGAINST *C. ALBICANS*

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Enhanced Immunogenicity of a Tricomponent Mannan Tetanus Toxoid Conjugate Vaccine Targeted to Dendritic Cells via Dectin-1 by Incorporating β-Glucan

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Contribution to the chapter:

As highlighted in the footnotes of the front page of the paper, I am the co-lead author of this article with Tomasz Lipinski (formerly a post-doctoral fellow in Dr. David Bundle's lab, Dept. of Chemistry, Faculty of Science, U of A). I have mainly conceived, designed, performed, interpreted, and analyzed the immunological, biochemical, cell biology and molecular biology experiments of this paper. The main work done by Tomasz Lipinski in the paper was designing, synthetizing the vaccine conjugates, performing the animal/mice vaccination and characterizing the antibody titer. In summary, I did the biochemical, cell biological, and immunological

characterization of the vaccine, and overall succeeded in this paper to provide valuable mechanistic insight into the molecular and biochemical basis of the enhanced immune response provided by the vaccine. I would like to acknowledge Dr. Tomasz Lipinski for initiating this project, which was initiated through discussion between the group of Dr. Bundle and our group and based on our preliminary findings that multivalent ligands are more potent stimulator of Dectin-1 signaling. I would also want to acknowledge and thank Dr. Hanne Ostergaard for providing her laboratory space and experimental mice, and for valuable feedback and ideas. Finally, I would like to thank Dr. Joëlle St. Pierre (then a fellow Ph.D. candidate) for teaching dissection of the animals and how to prepare BMDCs, as well as for her valuable insight.

4.1. Introduction

Despite huge prevention and control efforts, infectious diseases remain an important global problem in public health, causing 13 million deaths each year. Therefore, in recent decades, development of new vaccines against such diseases has become an important area of biomedical and public health research. Increasing occurrence of antibiotic resistance and high costs associated with implementation of new drugs justify efforts in this field. Vaccines have already been proven as effective agents to combat infectious disease, but despite a significant knowledge about mechanisms of vaccines action there is a paucity of successful approaches in the treatment of fungal infections. Fungal infections are ranked the seventh most common cause of infection-related death in North America. Current anti-fungal drug regimens are inefficient in curing patients with fungal infections, and at least 35% of affected individuals die of this disease. Because vaccines are one of the most cost effective modalities for prevention of microbial diseases, it is therefore a health care priority to develop efficacious antifungal vaccines.

Medical Importance of C. albicans.

Candida albicans *(C. albicans)* a medically important emerging fungus is a major threat to patients who are immunosuppressed and those who have undergone major surgical procedures, with mortality reaching 30-40% despite the availability of antifungal drugs. *Candida albicans (C. albicans*) is an emerging medically important fungal pathogen that poses a major threat to immunocompromised individuals e.g. subjects of surgery, transplantations, and chronically immunosuppressed patients, such as those with AIDS, autoimmune disease, and cancer patients treated with chemotherapy. *C. albicans,* although a commensal fungus, becomes an opportunistic

fungal pathogen that causes disease when exposure is combined with host susceptibility through immunodeficiency or a breach in normal barriers. A range of primary and secondary immunodeficiency syndromes are associated with susceptibility to mucosal infections caused by *C. albicans,* including the primary immunodeficiency hyper-IgE syndrome and a family of genetic diseases leading to chronic mucocutaneous candidiasis (CMC) (Farah et al., 2006; Hohl, 2014). Secondary immunodeficiencies are now the most common predisposing factor for mucosal Candida disease, which is well described in patients with HIV and is considered an AIDS-defining illness. Cancer chemotherapy, immunosuppression for transplant recipients, renal failure, and high-dose corticosteroids increase the risk for candidiasis (Conti and Gaffen, 2010). Invasive infections with opportunistic *C. albicans* is one of the leading causes of hospital-acquired bloodstream infections, and according to published data, *C. albicans* is responsible for every fourth incident of blood infection and mortality rate in the case of systemic infections may approach 40% despite the availability of antifungal drugs (Gudlaugsson et al.; Wisplinghoff et al., 2004).

Need for Anti-Candida Vaccine

Therefore it is of great importance to develop novel treatment strategies for infections with *C. albicans*, such as adjunctive immunotherapy as well as novel anti-Candida vaccines, which are promising, yet unfulfilled therapeutic and preventive strategies. With that goal in mind, much research has been done to elucidate the host defense mechanisms against systemic *C. albicans*. The importance of phagocytosis and the elimination of *C. albicans* by cells of the innate immune, especially neutrophils, and macrophages, as well as the modulation of the activity of these cells by proinflammatory mediators and anti-inflammatory cytokines has been firmly established (Gow and Hube, 2012; Gow et al., 2007; Kullberg et al., 1999; Netea et al., 2006b; Netea et al., 1999; van Enckevort et al., 1999). Nevertheless, to date available anti-Candida drugs are still ineffective, costly and pose serious toxicity issues, and accordingly introduction of effective anti-Candida vaccine is highly demanded for public health. Carbohydrate conjugate vaccines have been immensely successful in preventing diseases such as bacterial meningitis and pneumococcal pneumonia. In fact Prevnar 13, a pneumococcal conjugate vaccine, is now a multibillion-dollar drug for Pfizer. The dogma in the field of conjugate vaccines has held that large oligo or even large polysaccharides are essential for protective vaccines. Smaller structures such as a trisaccharide typically do not afford protection. In earlier studies, it has been shown that a

bicomponent vaccine conjugate of tetanus toxoid with synthetic β-mannan trisaccharides (a protective epitope of C. albicans) was effective against disseminated candidiasis in a rabbit model. However the same conjugate was poorly immunogenic in mice.

Our Hypothesis

In order to improve vaccine immunogenicity we decided to explore a modern concept in vaccinology that targets vaccines to dendritic cells (DCs). In our JI research paper, we were able to achieve DC targeting by covalently adding a third component, a second carbohydrate to the conjugate vaccine. This carbohydrate is β-glucan, which is a ligand for Dectin-1, a pattern recognition immune receptor that occurs on DCs and is key to antifungal immunity. We succeeded to develop a tricomponent vaccine that is not only delivered to the DC surface, but is also able to activate Dectin-1, resulting in production of cytokines beneficial for antifungal response, and key to clearance of the Candida infection. Moreover, we have shown that by targeting a trisaccharide-tetanus toxoid conjugate vaccine to DCs, the immune response is enhanced, and the antibody isotype distribution reflects DC processing. Overall, this elevated immune response would afford protection against Candida infection.

Vaccine targeting to innate immune cells rationale

Antigen-presenting cells (APCs), particularly dendritic cells (DCs) and macrophages, play a crucial role in the initiation and regulation of antigen-specific immunity (Roy and Klein, 2012). In general, the immune responses are the outcomes of the following events: foreign proteins or antigens are taken up by APCs, processed into small peptides and displayed on the surface of APCs as a major histocompatibility complex (MHC) (Reis e Sousa and Unanue, 2014). Naive T cells then recognize the MHC/antigen complex, and this recognition eventually leads to T cell activation and evokes adaptive immunity, including humoral immune response and cellular immune response.

Given the importance of APCs in triggering and regulating immune systems, strategies for targeting these cells in vivo will greatly advance immunotherapy and facilitate the design of more effective vaccines. One approach to accessing APCs in vivo is to target APC-specific surface receptors with ligand–antigen conjugates that deliver targeted antigens to the antigen-processing/presentation machinery of APCs via receptor-mediated endocytosis. This can be done by complexing antigenic proteins to antibodies that are specific for surface receptors on APCs.

Recently, of specific interest, is targeting CLRs in vaccine studies to increase Ag-specific immune responses. The CLR DEC-205 was one of the first targets in these studies, and later antibody-mediated targeting of antigen to DCs via the C-type lectin DC-SIGN effectively induced antigen-specific naive as well as recall/memory T cell responses (Geijtenbeek et al., 2009; Geijtenbeek and Gringhuis, 2009; Tacken et al., 2005). Examples of other CLRs that have been recently exploited for antigen/vaccine or drug delivery include, DC/macrophage expressed Dectin-1 that has been targeted by the β-glucans laminarin (Xie et al., 2010) and schizophyllan (Mochizuki and Sakurai, 2011), and selectins, as well as CD69 an early activation marker expressed by activated T cells and NK cells (Lepenies et al., 2013). Interestingly *in vivo* immunization using a Dectin-1 agonist or *C. albicans* infection promotes the differentiation of Th1 and Th17.

Furthermore, several studies have demonstrated that targeting APCs can induce defined and tailored immune responses depending on the type of CLR targeted. It is widely accepted that the nature of an innate immune response to a microbe is defined by the types of pattern-recognition receptors that detect it. Accordingly, this suggests that choosing different target receptors may produce different outcomes, which is a critical consideration that needs to be in taken into account when designing multicomponent vaccine conjugates. In this context, it should be carefully considered which ligand will be incorporated into the vaccine conjugate so that it is specifically targeted to the CLR most suitable for mounting a desirable and specific immune response tailored against the pathogenic antigen of interest that is present in the vaccine.

An intriguing application of the adjuvant/immunomodulatory activity of β-glucans that has attracted the attention of researchers and been exploited in the development of glucan-based adjuvants as part of conjugate vaccines for the purpose of enhancing the host immune response. Moreover it is of immense potential for immunotherapy and vaccination to target antigens to antigen-presenting cells (APCs) especially DCs. Here we described a method for delivering a *Candida albicans* cell wall-derived carbohydrate antigen, β-mannan, to APCs via carbohydrate-mediated targeting to a C-type lectin. Therefore for the purpose of vaccine and antigen targeting to APCs (mainly DCs), laminarin, which is a β-(1,3)-glucan with occasional β-(1,6)-linked branches and a typical ligand for Dectin-1, was chemically coupled to beta-mannan TT-TS. Additionally, for the benefit of harnessing our results from Chapter 3 regarding Dectin-1 activation in reponse to clustering by multivalent ligands, we added few laminarin molecules

(three or four) to this conjugate, in order to enhance the induction of Dectin-1–mediated cellular and immune responses, and thereby its immunogenicity and adjuvanticity.

Intriguingly, attaching laminarin (3 laminarin molecules) to this conjugate led to significant enhancement of DC cytokine response towards a cytokine profile that favours development of naïve T–cells into Th17, and therefore laminarin was not only acting as a β-glucan targeting the vaccine conjugate to DCs via binding to CLR Dectin-1 but also served as a β-glucan adjuvant with immunostimulatory properties. Moreover, when used to immunize mice, the laminarin-TT-TS-conjugate enhanced the primary IgG antibody response. Taken together, our data suggest that APCs targeting based on glucan–Dectin-1 interaction is a promising approach to improve vaccines.

In summary, our paper has shown for the very first time that introducing a β-glucan ligand to the vaccine construct can augment the immune response to a small carbohydrate hapten (in this case the β-mannan trisaccharide). Ultimately, results from our paper will provide a framework for the development and novel design of more effective anti-fungal vaccines.

4.2. Results

4.2.1. Generation of Trisaccharide Tetanus-toxoid (TS-TT) Vaccine Conjugates & Characterization of RAW Dectin-1 Cells

A tetanus-toxoid vaccine bearing β-mannan (a cell wall epitope of *C. albicans*) was generated by Dr. Tomasz Lipinski (Dr. David Bundle's laboratory, Dept. of Chemistry, Faculty of Science, U of A, Edmonton, AB), as initially described in Lipinski *et al.* (Lipinski et al., 2013; Lipinski et al., 2011), and conjugated to additional laminarin molecules (Figure 78**)**. As described in section **(1.5.2)**, laminarin is a small soluble β-glucan (5-6 kDa), which is recognized by Dectin-1 on DCs and macrophages, yet doesn't fully activate Dectin-1 signaling (see data in Chapter 3) (Adams et al., 2008; Brown et al., 2002b; Goodridge et al., 2011).

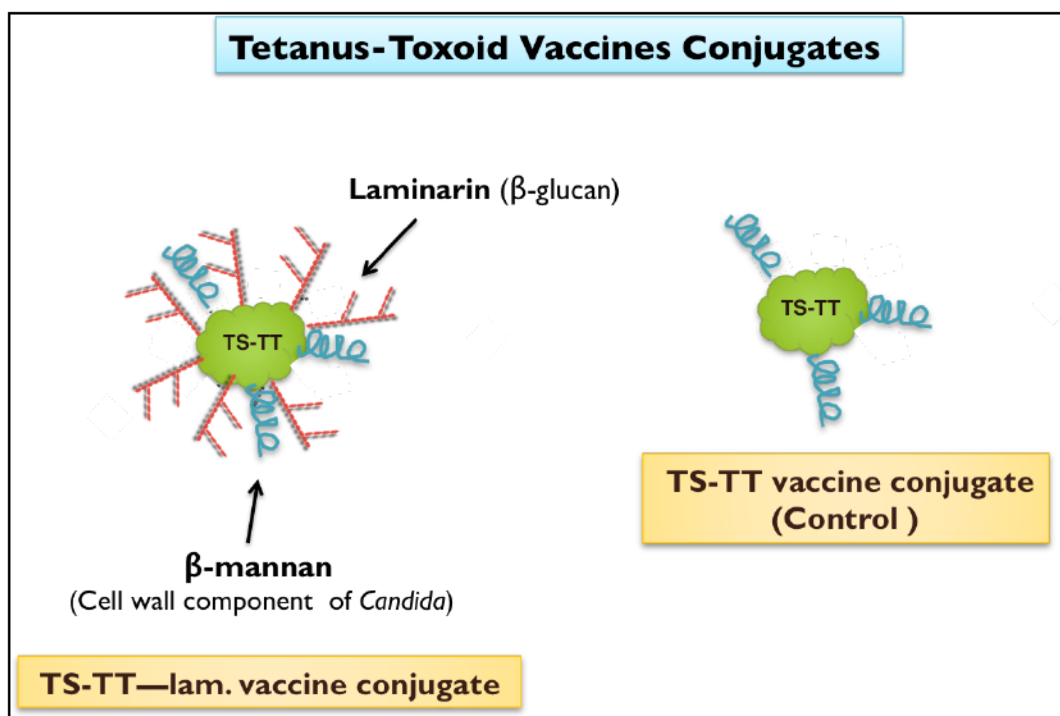

Figure 78: Tetanus-Toxoid Trisaccharide Vaccine Conjugates

Two types of β-mannan trisaccharide vaccine conjugates were prepared in this study by Dr. Tomasz Lipinski in the Bundle laboratory (Dept. of Chemistry, U of A). In the first one β-mannan trisaccharide epitope derived from the cell wall of *Candida albicans* was conjugated to tetanus-toxoid carrier protein to form the vaccine conjugate was to refered to as TS-TT. This vaccine conjugate (TS-TT) and was was used as a reference vaccine (control vaccine) for this study. On the other hand laminarin molecules (a small β-glucan of 5-6 kDa) were chemically linked to the control vaccine TS-TT to generate the tricomponent vaccine of interest in this study, named TS-TT-Lam., consisting of the three components, β-mannan, laminarin and Tetanus toxoid. Adapted with permission from (Lipinski et al., 2013).

Briefly, Tetanus-toxoid was reacted with the diazo transfer reagent, imidazole-1-sulfonyl azide hydrochloride, to convert amino groups into azide groups. Transformation of amines into azides prevented cross-linking of the protein following activation of aspartic and glutamine side chain carboxylic acids for coupling with the amino-terminated trisaccharide hapten. The resulting conjugate was divided into 2 portions, one was conjugated to propargylated laminarin using click chemistry (see materials and methods, **Chapter 2)** and the other one remained unattached to laminarin. The residual azide groups in each conjugate were reduced back to the original amino forms to produce two conjugates designated TS-TT and TS-TT-Lam (Figure 78**)**. The latter conjugate vaccine named “TS-TT-lam” is a tricomponent conjugate composed of 3 constituents: the TS-TT carrier protein, β-mannan and laminarin, where as the other TS-TT conjugate vaccine was used as the control vaccine **(**Figure 78**)**.

We first decided to validate the Dectin-1 specific targeting of this tri-functional vaccine using the murine macrophage cell line 'Raw-Dectin-1A' stably expressing Dectin-1A, which was generated as described above in (Chapter 2). The human Dectin-1A isoform was readily detectable at the cell surface upon immunostaining of non-permeabilized cells **(**Figure 79**)** and not detected in 'RAW WT' control cells not expressing Dectin-1. Similarly, immunoblotting of whole cell lysates prepared from cells expressing Dectin-1 demonstrated expression of the mature glycosylated form of human Dectin-1 as a higher molecular weight band at approximately 40-50 kDa confirming the appropriate folding, targeting and surface expression of Dectin-1 in the RAW macrophages ***(***Figure 24, *Chapter 3).*

4.2.1. Cellular Response of RAW Dectin-1 Macrophages to Laminarin-containing Vaccine conjugate versus Control Vaccine

We next investigated whether the presence of the laminarin polysaccharide on the tetanus toxoid (TS-TT-Lam) would allow for a better binding of the vaccine by macrophages expressing Dectin-1 compared to the TS-TT compound (Tetanus toxoid with β-mannan only). To visualize the binding of the vaccine conjugate to RAW Dectin-1 cells, these compounds were coupled to AF546 (see Materials and Methods, Chapter 2). Binding was achieved by incubation of the RAW Dectin-1 cells with the fluorescent compounds, TS-TT-AF546 or TS-TT-Lam-AF546 for 10 minutes followed by washes with PBS and fixation with 4 % PFA. Dectin-1 immunostaining was performed in parallel and as shown on (Figure 80), only the vaccine coupled to laminarin

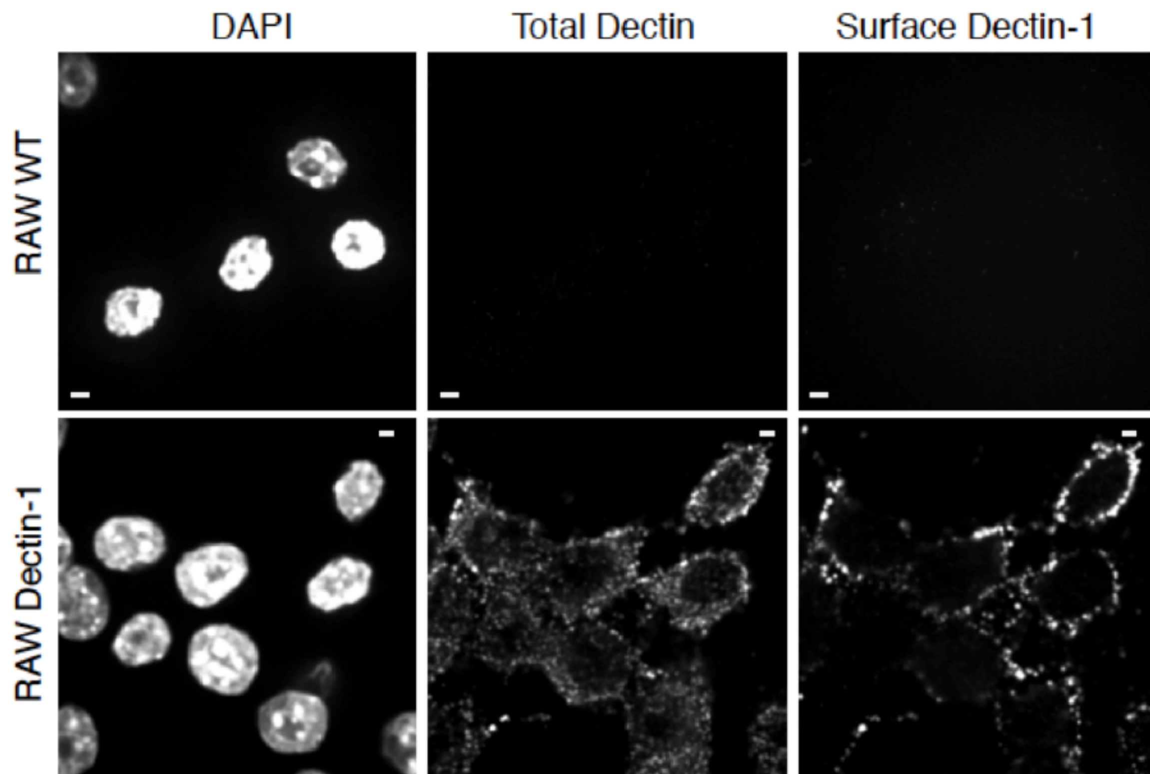

Figure 79: Characterization of Surface Expression of Dectin-1 in RAW Dectin-1 Cells for the Vaccine Study

Immunofluorescence staining on nonpermeabilized cells (surface Dectin-1) with a mouse anti-Dectin-1 Ab, followed by a donkey anti-mouse coupled to Cy3, revealed the membrane targeting of the receptor. Following permeabilization with 0.1% Triton X-100, the same cells were stained with the same Ab, except that the secondary was coupled to Alexa Fluor 488, revealing internal structures. DAPI was used to localize the cells' nuclei. Data are representative of five independent experiments. Images were acquired on the confocal microscope. Adapted with permission from (Lipinski et al., 2013). Scale bars, 2 μm.

was able to bind RAW Dectin-1 cells. We also confirmed that the binding of the TS-TT-Lam-AF546 was specific to the cells expressing Dectin-1, as RAW WT were not capable of recognizing the laminarin conjugate (data not shown).

As mentioned in Chapter 1, Dectin-1 signaling requires phosphorylation of its cytosolic hemITAM motif on tyrosine 15 (Tyr) by a Src-family-kinase (SFK) allowing the phospho-ITAM-like domain to recruit the spleen tyrosine kinase, SYK, to mediate further downstream signaling. To verify that TS-TT-Lam glycoconjugate was indeed able to activate SFK, we tested the appearance and colocalization of the active form of SFK with Dectin-1 following binding of fluorescently-labeled TS-TT-Lam. Following stimulation with TS-TT or TS-TT-Lam, cells were fixed, permeabilized and immunostained with anti-Dectin-1 antibody and an anti-

phospho-(Y^{416}) Src antibody that reports SFK (Src family kinase) activation. Cells stimulated with TS-TT compound did not show significant staining for the phosphorylated form of Src (Figure 81). In contrast, the laminarin-carrying vaccine showed a pronounced increase in phosphorylated SFK (Figure 81).

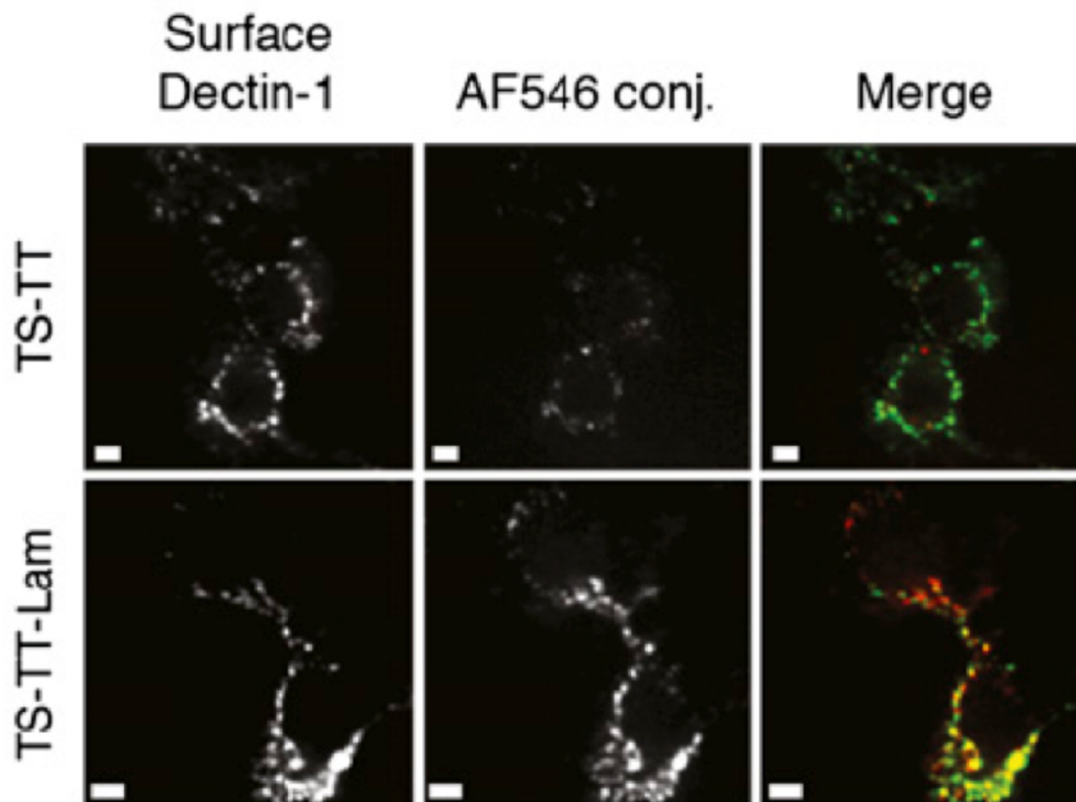

Figure 80: Characterization of the Binding of Vaccine Conjugates to Dectin-1 in RAW Dectin-1 cells (Vaccine Binding assay).

RAW Dectin-1 Cells RAW Dectin-1 cells were incubated on ice (to minimize internalization of vaccine conjugates) for 10 min with AF546 (red) fluorescently-labeled vaccine conjugates TS-TT-AF546 (top row), or TS-TT-Lam-AF546 (lower row), followed by 4% paraformaldehyde (PFA) fixation. Dectin-1 was immunostained in non-permeabilized macrophages with the mouse anti–Dectin-1 and AF488-conjugated anti-mouse secondary Abs. Images were acquired on the confocal microcope. Images revealed that only the TS-TT-Lam-AF546, and not the control vaccine vaccine, is able to bind to the cell membrane and colocalizes with surface Dectin-1. Data is representative of three similar experiments. Adapted with permission from (Lipinski et al., 2013). Scale bars, 2 μm.

We next confirmed the recruitment and activation of SYK by immunostaining and immunoblotting (Figure 82 & Figure 83). Cells incubated with TS-TT or TS-TT-Lam for 10 min were either fixed and immunostained for Dectin-1 and phospho-SYK or lysed and analyzed by western blotting with anti-phospho-SYK antibodies, which are specific for Y525/526 or Y352 in

SYK. Phosphorylation of these tyrosine residues is activatory and leads to stimulation of the kinase activity of SYK (Mocsai et al., 2010). By immunostaining (Figure 82) activation of SYK could be detected only upon stimulation with TS-TT-Lam, but not with TS-TT. This result was confirmed by the western blot experiment showing that TS-TT-Lam activates SYK in a Dectin-1–dependent manner. In RAW cells that do not express Dectin-1, addition of either TS-TT or TS-TT-Lam did not increase SYK phosphorylation on either Y525/526 or Y352 (Figure 83).

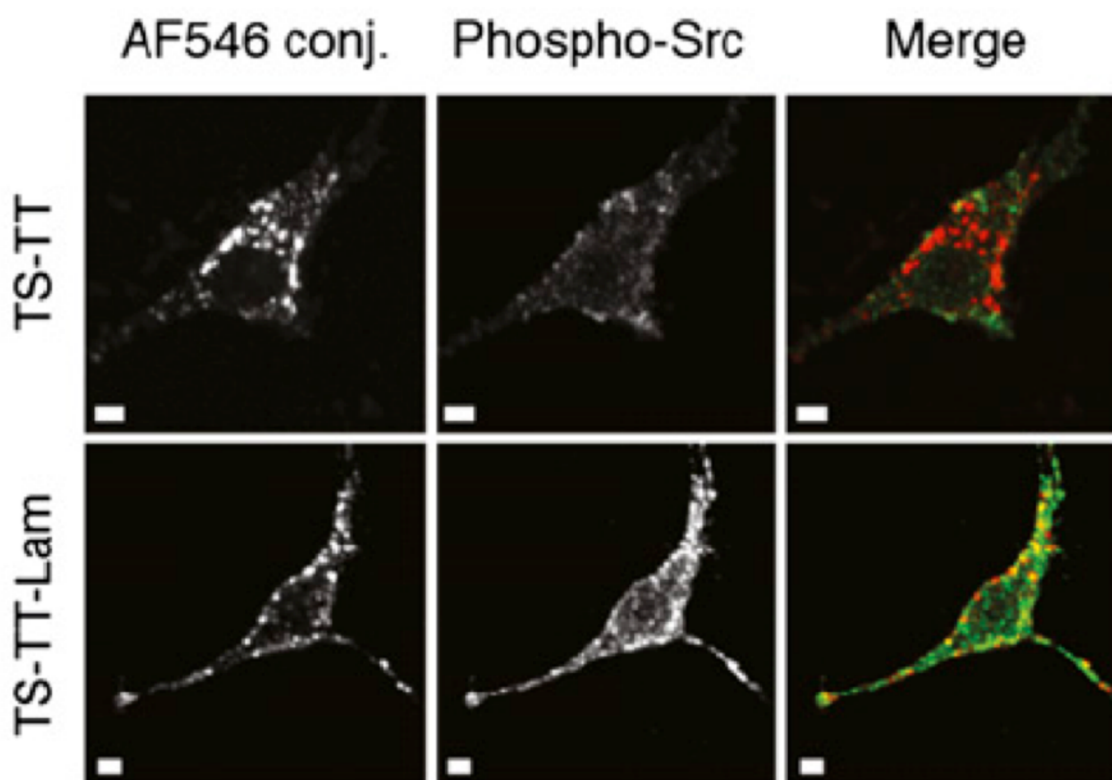

Figure 81: Src Activation in Response to Vaccine Conjugates

RAW Dectin-1 cells were incubated on ice for 10 min at 37°C with 100 μg/ml of the fluorescently labeled vaccine conjugates TS-TT-AF546 (top row) or TS-TT-Lam-AF546, (lower row), followed by 4% PFA (paraformaldehyde) fixation. Dectin-1 was immunostained in nonpermeabilized macrophages with the mouse anti–Dectin-1 and AF488-conjugated anti-mouse secondary antibody. Activation of SFK was visualized using rabbit anti-phospho Src (Y416) antibody coupled to AF488 (Invitrogen). As seen in figure RAW Dectin-1 macrophages respond to laminarin-containing vaccine as seen by activation and recruitment of P-src (green channel) to the membrane and its colocalization with the AF546-labeled vaccine (red). Images were acquired on the confocal microscope. Data is representative of three similar experiments. Adapted with permission from (Lipinski et al., 2013). Scale bars, 2 μm.

Similarly, SYK was not activated in the Dectin-1 expressing cells upon addition of the control vaccine conjugate TS-TT. However, SYK phosphorylation of Y525/526 and Y352 could only be detected when the laminarin conjugate was used on Dectin-1–expressing cells (Figure 83). This

result indicates that following binding to the β-glucan receptor, the oligovalent TS-TT-Lam molecules are capable of triggering Dectin-1 activation and in particular the SYK pathways. Moreover, these results confirm that the laminarin conjugate is able to activate Src-family-kinases (SFKs) and the SYK adaptor proteins, which are two crucial effectors of the Dectin-1 signaling pathway. Finally, we tested the activation and translocation of the nuclear factor NF-κB

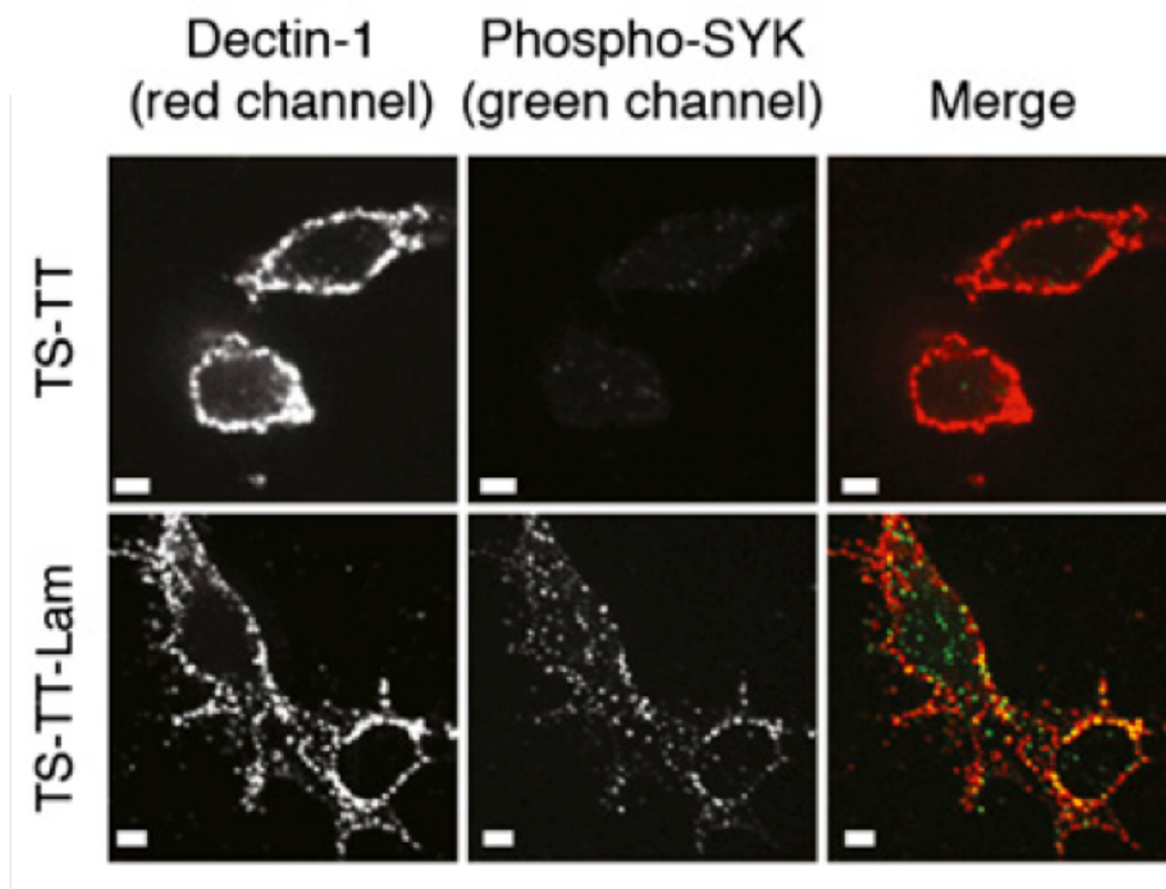

Figure 82: Effect of Vaccine Conjugates on SYK Activation

RAW Dectin-1 cells were incubated with 100 μg/ml of the vaccine conjugates TS-TT-AF546 or TS-TT-Lam-AF546 for 10 min at 37°C before fixation with 4% PFA. Activation of SFK was monitored by observing SYK phosphorylation in these cells via immunofluorescence using rabbit antiphospho-Src (Y418) coupled to AF488 (Invitrogen). SYK recruitment and activation were observed using a rabbit anti-phospho-SYK(Tyr352) and an anti-rabbit AF488. Images were acquired on the confocal microscope. Data is representative of three similar experiments. Adapted with permission from (Lipinski et al., 2013). Scale bars, 2 μm.

in fixed RAW Dectin-1 macrophages by immunostaining for the p65 subunit of NF-κB in parallel with DAPI staining following 10 min of incubation with either TS-TT or TS-TT-Lam. Although nuclear translocation of NF-κB was not seen in cells treated with TS-TT, TS-TT-Lam led to its pronounced translocation (Figure 84). Activation of NF-κB by the laminarin-containing vaccine

further illustrates the capacity of this compound to stimulate innate immune cells in a Dectin-1–dependent fashion.

Using macrophages stably expressing Dectin-1 at the cell surface, we have validated that the laminarin-containing vaccine, TS-TT-Lam, was able to specifically interact with the β-glucan receptor, Dectin-1, and activate well-characterized downstream effectors.

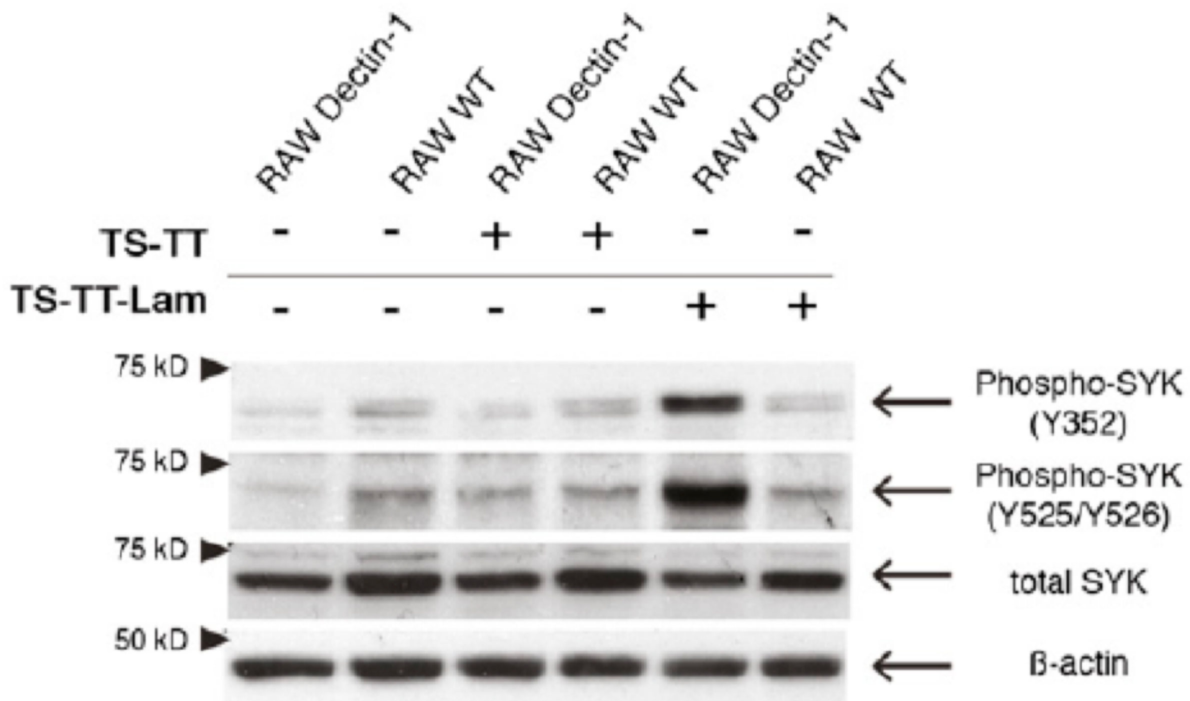

Figure 83: Western Blot of SYK Phosphorylation in Response to Vaccine Conjugates

RAW Dectin-1 or RAW WT cells were treated with TS-TT or TS-TT-Lam for 10 min at 37°C, and protein lysates were prepared, separated by SDS-PAGE, transferred on nitrocellulose membrane, and blotted with Abs against phospho-(Y352)–SYK or phospho-(Y525/Y526)–SYK and against total SYK and β-actin as loading controls. Data is representative of three similar experiments. Adapted with permission from (Lipinski et al., 2013).

Before testing the vaccines directly in animals and to better define the immune response triggered by these compounds, we next examined the *in vitro* immunological capacity of the TS-TT and TS-TT-Lam vaccine conjugates in inducing the activation and maturation of BMDCs; i.e., determining the *in vitro* immunogenicity of these vaccine compounds.

4.2.2. Analysis of the Cellular Response of Primary BMDCs to Dectin-1 ligands

As mentioned in section **(1.5.1)** Dectin-1 is expressed on cells from the monocytic lineage,

especially on dendritic cells (DCs) (Taylor et al., 2002), which are responsible for promoting anti-fungal immunity during pathogenic challenges (Marakalala et al., 2011; Taylor et al., 2007; van de Veerdonk et al., 2008; Wevers et al., 2013). To determine the potential of TS-TT-Lam conjugates in stimulating immune responses in BMDMs we first characterized the response of primary bone marrow-derived dendritic cells (BMDCs) to a well-established Dectin-1 ligand, phospho-curdlan (P-curdlan; described in Chapter 2, "Materials and Methods") that is known to stimulate Dectin-1 responses. Indeed, P-curdlan, a pure and soluble linear β-glucan of high molecular weight (see materials and methods, Chapter 2), is a potent stimulator of Dectin-1 activity as described in our results in Chapter 1.

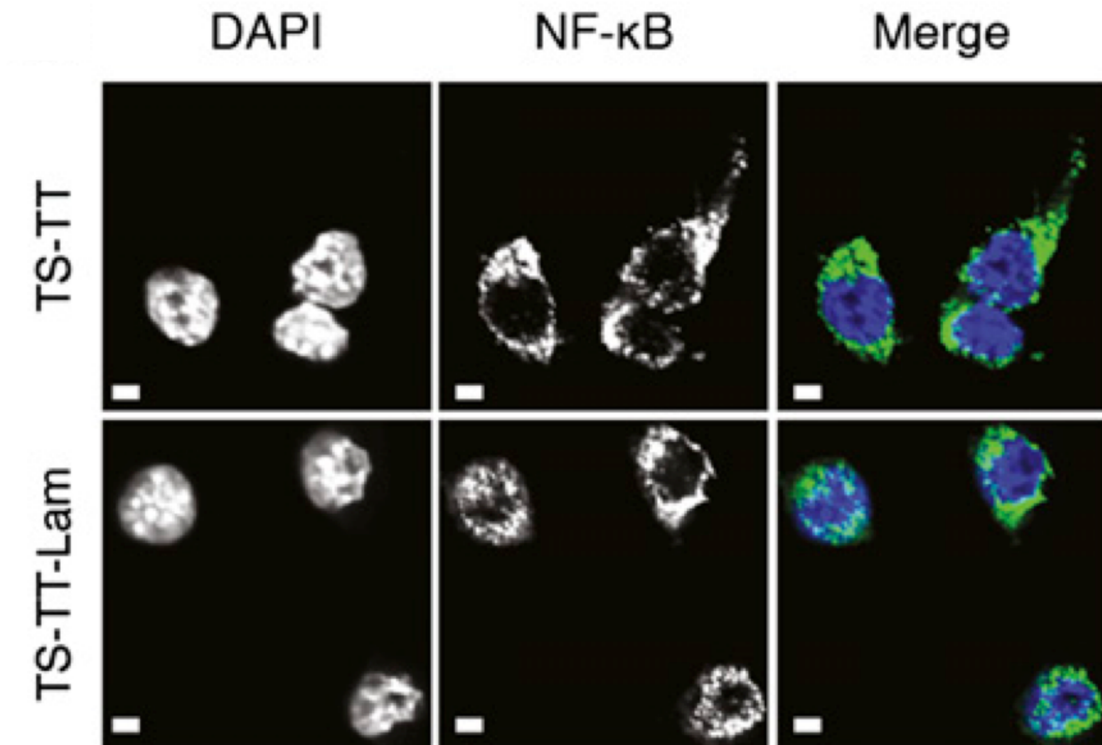

Figure 84: NF-κB Nuclear Translocation in RAW Dectin-1 Cells Treated by Vaccine Conjugates

RAW Dectin-1 macrophages were treated with TS-TT-AF546 or TS-TT-Lam-AF546 for 10 min at 37°C before fixation with 4% PFA. NF-κB translocation to the nucleus was determined upon an additional 20-min incubation of the cells following ligand incubation. Cells were fixed and permeabilized, and NF-κB was labeled using the rabbit anti-p65 Ab (Santa Cruz Biotechnology), followed by an anti-rabbit AF488 (green) Ab in parallel to DAPI (blue). Images were acquired on the confocal microscope. Data is representative of three similar experiments. Adapted with permission from (Lipinski et al., 2013). Scale bars, 2 μm.

Preparation of DCs was performed from bone marrow lavages of C57BL/6 mice that were

differentiated in presence of GM-CSF and IL-4. Dendritic cell (DC) surface marker characterization was performed by flow cytometry (Figure 85) with a series of Abs for different surface markers (Ags; antigens) described in Table 4 (See Material and Methods, Chapter 2).

Ag	CD11c	CD11b	CD80 (B7-1)	CD86 (B7-2)	F4/80	MHC-II
Fluorophore	PE-Cy7	Alexa Fluor 700	PE	FITC	FITC	eFluor 450
Final dilutions	1/1000	1/500	1/1000	1/800	1/500	1/1000
Isotype controls	Hamster IgG	Rat IgG2b, κ	Rat IgG2a, κ	Rat IgG2b, κ	Rat IgG2b, κ	Rat IgG2b, κ

Table 4: List of Fluorophores Used for Surface Marker Characterization of BMDCs by Flow Cytometry Analysis

Table lists the different surface markers (Antigens or Ags) tested in this study and the corresponding fluorophores for Antibodys used against these markers or antigens (Ags). The table also demonstrates the dilutions of each fluorphore used for the flow cytometry analysis, as well as the corresponding isotype control utilized for each Ab/flurophore (See materials and methods (Chapter 2) for further details of the methodology of the staining performed for this analysis. Adapted with permission from (Lipinski et al., 2013).

As expected for BMDCs, the majority of cells showed expression of CD11b and CD11c surface proteins. A small proportion (~ 9 %) of the culture expressed the macrophages marker F4/80, but the majority of the cells presented an immature DC phenotype with a moderate expression of MHC-II and CD86, and high expression of CD80. Additionally, Dectin-1 immunofluorescence

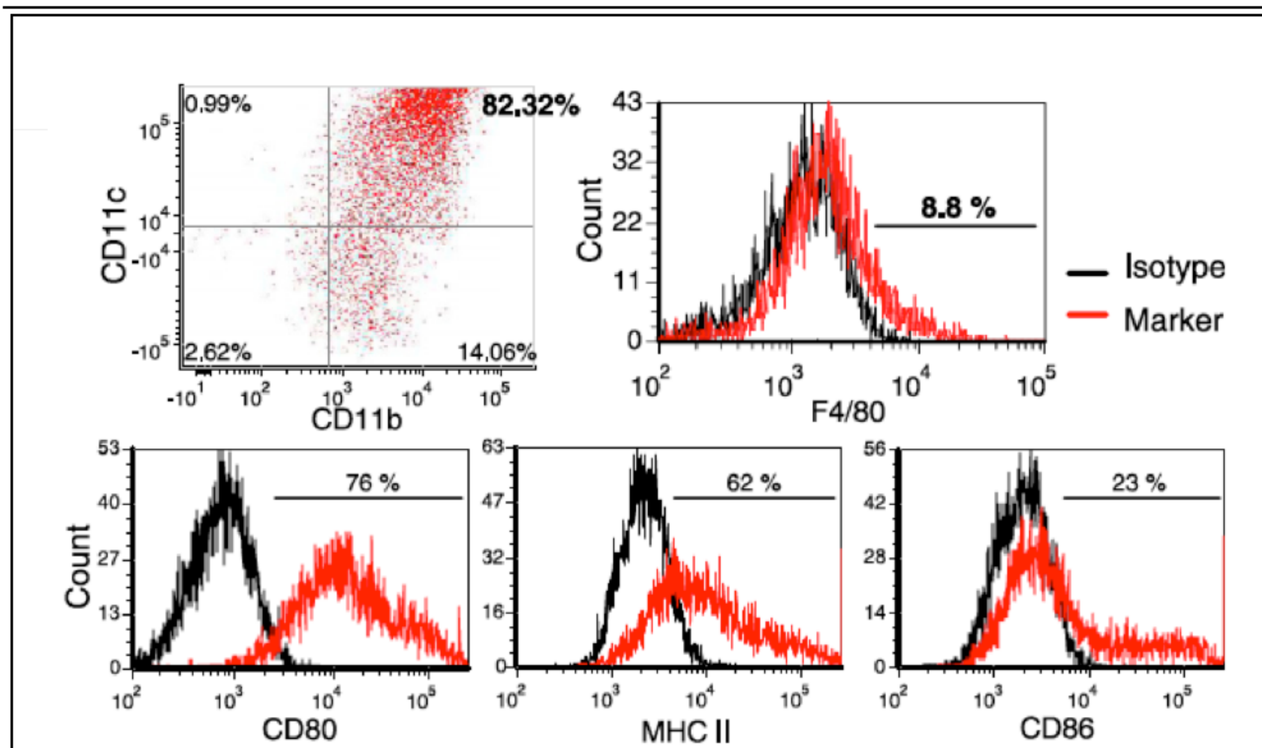

Figure 85: Flow Cytometry Profile Confirming the Phenotype of the Immature BMDCs

Histograms in black indicate isotype controls. More than 80% of CD11b$^{+/}$CD11c^{+} also express CD80. The moderate expression of CD86 and MHC-II indicates that these DCs are immature. The low expression of the macrophage

marker F4/80 indicates that the cell populations consist mainly of DCs. Data is representative of three independent experiments. Adapted with permission from (Lipinski et al., 2013).

staining further confirmed the expression of the β-glucan receptor, Dectin-1, in these immature BMDCs.

Cells plated on glass coverslips were fixed and non-permeabilized before addition of a rat anti-mouse Dectin-1 antibody (Figure 86). As described in **section 1.5.3,** the expression of Dectin-1 in primary DCs has been shown by several studies to promote several cell responses upon binding of particulate β-glucans. Additionally, we have shown in **(Chapter 3)** that DCs are activated in response to large soluble β-glucans such as P-curdlan (Brown, 2006a; Brown et al., 2003; LeibundGut-Landmann et al., 2007; Willment et al., 2005). We performed a series of experiments to test the responsiveness of immature DCs to Dectin-1 ligands. BMDCs were incubated for 10 minutes with P-curdlan and activation of SYK and nuclear translocation of NF-κB were examined. Using the anti-phospho-SYK (Y525/Y526) antibody, we verified P-curdlan was able to potently trigger SYK activation immediately following incubation with the ligand. Incubation of BMDCs with P-curdlan for 10 minutes resulted in the appearance and recruitment of a phosphorylated form of the kinase as shown by the strong near-membrane localization of phospho-SYK (as shown in the *middle panel* of (Figure 86). NF-κB translocation was analyzed after 10 minutes of ligand treatment followed by another 20 minutes of incubation. Immunofluorescence staining of NF-κB in BMDCs untreated or treated with the soluble Dectin-1 ligand, P-curdlan, demonstrated the responsiveness of DCs to Dectin-1 ligand **(**Figure 87**).** In unstimulated cells, NF-κB is dispersed throughout the cytosol, while cells incubated P-curdlan showed a strong localization in the nucleus as demonstrated by the DNA-binding fluorophores DAPI **(**Figure 87**).** We have therefore demonstrated so far that immature DCs express Dectin-1 and that they respond to treatment with a highly pure and soluble β-glucan (P-curdlan) by triggering SYK activation and NF-κB translocation in the nucleus.

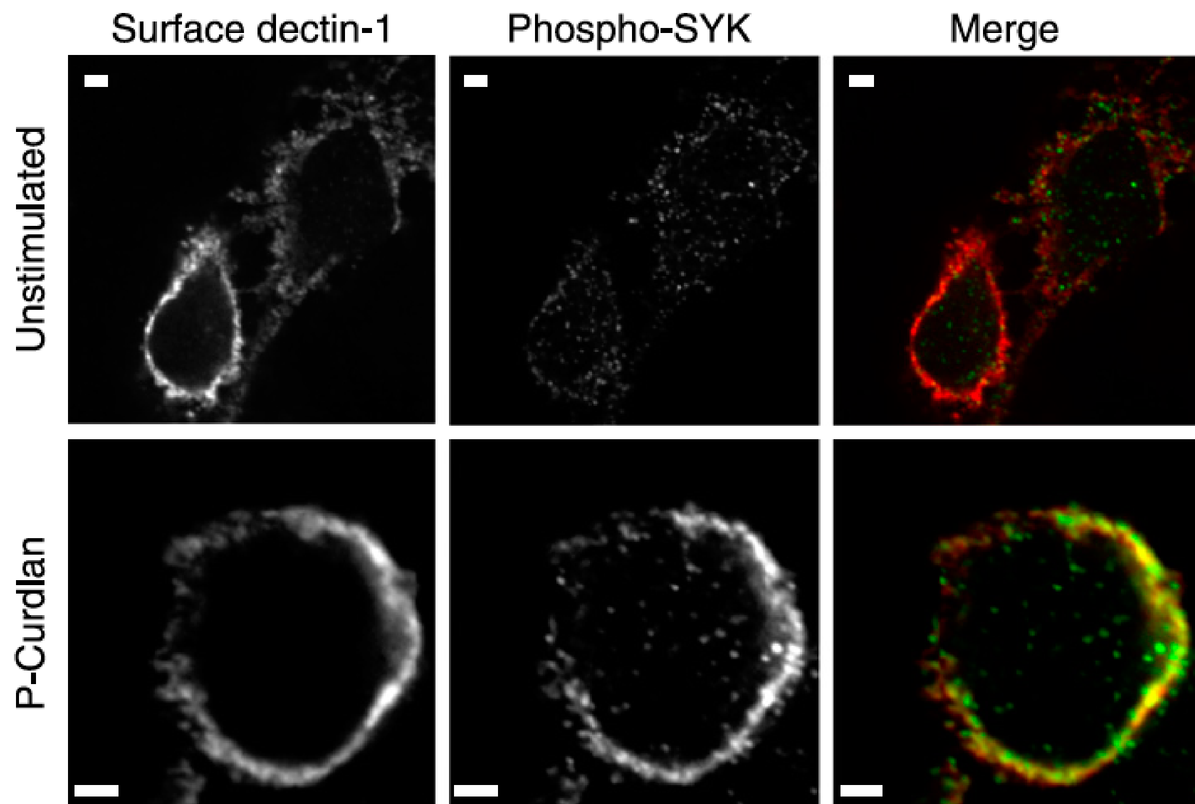

Figure 86: SYK Activation Following BMDC Treatment with Soluble P-curdlan or Vehicle Control (Unstimulated).

P-curdlan was solubilized at a concentration of 10mg/ml in PBS followed by resuspension at a concentration of 100µg/ml in hRPMI cell culture medium. Plain cell culture medium was used as a vehicle control for cells that were unstimulated. Primary DCs were then washed 2X with warm PBS and incubated for 10 min at 37°C with or without the ligand, and fixed with 4% PFA for 10 min at room temperature. Dectin-1 was immunostained first on nonpermeabilized cells with a rat anti-mouse Dectin-1 Ab followed by a Cy3-conjugated anti-rat Ab (red). Phospho-SYK (Y525/Y526) immunostaining was performed after permeabilization with 0.1% Triton X-100 using a rabbit anti-phospho SYK and AF488-conjugated secondary Ab. Images were acquired on a spinning disc confocal microscopy. Adapted with permission from (Lipinski et al., 2013). Scale bars, 2 µm.

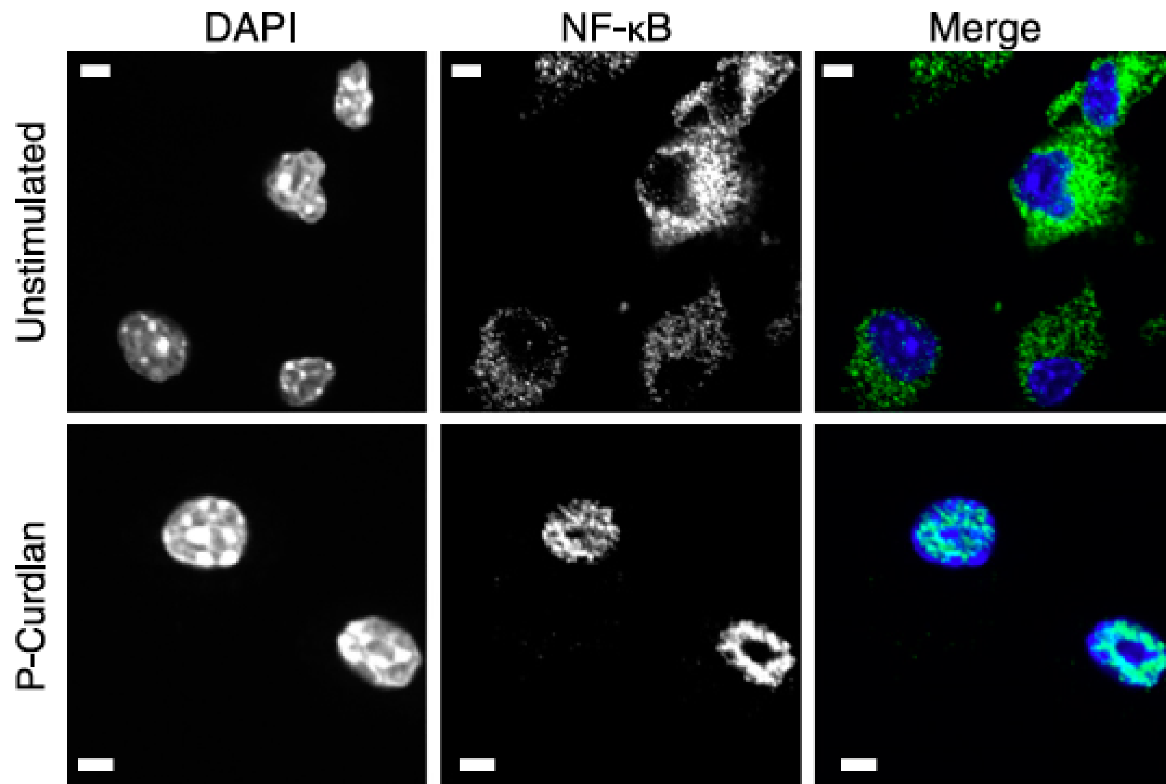

Figure 87: NF-κB Translocation in BMDCs in Response to P-curdlan

Following incubation without or with 100 mg/ml P-curdlan for 10 minutes 37°C, BMDCs were washed and incubated at 37°C for another 20 min, after which they were processed for immunostaining for NF-κB, as described in Materials and Methods (Chapter 2). Nuclear translocation of Dectin-1 is indicative of its activation. In the case of P-curdlan–stimulated cells cloclaization between NF-κB and DAPI revealed full nuclear translocation and activation of NF-κB. Images were acquired on a spinning disc confocal microscopy. Data is representative of five experiments. Adapted with permission from (Lipinski et al., 2013). Scale bars, 2 μm.

4.2.3. Activation of BMDCs and Secreted Cytokine Profile Upon Stimulation with Vaccine Conjugates

We next tested the *in vitro* immunogenic action of our vaccine conjugates on primary DCs by analyzing the secretion of a panel of cytokines, in addition to examining the appearance of surface markers specific to dendritic cell (DC) maturation (Figure 88). Immature BMDCs were treated with TS-TT or TS-TT-Lam, or LPS for 24 h before they were harvested. Cell supernatant was used for the determination of the secreted cytokines by specific ELISA assays, and the cells were gently scraped, stained and analyzed by flow cytometry for markers of maturation such as MHC-II and CD86. Figure 88 shows representative traces for the expression of CD86 and MHC-II and the quantification of the higher intensity section (M1) of the signals indicative of the

upregulation of these receptors during maturation. LPS is by far the strongest inducer of BMDCs maturation, as it promoted a significant increase in the expression level of CD86 and MHC-II after 1 d (one day) compared with control or vaccine treatment (Figure 88) Stimulation with the vaccine conjugates for the same period had milder effects on the up-regulation of the differentiation markers. TS-TT induced a small increase in the percent of cells expressing high levels of MHC-II compared with wild type (WT) (21.72 ± 2.28 vs. 16.37 ± 0.17, p>0.05) and of CD86 (24.08 ± 0.81 versus 20.17 ± 0.93, p≤0.05).

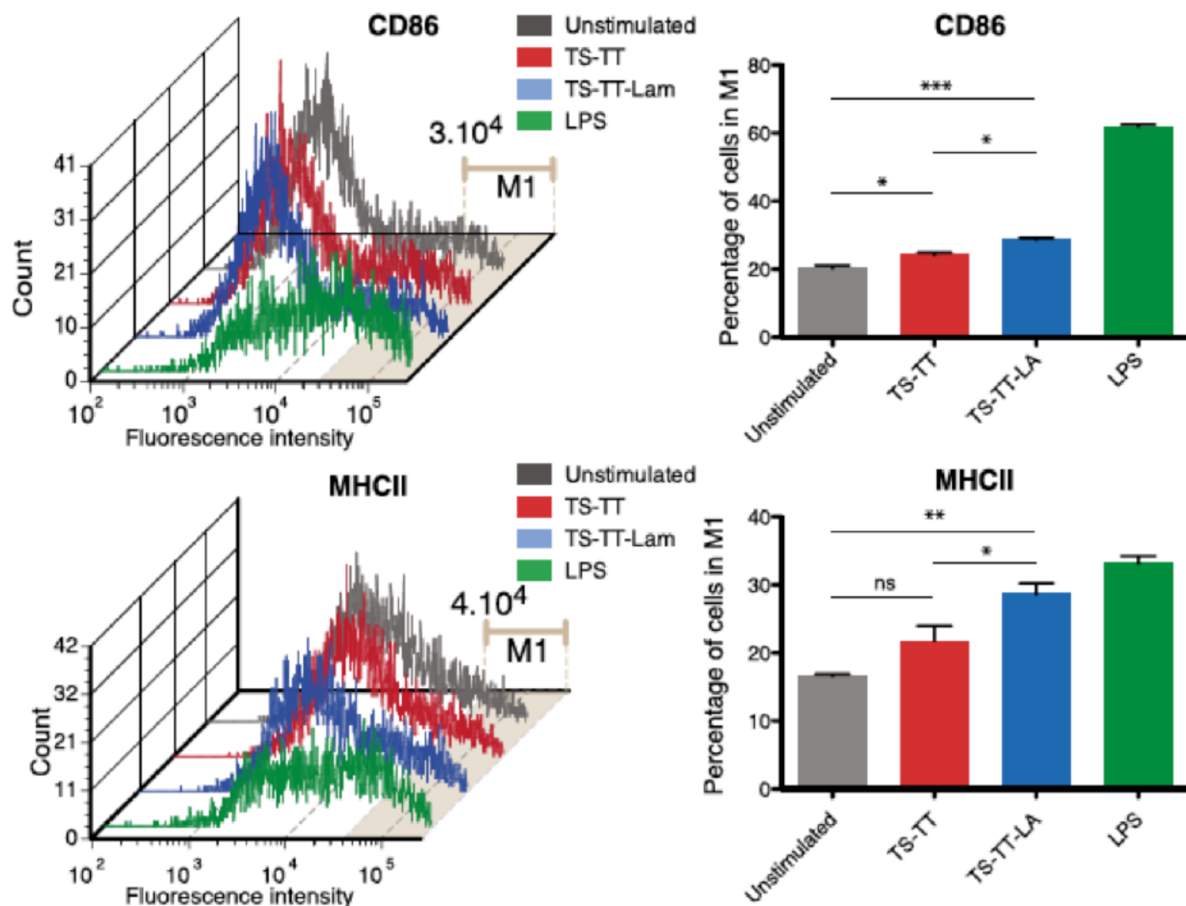

Figure 88: Flow cytometry Analysis of the Cell Surface Markers Expressed on BMDCs in Response to Vaccine Conjugates

BMDCs were treated for 24 h with TS-TT (red), TS-TT-Lam (blue), LPS (green), or just tissue culture medium (gray). BMDC activation was quantified (right histograms) by the increased expression of CD86 (top graphs) and MHC-II (lower graphs) using the percentage of cells appearing in the higher intensity level section of the graphs and marked M1. Data is mean ±SE of three independent experiments. ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$. ns: $p > 0.05$, calculated using Student *t* test. Adapted with permission from (Lipinski et al., 2013).

By contrast, TS-TT-Lam induced a more pronounced induction as supported by observations of a significantly greater fraction of cells expressing these markers in treated cells compared with

unstimulated (MHC-II: 28.79 ± 1.31 for TS-TT-Lam versus 16.37± 0.17 for unstimulated, p≤0.01 and CD86: 28.55 ± 0.59 for TS-TT-Lam versus 20.17 ± 0.93 for unstimulated, p≤0.001). Overall, these results indicate that the vaccine conjugates have the capacity to induce the differentiation of immature DCs.

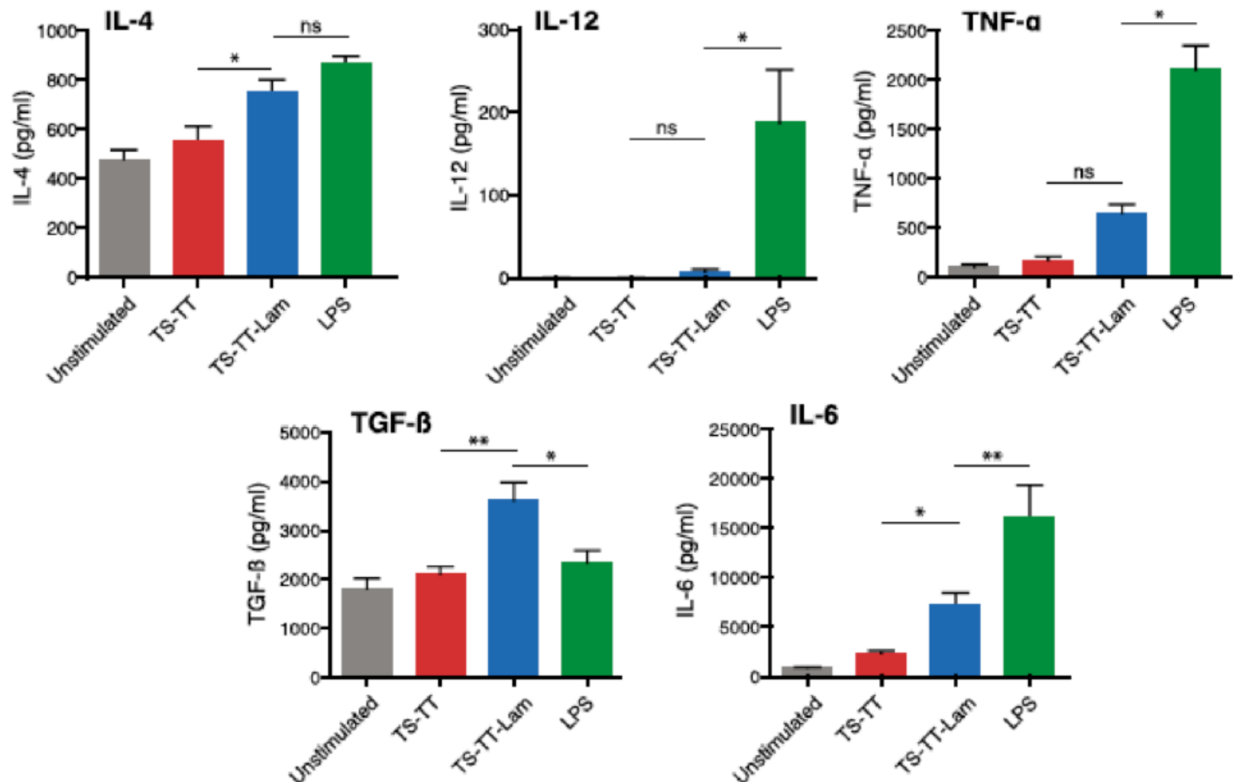

Figure 89: Cytokine Measurements Performed by ELISAs on BMDCs Treated for 24 h.

The level of IL-4, IL-12, TNF-α, TGF- β and IL-6 were determined by comparison with protein standards provided by the manufacturer and as described in Materials and Methods. Data is mean ±SE of three independent experiments. ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$. ns: $p > 0.05$, calculated using Student *t* test. Adapted with permission from (Lipinski et al., 2013).

We next looked at the secretion of several cytokines in the culture medium recovered from BMDCs after 24 h of stimulation with TS-TT or TS-TT-Lam. Figure 89 shows the ELISA quantification of the following five cytokines: IL-4, IL-12, TNF-α, TGF- β and IL-6. Stimulation with the TLR4 ligands LPS led to secretion of larger amounts of cytokines, especially of IL-4, IL-12, TNF-α and IL-6. Although TS-TT conjugate vaccine did not result in higher cytokine production when compared to untreated cells, TS-TT-Lam augmented the secretion of IL-4, IL-6 and TGF-β. Taken together, we show that conjugating β-glucans to the TS-TT vaccine boosted

its effects and especially raised the secretion of IL-4, IL-6 and TGF-β. Taken together, our results to date confirmed that TS-TT-Lam binds to and activates the β-glucan receptor, Dectin-1, in RAW macrophages expressing Dectin-1 (RAW Dectin-1) and also in BMDCs. In the primary cells, this activation promotes the differentiation of DC and more significantly induces the production and secretion of cytokines that will act to stimulate the immune system. Response curves resulting after each booster injection

4.2.4. Examination of the *in vivo* Immunization Potential of the TS-TT-Lam Vaccine Conjugate in Mice

The following experiments described in this section were performed by Dr. Tomasz Lipinski, in the Bundle Laboratory, at the Department of Chemistry, U of A (Edmonton Alberta). As mentioned above figures of this section were adapted with permission from Lipinski et al. (2013), Journal of Immunology.

Validation of the immunogenicity (immunization capacity) of the TS-TT-Lam vaccine was carried out in animal models (performed by Dr. Tomasz Lipinski in the Bundle lab.). Groups of 10 mice from three different mouse strains (BALB/c, C57/BL6, and outbred CD1) were vaccinated with either TS-TT-Lam or TS-TT conjugates four times (prime injection and three boosts. Serum samples were taken 10 days (10 d) after each booster injection and analyzed using ELISA for titers against the trisaccharide hapten attached to a heterologous carrier protein (TS-BSA) and against *C. albicans* cell wall phosphomannan (PMC). Results of the sera titers for the 3 mouse strains after each boost are shown on (Figure 90A), and demonstrate that antibody titers increased following each challenge.

Compared with TS-TT–vaccinated mice, TS-TT-Lam conjugate vaccination resulted in 5- to 10-fold higher titers against both synthetic Ag and cell wall phosphomannan (median values are indicated on the graph) in all tested mice strains (Figure 90B). Because the vaccine was derived from a single batch, each conjugate had exactly the same number of trisaccharide epitopes. Ab IgG subisotype distribution (Figure 91) was also influenced by the conjugate used during the vaccination protocol. Vaccination with TS-TT-Lam resulted in an increased level of IgG1 (~50%) and decrease of other subclasses, particularly IgG2a, in BALB/c and C57BL/6 strains. The changes in subclass distribution were significant in both BALB/c and C57BL/6 strains (p = 0.04676 and 0.00375, respectively), but the results in CD1 mice were inconclusive due possibly

to the low number of animals and high variation between individual mice.

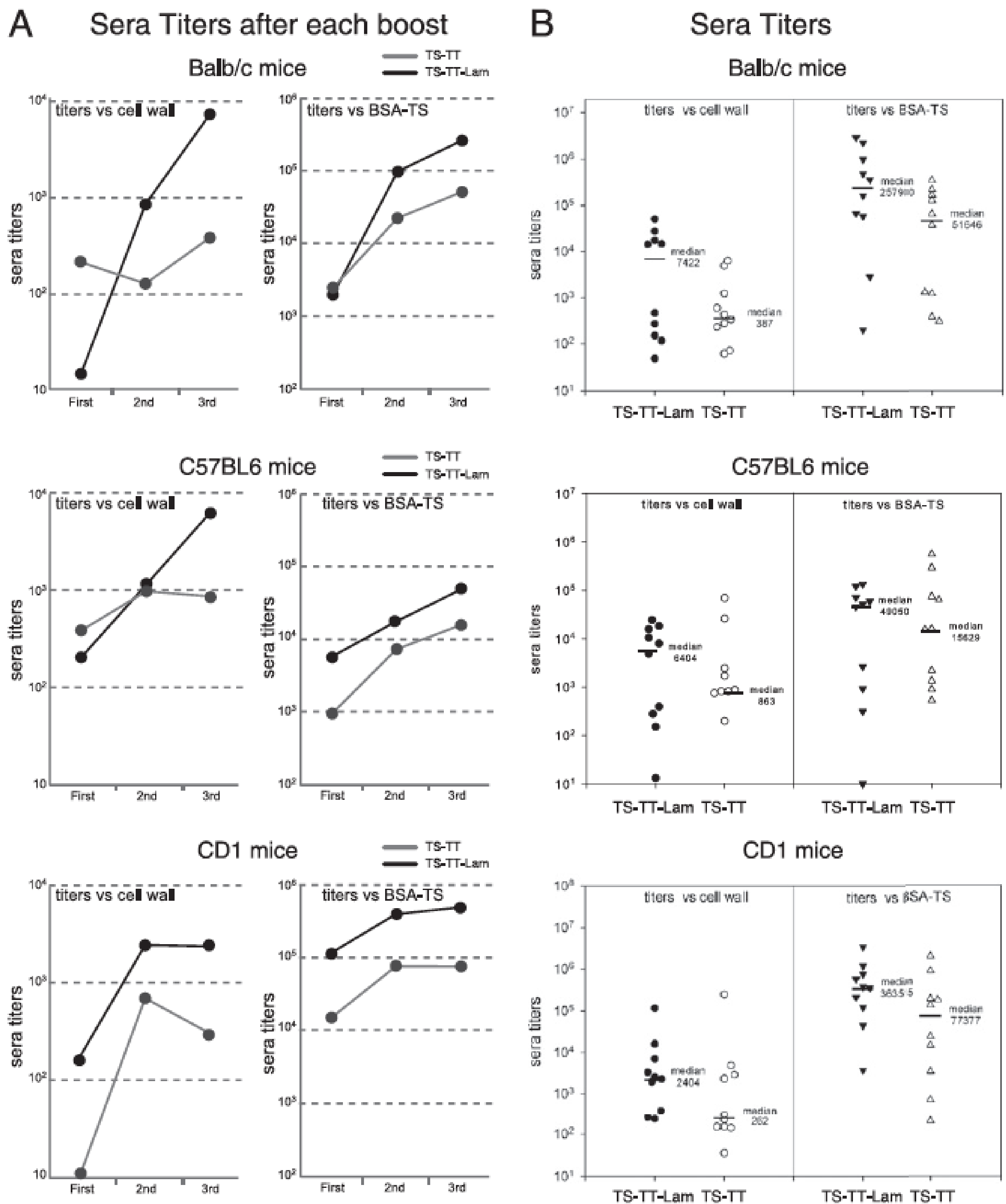

Figure 90: Characterization and Statistical Analysis of the Sera Antibody (Ab) Titers produced in Response to Vaccine Conjugates

(A) Statistical analysis of response curves resulting from data for average titer after each booster injection showed that curves for TS-TT-Lam and TS-TT conjugates are different for BALB/c (p = 0.0066 for BSA-TS, and p =

0.00012 for cell wall) and CD1 (p = 0.0432 for BSA-TS, and p = 0.0346 for cell wall) mice strains. **(B)** The end point (third boost) analysis included results from C57BL/6 mice and showed that TS-TT-Lam induced higher response to BSA-TS and cell wall in all tested strains (multivariate ANOVA test, p = 0.0336). Calculated effect size (Cohen's d) gave d = 0.448 for BSA-TS and d = 0.525 for cell wall that corresponds to moderate titer increase when vaccinating with TS-TT-Lam conjugate. Adapted with permission from (Lipinski et al., 2013).

In summary, we demonstrated that the novel laminarin- bearing vaccine conjugate tested in this study is recognized by and activates Dectin-1 cells. In the case of primary DCs, this activation leads to the production of several regulatory cytokines such as IL-4, IL-6 and TGF-β. Finally, animals challenged with TS-TT-Lam showed increased antibody (Ab) responses, in the form of IgG isotypes, directed against the β-mannan trisaccharide and the tetanus toxoid as compared with the TS-TT conjugate.

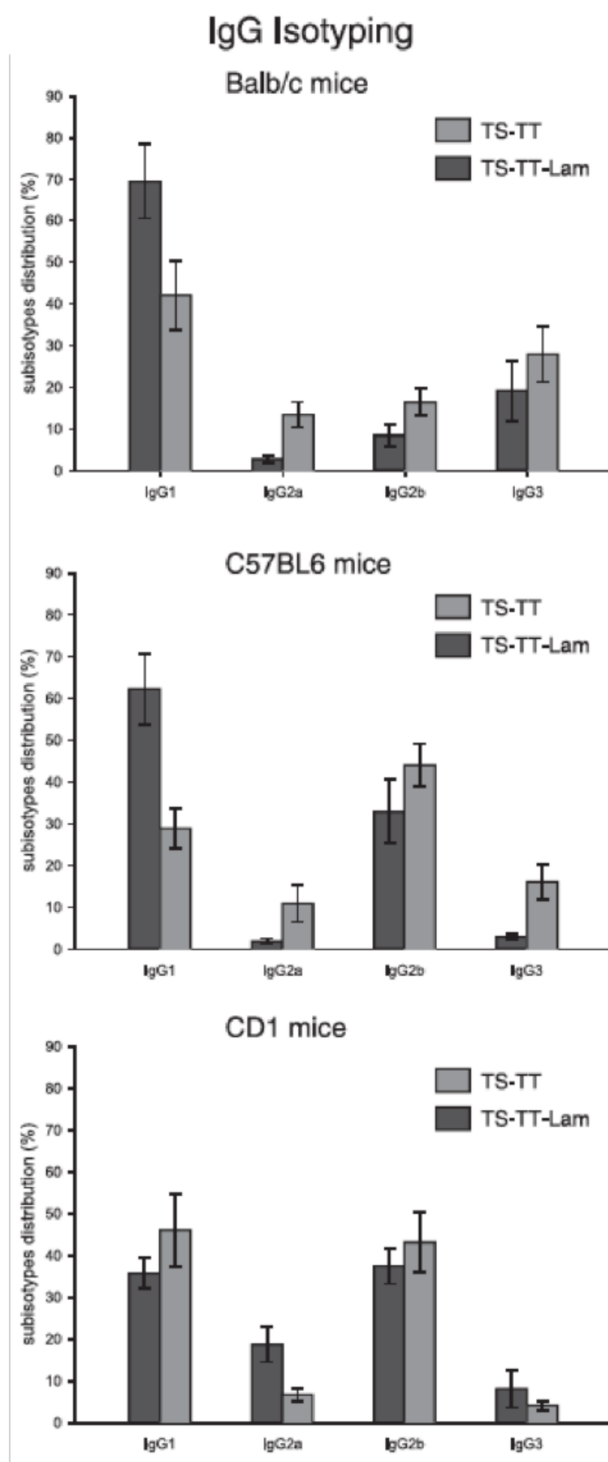

Figure 91: IgG Isotype Subclasses Analysis in End Point (Third boost) Mouse Sera.

Statistical analysis for this figure was performed by Dr.Tomasz Lipinski described in *Materials and Methods*. Adapted with permission from (Lipinski et al., 2013).

4.3. Discussion

Reproduced and adapted with permission from (Lipinski et al., 2013)

It has been previously shown that five or six synthetic β-mannotriose-peptide conjugates conferred good to excellent protection against *C. albicans* challenge if the glycopeptides were administered via antigen-pulsed dendritic cell vaccination (Xie et al., 2010). This was in sharp contrast to the poor immunogenicity of β-mannotriose-tetanus toxoid conjugate in mice (Lipinski *et al.,* 2011; Wu *et al.*, 2007; Lipinski *et al.*, 2011). These observations prompted us to consider dendritic cell targeting as a mean to enhance the antigenicity of the β-mannan-tetanus toxoid conjugate.

In addition to TLR agonists, ligands for the CLR Dectin-1 have been demonstrated to strongly activate antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages, and to promote innate and adaptive immune responses induced by these cells. Therefore β-glucans as ligands of Dectin-1 have been exploited for use as potential adjuvants to enhance immune responses conferred by vaccine conjugates (Leibundgut-Landmann et al., 2008). Indeed, recent studies have demonstrated β-glucans can function as potent adjuvants that target Dectin-1 to stimulate innate and adaptive immune responses (LeibundGut-Landmann et al., 2007; Leibundgut-Landmann et al., 2008; Li et al., 2010). In a recent study, Dectin-1 antibody mediated targeting of the protein antigen OVA to DC has been reported to yield superior response to the protein antigen OVA, whereas targeting via DEC205 did not (Carter et al., 2006). Here we elected to target DCs by attaching β-glucan–a ligand for the C-type lectin receptor Dectin-1–to the β- mannan-tetanus toxoid conjugate. This approach has the potential for a dual outcome: by elevating the response to the β-mannan of *C. albicans* while taking advantage of the proven targeting and antifungal response of β-glucan conjugates.

The ability of PRRs to influence adaptive immunity and to exploit them as vaccine targets has been of great interest recently. In particular, the study of Dectin-1 has provided new insights into the development of Th17 responses, which are Th responses central to host protection against fungal infection. Dectin-1 can induce several Th17-associated cytokines (e.g. IL-1β, IL-6 and IL-23, and TGF-β), and downregulate IL-12 (Th1-associated) induced by the TLRs (Dennehy et al., 2009), influencing the differentiation of activated CD4+ T cells. Indeed, stimulation of Dectin-1 in DCs with the purified agonist curdlan has been shown to induce the production of Th17

responses both *in vitro* (Osorio et al., 2008) and *in vivo* (Huang et al., 2010; LeibundGut-Landmann et al., 2007). Dectin-1-activated DCs by curdlan secrete a proinflammatory cytokine profile (including IL-6, TNF-α, Il-23, TGF-β but little IL-12) (LeibundGut-Landmann et al., 2007) that instructs the differentiation of T helper (Th) cells into IL-17–producing Th17 cells, as well as Th1 cells (Gringhuis et al., 2009a; LeibundGut-Landmann et al., 2007; Leibundgut-Landmann et al., 2008).

DCs targeting of the vaccine resulted in antibody titers that were 5-10 fold higher than those observed for a TT conjugate bearing only the β-mannan B cell epitope. The much higher antibody levels observed in Balb/c mice were also reflected in the production of protective β-mannan specific mAbs, which is in sharp contrast to numerous unsuccessful attempts when mice were immunized with the trisaccharide-TT conjugate (unpublished results).

The role of Dectin-1 in the binding and activation of innate immune cells was initially confirmed by *in vitro* experiments on a cell line of macrophages expressing a human isoform of Dectin-1. Stable Dectin-1 expressing cells were obtained after viral transduction of RAW 264.7 macrophages, and Dectin-1 was detected on their cell surface (Figure 26). This provided us with a system that allowed testing of ligand binding and activation of downstream Dectin-1 effectors in comparison with the wild-type cells (RAW WT). The laminarin containing conjugate (TS-TT-Lam) could bind to RAW Dectin-1 cells only, while the β-mannan only vaccine (TS-TT) did not show any significant interaction (Figure 80).

Laminarin, a small soluble ligand has been reported to bind Dectin-1 but it remains unclear whether the small β-glucan can produce specific Dectin-1 mediated responses. Goodridge *et al.* (Goodridge et al., 2011) recently reported that soluble β-glucans are also ineffective in triggering Dectin-1 activation. Because our TS-TT-Lam conjugate carries about 3 laminarin chains per vaccine molecule, we surmised that this oligovalent presentation, in contrast to the soluble form, could activate Dectin-1 by engaging a series of receptors. If so, this would suggest that even relatively short β-glucan sequences might be capable of activating Dectin-1, provided that they are presented in multivalent format. In accordance with this notion, we showed here that only the β-glucan containing conjugate (TS-TT-Lam) was able to stimulate effectors of the Dectin-1 pathway (Figure 81, Figure 82, Figure 83, Figure 84). Upon ligand binding, tyrosine 15 phosphorylation of Dectin-1 by Src-family kinases enables the recruitment of SYK on the

resulting phospho hemi-ITAM motif. In RAW Dectin-1 macrophages, TS-TT-Lam was able to 1) increase phospho-SFK in the vicinity of bound conjugates, leading to 2) activation of SYK and 3) inducing the translocation of NF-κB to the cell nucleus (Figure 81, Figure 82, Figure 83, Figure 84). Activation of Src-family-kinases, SYK and NF-κB only occurred in Dectin-1 expressing cells with the laminarin containing conjugate demonstrating the specificity of this Dectin-1 dependent signaling pathway to β-glucans. Following binding of the β-glucan conjugated vaccine TS-TT-Lam, it is taken up by RAW Dectin-1 macrophages resulting in stimulation of the cells.

To further confirm the immune stimulation potential of these glycoconjugates, we tested their effects on immature bone marrow derived dendritic cells. Dectin-1 expression and binding of the conjugate vaccine TS-TT-Lam on BMDCs were confirmed (Figure 88). The poor immunogenicity of the TS- TT conjugate can be explained by the inability of TS-TT to bind to macrophages or BMDCs presumably resulting in poor uptake and processing for presentation by DCs. To validate the potency of TS-TT-Lam in inducing an immune response, we determined its effect on primary innate immune cells. We used bone marrow derived dendritic cells grown in medium containing IL-4 and GM-CSF. After 8 days of culture, BMDCs were mainly immature as shown by the following flow cytometry profile: majority of $CD11c^{+}$-$CD11b^{+}$-$CD80^{+}$ and moderate expression levels of MHC-II and CD86 (Figure 88). In response to 24 h incubation with the TS-TT-Lam conjugate, a significant fraction of cells expressed higher levels of MHC-II and CD86 (Figure 89) compared to unstimulated conditions, indicating an induction of the differentiation of the DCs towards a more mature phenotype. These results are in agreement with reports demonstrating that laminarin and curdlan can activate immature dendritic cells (Yoshimoto *et al.*, 2005). In addition, curdlan was shown to induce cytotoxic T cell priming through the stimulation of IFNγ secretion by DCs (Leibundgut-Landmann et al., 2008). DCs are immune regulatory cells that can produce selective molecules that will influence the overall immune response (Banchereau and Steinman, 1998; Steinman, 2007; Tacken and Figdor, 2011).

Cytokines secreted by BMDCs stimulated with the TS-TT-Lam vaccine showed a modest increase in TNF-α and IL-12. Instead, we measured a significant augmentation of IL-4 secretion ($p \leq 0.05$), and a stronger induction of TGF-β and IL-6. IL-4 secretion favors the differentiation of

type 2 helper T cells, which in turn promotes antibody production B cell and isotype class switching (Paul et al., 2011). TGF-β is an important regulatory signal for Foxp3+ regulatory T cells (T_{reg}) (Bommireddy and Doetschman, 2007). However, when secreted in conjunction with IL-6, these cytokines initiate the differentiation of naïve T cell into IL-17 secreting T- cells called Th17 cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen and Stockinger, 2006). In contrast to the potential induction of T_{reg} by TGF-β, our measurements of increased IgGs titers following each vaccination boosts support the latter alternative. While, we did not investigated the exact nature of the T cell response triggered *in vivo*, our results indicate that T_{reg} cells are not being activated. The concomitant increased secretion of TGF-β and IL-6 by BMDCs stimulated by the laminarin containing vaccine suggests that T helper 17 cells are possibly activated. In the context of Dectin-1, several groups have reported the capacity of DCs to stimulate a T cell mediated immune responses (Carter et al., 2006; Leibundgut-Landmann et al., 2008) and can in addition activate Th17 T-helper cells through the secretion of IL-6 and TGF-β (Gringhuis et al., 2009b; Gringhuis et al., 2011; LeibundGut-Landmann et al., 2007). This Th17 immune response is promoted by Dectin-1 and leads to a more efficient anti-fungal immunity through the recruitment of neutrophils to the infection site (Curtis and Way, 2009; Gagliardi et al., 2010; Hung et al., 2011; Lin et al., 2009; Wuthrich et al., 2011b).

Compared to bi-component conjugate vaccines, such as OVA-Laminarin (Xie et al., 2010) or our previous TS- TT compound (Lipinski et al., 2011), the tri-component TS-TT-Lam promotes multiple immune pathways and enables a more robust immune response. Indeed, immunization of 3 strains of mice resulted in an improved vaccination documented on Figure 90 by higher IgG antibody titers against the *C. albicans* cell wall β- mannan and the tetanus toxoid. While carbohydrates are T cell independent antigens, recent studies of polysaccharide conjugate vaccines demonstrated their capacity to promote T cell dependent responses with induction of memory and IgM to IgG antibody class switching (Avci et al., 2011; Xie et al., 2010). The same antibody class switching was induced by the TS-TT-Lam following mice immunization indicating T cell supported B- cell differentiation and immune memory (Figure 90). Taken together, our results suggest that at least 2 distinct immunoregulatory mechanisms are stimulated by TS-TT-Lam: 1) an antibody response with isotype class switching, possibly induced by IL-4 secretion and 2) an proinflammatory response through the activation of Th17 cells induced by the concomitant production of TGF-β and IL-6. We conclude that our synthetic tri-partite

oligosaccharide conjugate vaccine equally promotes DCs activation, the induction of the Th17 response and potent immunization.

Several lines of evidence suggest that TS-TT-Lam will afford protection. The isotype shift that results form DC targeting of the vaccine is important. It has been shown by several indirect and direct measures, that in contrast to nonprotective mAbs, antibodies that protect mice against *C. albicans* more efficiently bind complement factor C3 to the yeast cell and that the mechanism of protection appears to be associated with enhanced phagocytosis and killing of the fungus (Han et al., 2001). Our analysis of the IgG isotype distribution specific for the beta-mannan trisaccharide, while variable across 3 strains of mice, shows a general shift toward IgG1 and significant levels of IgG2b with some IgG2a and IgG3. Literature data (Brekke et al., 1995; Neuberger and Rajewsky, 1981; Unkeless et al., 1988) indicates that IgG1 does not fix complement but IgG2b does as do the other two isotypes. Our own work (Xin et al., 2012) demonstrated that the glycopeptide in Xin *et al.(Xin et al., 2008)* when conjugated to tetanus toxin yields anti-trisaccharide antibodies with IgG2a and IgG3 subclass distribution and confers protection without adjuvant, presumably via the ability of the toxoid or its fragments to be presented by DCs. The literature also reports that monocyte responses to *C. albicans* are enhanced when *C. albicans* is opsonized by specific antibodies (Wellington et al., 2007).

Conjugate vaccines based on polysaccharides that combat pneumococcal pneumonia, bacterial meningitis caused by N. meningiiditis or Hemophilus influenza have been extremely successful in reducing disease (Ada and Isaacs, 2003). While these polysaccharides conjugated to carrier protein are successful in raising protective antibodies, smaller oligosaccharide epitopes are far less successful in conferring protection. Similar observations apply to tumour associated carbohydrate antigens. A notable success in raising high titer of protective antibodies has employed targeting of the innate immune system via Toll like receptors. The approach we have adopted here does not require the activation of TLRs and is consistent with reports of DC targeting of protein antigens.

Avci *et al*. have identified how glycoconjugate vaccines are degraded after injection and then presented by cells of the immune system (Avci et al., 2011). These conclusions show that polysaccharide is degraded to ~10 kDa fragments with attached peptide fragments and that these fragments are recognized by T cell receptors. When conjugates were created that maximized the

presentation of these essential elements a superior immune response was observed. This discovery raises the prospect of designing fully synthetic three component conjugate vaccines composed of the optimum B cell epitope, a T cell peptide possibility replacing carrier protein with the third component, a glycan able to direct the vaccine to DCs for effective presentation. A number of glycan receptors displayed on the DC membrane are known to modulate immunological responses (Geijtenbeek and Gringhuis, 2009). Consequently it might be expected that the most appropriate ligand for targeting may vary depending on the particular vaccine and B cell epitope.

Our combined approach of cellular immunology and *in vivo* vaccination challenges confirm the importance of DCs in the modulation of immune responses. By carefully defining the pathways stimulated by multicomponent vaccines, specific responses could be induced and regulated. While further characterization of the T cells activated by these compounds is required, we will first focus on optimizing vaccine conjugates. In the context of a practical *C. albicans* vaccine we consider a trisaccharide B cell epitope (Johnson et al., 2012) conjugated to the Fba 14 peptide (Xin et al., 2008) with attached multivalent β- glucan may provide an even more potent immunogenic compound.

Personal Conclusion (by Amira Fitieh):

CLRs are a vital part of the innate immune response, to fungal pathogens and playing a key role in the immediate recognition of these pathogens and shaping how the adaptive immune response develops. CLRs may synergize with other PRRs to coordinate the developing immune response, ensuring the appropriate discrimination of pathogens and activating the leukocytes needed to fight infection. CLRs may also contribute to the development of autoimmune disorders, and some CLRs may play a role in wound healing and the clearance of dead cells. Targeting CLRs has shown to be an effective means of improving vaccine efficacy, but may also work to induce tolerance in some situations. CLR targeting has also been used to improve anti-tumor responses. Targeting ovalbumin antigen to the human mannose receptor was shown to reduce tumor burden in human mannose receptor-expressing mice, when the mice were inoculated with ovalbumin expressing melanoma cells (He et al., 2007). In summary, various CLRs may be targeted to enhance the immune response to infectious agents or even tumors, and further research is needed to identify the most promising vaccine strategies. There are many CLR receptors with unidentified ligands. Identifying the ligands that trigger receptor signaling will provide new

targets for therapies, and will contribute to our understanding of how these receptors influence the immune response. Identifying other molecular players in CLR signaling will provide additional targets for immunomodulatory therapies. Finally, how can CLRs be targeted to improve vaccine efficacy, or to induce tolerance in autoimmune disorders. Promising work with DEC205 and in our case Dectin-1 has been performed, but can other CLRs be targeted to the same or greater effect? It will be exciting to see how these and other questions are answered in the years to come.

Several Dectin-1-mediated pathways have been shown to influence the production of polarising cytokines (reviewed in (Vautier et al., 2012; Vautier et al., 2010)). For example, it was recently shown that activation of specific NF-κB, subunits by Dectin-1 was important for IL-23 and IL-1β induction in response to curdlan (Gringhuis et al., 2011). This pathway could in turn be enhanced by Dectin-2, indicating that integrative responses between PRRs may influence the adaptive outcome (Gringhuis et al., 2011). Th17 cells play important roles for the host protection against fungal infection and are implicated in the pathogenesis of autoimmune and inflammatory diseases, such as arthritis (Diveu et al., 2008; Ghilardi and Ouyang, 2007; Ouyang et al., 2008). Dectin-1 triggering generally favours a Th17 response. However, the exact mechanisms linking Dectin-1 signalling with Th17 responses is unclear, but is thought to mainly depend on the ability of Dectin-1 to induce the production of cytokines such as Il-6, TGF-β, as well as Il-23 (LeibundGut-Landmann et al., 2007; Lipinski et al., 2013). Furthermore, the tendency of DCs stimulated via Dectin-1 to produce IL-23 as opposed to IL-12, might contribute to limiting Th1 cell differentiation and consequently reduce negative feedback on Th17 cell differentiation (LeibundGut-Landmann et al., 2007).

Chapter 5. GENERAL DISCUSSION & CONCLUSIONS

Results from this thesis provide unique insight into the molecular events that take place during the activation of the innate immune receptor Dectin-1. My data demonstrate that receptor clustering is the mechanism by which Dectin-1 is activated and that using a vaccine that targets Dectin-1 by clustering would enhance antifungal immunity. More specifically, studies from this thesis provide valuable insights into the effect of Dectin-1 clustering on both early and downstream signaling events and how Dectin-1 clustering could initiate beneficial immunomodulatory responses and enhance the immunogenicity of a vaccine containing multivalent β-glucan. These results provide deeper insight into the precise molecular mechanisms of how Dectin-1-dependent signaling is initiated when challenged by fungal pathogens, thereby enhancing our understanding of host protection against fungal infection.

The last few years have seen substantial advances in our understanding of antifungal immunity. Although a previously ‘‘neglected’’ area, these developments have promoted renewed interest in fungal infections and a rapid expansion of the field. Despite the important discoveries and successes of the last years, many of which were not discussed in this thesis, considerable challenges still remain. Developing vaccines that will be effective in immunocompromised patients is just one example. Furthermore, most attention has been focused on understanding the immunopathological mechanisms of the major invasive fungal pathogens that occur in immunocompromised patients in the developed societies, yet we still know little about the terrible endemic mycoses that affect thousands of individuals in the developing world or the common mucocutaneous infections that affect millions worldwide. No doubt exciting developments in these areas will be forthcoming in the future.

An increased interest in Dectin-1 (and other PRRs) involved in host defense against fungal infections has come at a time when there has been a resurgence of research into understanding the mechanisms underlying protective immunity to fungal pathogens. This interest has been largely influenced by the worrying rise in fungal infections over the last few decades, because of rising numbers of immunosuppressed patients, due to an increased population of individuals with AIDS, as well as an increase in the use of modern medical interventions such as chemotherapy treatment

of cancer, and organ-transplants. Furthermore, despite the availability of effective antifungal drugs, systemic fungal infections have unacceptably high mortality rates, and infections with these pathogens can cause substantial morbidity in otherwise healthy individuals (Brown et al., 2012a). It is therefore hoped that the better understanding of protective antifungal responses will ultimately lead to the development of novel approaches to boost host resistance.

Given the complexities of Dectin-1 signalling, along with the current ambiguities including different results in different cell types and subpopulations, further study is required to precisely delineate the downstream pathways in various cell types.

In recent years, there have been several studies, which have sought to decipher the Dectin-1 induced signaling pathways that underlie various cellular responses. It is worth noting that as evidence of cross-talk and branching in various systems is emerging, the traditional visualization of a linear pathways are in fact much more intricate (Drummond et al., 2011; Geijtenbeek and Gringhuis, 2009). This holds true for Dectin-1 induced signaling, which in some cases can trigger a response directly but in other cases requires cooperative signaling with MyD88-coupled TLRs. An added layer of complexity was revealed by recent evidence, which shows variability in Dectin-1 signaling in different myeloid cell types (Gross et al., 2006; Rosas et al., 2008). For instance, it has recently come to light that Dectin-1 signaling via the CARD9 pathway (described above **1.5.4**) is differentially regulated in myeloid cells (Gross et al., 2006; Rosas et al., 2008). This variability in Dectin-1 signaling in different cell populations is potentially a novel mechanism by which myeloid cells can be tweaked to regulate immune responses and may have implications for therapeutic uses (Gross et al., 2006; Rosas et al., 2008).

5.1. Limitation of the Study and Questions Open to the Future

Although extensive research on Dectin-1-mediated signaling has been done in the last few years, the exact mechanisms of Dectin-1 signal transduction by ligand stimulation are not yet understood. Despite my results, demonstrating that Dectin-1 is activated by clustering, a question that still remains open is what are the exact molecular features, other than the size of the ligand that induce the Dectin-1 responsible for fighting these infections. The molecular and structural features that enable Dectin-1 to promote signaling have still to be elucidated. Also there are several missing gaps in the downstream signaling pathways triggered in response to Dectin-1 activation, and some of the pathways are still not fully characterized. For instance, the exact

molecular mechanism of how signals are transmitted from the CARD9 ⁄ Bcl10 ⁄ MALT1 (CBM) complex to NF-κB is not yet described (see section **1.5.4**). However, if one looks into analogous well-defined pathways of other immune receptors that lead to canonical activation of NF-κB (e.g., TLR signaling pathway), as well as those pathways in lymphocytes that utilize CARMA1, an adaptor protein closely related to CARD9, it seems reasonable to consider that the direct interaction of MALT1 with the adapters TRAF2 and TRAF6 may lead to activation of IKK, which results in translocation of NF-κB to the nucleus where it influences gene transcription (section **1.5.4**) (Geijtenbeek and Gringhuis, 2009; Hara and Saito, 2009; Plato et al., 2013).

β-Glucans are well-known biological response modifiers that have been long known to function as immunostimulants with therapeutic effects on a variety of infectious diseases, and foremost on tumour growth (Brown et al., 2003; Novak and Vetvicka, 2008). Natural products useful in preventing or treating disease have been highly sought after throughout human history, however a major problem in characterizing many natural products is that they represent a complex mixture of ingredients, and each one may contribute to their bioactivity. Unlike most other natural products, purified β-glucans retain their bioactivity, which permits the characterization of how β-glucans work on a cellular and molecular level. Nevertheless the precise mechanisms of action of β-glucans have only recently been unraveled.

Furthermore, β-glucans unlike other immunomodulators demonstrate low-to-negligible toxicity. Accordingly, there was a time when faintly enlightened non-specialists in the field of traditional medicine, would commonly offer β-glucans as alternative remedies and immunomodulatory supplements. However, while the predominant pharmacological effects of β-glucans may be positive, their unfavorable side effects cannot be overlooked and have not been thoroughly investigated. At this time, few adverse effects have been described. For instance, intramuscularly administered β-glucan induces an inflammatory reaction and granuloma formation at the puncture site. This is a painful method of application, and the fact that β-glucan can be the cause of the inflammatory reaction itself represents a certain risk. At some point, β-glucans were criticized by many regulatory authorities. The main reasons being because of its insufficiently defined preparations and non-specific and/or complex effects. Moreover, the literature on immune responses to glucans can be quite confusing as what is observed for one preparation of glucan is often inappropriately extrapolated to all glucans. It can be presumed, however, that with improved knowledge of mechanism of action of β-glucans, this area will broaden. Indeed, the key

discovery of Dectin-1 as the major leukocyte receptor of β-glucans by Brown *et al.* in 2001 (Brown and Gordon, 2001) has greatly enhanced our understanding of some of the immunomodulatory effects of these sugar polymers. Moreover, fortunately, in the last ten years, extensive research in reputable laboratories in the fields of both β-glucans as well as Dectin-1, reached a phase where the basic mechanisms of glucan effects as well as the relationship between structure and activity have just started to unravel. However, the precise mechanisms of the recognition of β-glucans by Dectin-1 and induction of signaling and immune responses are still unclear to date.

Various natural sources, polymer character, methods of isolation, insolubility or limited solubility of preparations (and therefore unrealistic fractionation of sample), all result in the fact that each preparation of β-glucan, especially the particulate one, is necessarily heterogeneous. Eventhough in some cases the heterogeneity of β-glucan (from the point of view of molecular size, branching, and crystalline or amorphous structure) does not principally change the *in vivo* activities of β-glucans, for trustworthy pharmacological research as well as for regulatory authorities (such as the FDA) it represents substantial complications. It follows then that most preparations containing β-glucan – with the aim of avoiding complications – are classified as healthy food or nutritional supplements. As a result, the market is saturated with many products containing greater or lesser amounts of β-glucan that are often of questionable quality, with uncertain effects, and recommended by misleading advertisement.

Reliable research techniques allow the problems of heterogeneity and nonexistent standards of various natural β-glucans to be solved. A first possibility is to improve isolation techniques to obtain products with closely defined chemical composition and to use up-to-date physical chemistry methods and biophysical techniques for infallible identification and analyses of the physicochemical properties of these products. Chemically modified β-glucans, which due to their solubility can be easily fractionated represents a second possibility. Unfortunately, chemical modification of β-glucans could affect and even decrease the biological activity of some of these products. Recently, an attempt has been made to solve this problem by construction of semi-synthetic or synthetic probes that are suitable for exact immunological research (Adams et al., 2008; Jamois et al., 2005; Palma et al., 2006; Vos et al., 2007c). A general solution is the binding of short glucan containing β-(1,3) and β-(1,6) linkages to a polymer carrier of defined size and structure, for instance to a linear strand of polyacrylamide gel. A reasonable assumption is that

such “synthetic” β-glucans will interact with receptors of immunocompetent cells and elicit analogous reactions as natural β-glucan (Descroix et al., 2006). From the immunopharmacological point of view, such probes could not only be of great value to research in the β-glucan field but eventually could replace natural β-glucans.

Therefore, it seems now that intensive and up to date research in the β-glucan field will finally place β-glucans in the position, which was ascribed to them more than fifty years ago.

One aspect that is still unclear for the role of Dectin-1 in β-glucan-induced immune modulation, is the role of Dectin-1 in the biological activity of soluble β-glucans *in vivo*. Much of the data on Dectin-1 have been generated using particulate glucans, however some soluble β-glucans, such as laminarin are used as inhibitors. However, only recently there has been an increasing interest in studying the effects of soluble β-glucans, especially of those derived from edible mushrooms, such as Grifola frondosa, on Dectin-1-induced signaling pathways (Ishibashi et al., 2001; Masuda et al., 2009; Masuda et al., 2008; Masuda et al., 2012; Mayell, 2001). Hopefully, future studies of the effect of the biological effects of soluble β-glucans in Dectin-1 knockout mouse will help to resolve this issue.

Although Dectin-1 signaling has been extensively studied, downstream signaling of Dectin-1 is complex and not fully understood and many of the early steps are still unclear. However, insights into the signalling mechanisms into related SYK-coupled CLRs with hemITAM motifs such as, CLEC-2 (an NKCL receptor of ‘group V’ CLRs may help explain these gaps (Hughes et al., 2010a; Severin et al., 2011). Furthermore, given the complexities of Dectin-1 signalling, along with the current ambiguities including different results in different cell types and subpopulations, further study is required to precisely delineate the downstream pathways in various cell types.

In my PhD study in preparation for this thesis, we were able to show that the reason why some high molecular weight beta-glucans such as phospho-curdlan could activate immune responses, stems from its large size, and ability to cluster Dectin-1. This is in contrast to laminarin, a smaller soluble glucan, which fails to activate Dectin-1–mediated immune responses. Therefore, we have shown that it is the size of the β-glucan ligand rather than its state of solubility (whether it is particulate or not) that determines whether the ligand can activate or inhibit Dectin-1 signaling. (Figure 92). This is totally in contrast to a recent study by (Goodridge et al., 2011) that led to the belief in the Dectin-1 literature that only particulate glucans are capable of activating Dectin-1

(Figure 92). So indeed we are the first researchers to provide this data in the field of Dectin-1 signaling and how Dectin-1 is activated by truly soluble Dectin-1 ligands. Indeed one of the advantages we had for this study was receiving highly soluble and pure β-glucan ligands for dectin-1 from the Bundle group. These glucans were able to give us precise results about the exact mechanisms of how Dectin-1 is activated by β-glucans, as these compounds were highly water-soluble, unlike the crude, impure and largely insoluble preparations of β-glucan that have been used in previous studies yielding misleading results and controversies in the field of β-glucans and Dectin-1. Figure 92 shows a schematic of our final model that we concluded from this thesis study for the molecular mechanism of Dectin-1 activation by β-glucans (Figure 92).

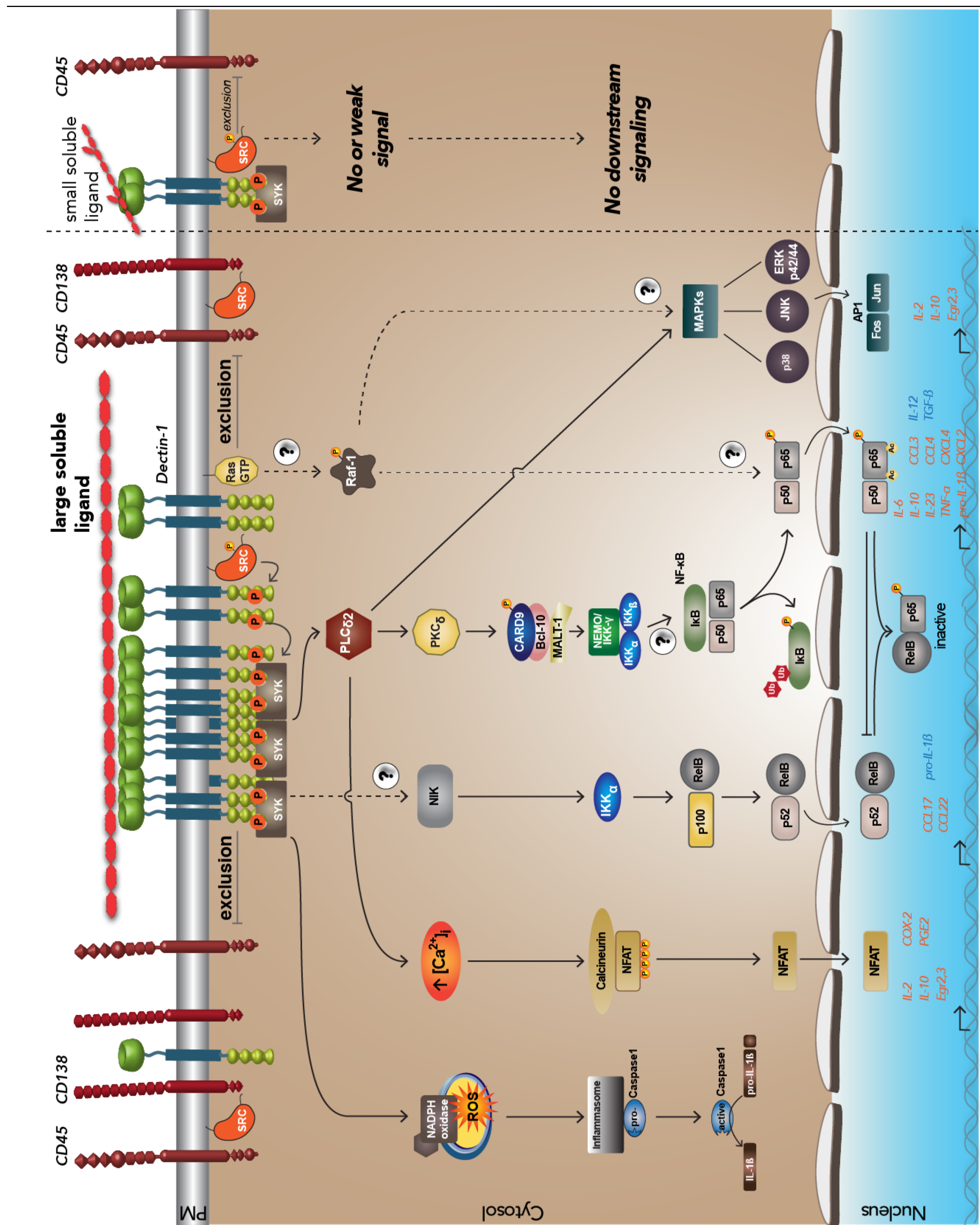

Figure 92: Proposed model for Dectin-1 signal transduction.

Similarly to the model illustrated in Chapter 1, Figure 16, our results demonstrated that soluble ligands are also capable of triggering downstream Dectin-1 signaling. However, small ligands (such as laminarin) will not be able to produce a complete immune stimulation, the strength of the activation of membrane proximal molecules appears

insufficient to be promoted downstream to the MAPK and NF-κB complexes. Larger soluble ligands (such as P-Curdlan or BSA conjugates carrying more than 10 laminarin) induce the formation of larger Dectin-1 clusters (about 150 nm compare to 66 nm at rest), which we propose result in the exclusion of phosphatases (CD45 and CD138) that initiates a stronger activation of SFKs and SYK resulting in a more complete stimulation of the MAPK and NF-κB pathways, leading to immunomodulatory effects. (Figure was conceived and designed by Amira Fitieh, and graphics were done in Adobe llustrator by Dr. Nicolas Touret).

5.2. Future Perspectives

To successfully fight off a fungal infection, our body has to coordinate a complex response involving signalling from multiple pathways and cell types, in a precise temporal window. The recognition of carbohydrate structures in pathogens through specific PRRs (i.e., Dectin-1 and 2) induces DC maturation into potent immuno-stimulatory cells endowed with the capacity to efficiently prime appropriate adaptive immune responses. While relationships between pairs of PRRs are becoming well defined, it is likely that antifungal immunity requires a precise cross-talk of multiple PRRs. This complex cross talk is very difficult to understand without a holistic, systems biology based approach. Thus integrating complex datasets coming from multiple sources with curated pathway information will be allowing to more definitalvely decipher the intricacies of the combination of multiple PRR pathways activation.

To further enhance our understanding of activation of anti-fungal immune responses, additional analysis of Dectin-1 signal transduction mechanisms are nessecary (Figure 93) and are detailed in the paragraphs below.

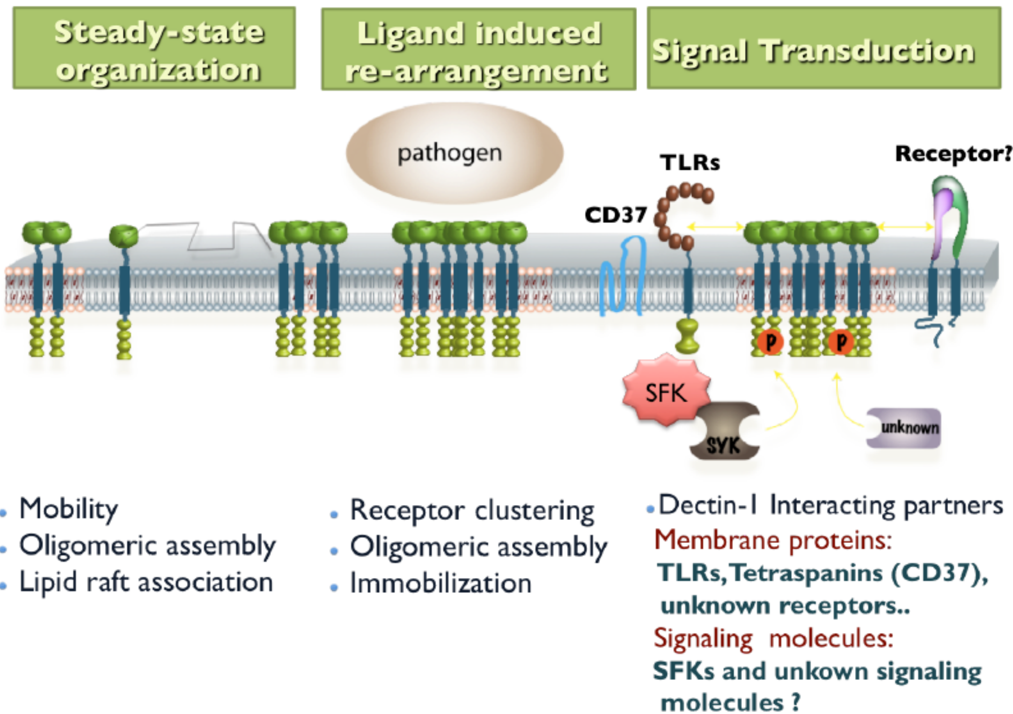

Figure 93: Future perspectives.

Future directions include the characterization of the dynamic behavior of Dectin-1, the examination of its relationship to know membrane organization centre (lipid nanodomains, actin cytoskeleton, tetraspanin web,…), the identification of co-signalling receptors or molecules.

Enhancing Vaccine Efficacy

CLRs, especially those limited to particular DC subsets, have been of interest as vaccine targets. One approach has been antibody-mediated targeting of these receptors and Dectin-1, Dectin-2, mannose receptor and DC-SIGN have all been examined in this way and shown to variably enhance CD4+ and CD8+ T cell responses to experimental antigens. However, any direct contribution of these receptors to the subsequent immune responses has not been examined. Another strategy has been to use carbohydrate ligands of CLRs to drive vaccine responses. This has been tested with several carbohydrates, including various mannose-based ligands aimed at targeting the mannose receptor and DC-SIGN (although the actual receptor specificity in these studies is always questionable) as well as β-glucans aimed at targeting Dectin-1. In all cases, such as in antibody-mediated targeting, the complexing of these antigens with carbohydrates enhanced CD4+ and CD8+ T cell responses. Notably, β-glucan particles have also been used to

systemically target macrophages via oral administration, but in this case complexed with small interfering RNA.

There has also been considerable interest in using these carbohydrates to develop antifungal vaccines, for which there is a desperate need as there are currently no clinically available vaccines. Several approaches have been tested including, for example, a conjugate vaccine composed of the β-glucan laminarin fused to diphtheria toxoid. This vaccine induced anti–β-glucan antibodies, which were protective against a range of fungi including *Candida*, *Aspergillus* and *Cryptococcus* species. Very recently, the particulate β-glucan curdlan was shown to act as a TH17-polarizing adjuvant when used with a novel epitope from *C. albicans*, which provided protection against infection with several species of *Candida*. The acquisition of vaccine immunity using a live attenuated pathogen, *Blastomyces dermatitidis*, also required Th17 immunity—a response that was induced through MyD88 but not Dectin-1, although the role of other CLRs was not examined. Mannose-based antifungal vaccines have also been tested in various models with various degrees of success, although mannosylation notably was essential for driving effective T cell responses against a recombinant *C. neoformans* protein.

Dectin-1 Dynamics during Signal Transduction

Our work has identified the molecular reorganization Dectin-1 is undergoing following large soluble ligand stimulation. The next step after determining that Dectin-1 activation occurs by clustering is to analyze the mobility of individual Dectin-1 molecules in the plasma membrane. The goal will be to determine whether the Dectin-1 clusters, at steady state or following ligand engagement are static or resulting from the dynamic metastable complexes. We will also follow the binding of fluorescently labeled Dectin-1 ligands and monitor their subsequent effects on receptor dynamics and mobility in intact Dectin-1 clusters. These unique readouts are essential to our understanding of the mechanism of Dectin-1 activation and signal transduction. To monitor the membrane organization, clustering state and mobility of the Dectin-1 receptor upon ligand stimulation, live cell imaging of Dectin-1 could be performed by Single Particle Tracking (SPT) PALM.

Decreased mobility of the receptor in the clusters could reflect interaction with other membrane proteins, signaling molecules or cell-structures. Stationary period could also indicate interaction with lipid rafts, tetraspanin microdomains or with the cytoskeleton (e.g., actin). The high spatio-

temporal resolution of SPT-PALM could help to discern any of these outcomes and also give us further details of the exact sequence of events taking place after Dectin-1 engagement with its ligand.

Dectin-1 Co-signaling Partners

Results from this thesis demonstrate that receptor clustering prmoted by ligand binding andtriggers signal transduction. Therfore it is of great interest to determine the mechanisms of Dectin-1 signal transduction by clustering i.e. what happens during and following Dectin-1 clustering that triggers subsequent intracellular signaling. We need to further investigate the function of associated receptor, membrane proteins and other signaling players and effectors in Dectin-1 signal transduction. Relevant to this goal is identifying new signaling players involved in Dectin-1 upstream signaling that could be play key roles in triggering membrane-proximal signaling events.

For instance determining which SFK member is important for initiating Dectin-1 signaling by phosphorylating the hemITAM motif Lyn, Fyn or Hck e.t.c. Dectin-1 clustering might mediate the activation of these SFKs to subsequently trigger the signaling cascade as demonstrated in the study by Goodridge *et al.* (2011). Also, it is not yet understood whether Ras GTPase or Rho GTPases are involved in Raf-1 activation or another small GTPase or membrane proximal signaling effector, which could be activated by Dectin-1 clustering. I suggest to develop biochemical techniques, such as co-immunoprecipitation of Dectin-1 to identify interacting proteins by mass spectrometry novel Dectin-1 interacting partners.

Investigating the nature of the Dectin-1 signaling complexes would be of great significance to understand how Dectin-1 could induce signaling in response to receptor clustering. As mentioned in the introduction, Dectin-1 is known to interact with other receptors on the plasma membrane that enhance Dectin-1 signaling. These include various TLRs (e.g TLR2) and the C-type lectin receptor, DC-SIGN. Although a functional collaboration of Dectin-1 with these receptors has been described, there is no evidence for physical interaction. However, Dectin-1 has been shown to physically interact with the fungal PRR Galectin-3, and the tetraspanins, CD37 and CD86. These interactions raise the possibility that Dectin-1 could be part of a tetraspanin-mediated supramolecular signaling complex at the cell surface, which may involve several different PRRs. Moreover, signaling effectors such as the SYK tyrosine kinase, are recruited to Dectin-1 after its

stimulation. We have also seen recruitment of SYK to Dectin-1 clusters. It is not yet fully understood though, how SYK is recruited to monophosphorylated Dectin-1. Src family kinases (SFKs) have been proposed to cause the phosphorylation of Dectin-1 hemITAM motif in the cytoplasmic tail. Indeed, we have observed the inhibition of Dectin-1 mediated SYK activation using the SFK inhibitor PP2. It is not yet known which of the SFKs (Hck, Src, Lyn or Fyn) triggers Dectin-1 signaling and how they activate Dectin-1. Examining this most upstream signaling event could give valuable mechanistic insights into how Dectin-1 is activated. To elucidate the nature of these Dectin-1 signaling complexes formed in response to Dectin-1 clustering, further examination the recruitment of known Dectin-1 partners (SYK, SFKs, TLR2, DC-SIGN) to the Dectin-1 clusters in real time and determine their exact role in Dectin-1 activation. Because the type of interactions that occur between receptors and signaling molecules are restricted in time and space, the high spatio-temporal resolution of single molecule tracking is well suited for the analysis of these events. Unique single molecule imaging approaches in two fluorescent channels simultaneously could be employed to analyze the relationships between Dectin-1 and these proteins. Simultaneous imaging of two fluorophores, one reporting Dectin-1 and the other reporting one of the Dectin-1 interacting proteins of interest, could be performed by dual-label PALM or dual SPT-PALM, using a dual-channel/dual- excitation microscope such as the one in the lab. For instance, interaction between Dectin-1 and the various players will be first studied in live cells in the absence of ligands. Next, Dectin-1 will be stimulated using the various conditions described in aim 1. The frequency and the duration of the interaction between Dectin-1 and associated molecules will help us define the exact steps underlying Dectin-1 signal transduction. Finally the recruitment of Dectin-1 and its partners to lipid microdomains (lipid rafts) or tetraspanin microdomains (CD37) as well as their interaction with cytoskeletal structures will be investigated. These microdomains and structures have been proposed to act as platforms that concentrate and anchor molecules in signaling complexes. A hypothetical outcome would be that, at the steady state, Dectin-1 is mobile and that upon clustering, it associates with the tetraspanin CD37 or lipid rafts and becomes immobile in these signaling platforms. The interaction of Dectin-1 with the different proteins as observed by SMI will be confirmed biochemically by their co-immunoprecipitation with Dectin-1. Indeed I have developed protocols in collaboration with the lab of Dr. Richard Fahlman (Department of Biochemistry, Faculty of Medicine & Dentistry, U of A, Alberta) followed by identification of the unknown interacting

proteins by mass spectrometry and we have obtained interesting preliminary results (Data not shown).

Signaling Trafficking Downstream of Dectin-1

In addition to better defining the dynamic events taking place during ligand stimulation and the intial activation of the receptors, special interest should be given to how the strength of the most Dectin-1 proximal signaling events is reflected in the conditional activation of key transcription factors.

As mentioned in section (1.5.4.1) Dectin-1 activation induces both the canonical (p65, p50, c-Rel) and non-canonical (ReLB, p52) NF-κB pathways (Figure 92). The differential activation and association of various NF-κB family subunits leads to the selective activation of specific cytokine programs (Geijtenbeek and Gringhuis, 2009; Gringhuis et al., 2011). I propose that Dectin-1 activation by ligands of different size or complexity, could affect this differential activation and thus skew the immune response towards certain responses. To test this, the activation of the different NF-κB subunits in response to Dectin-1 clustering should be investigated.

Dectin-1 is unique in that it can induce immune responses independent of other immune receptors, yet the capacity of the fungal pathogen to initiate an efficient immune response through its receptor Dectin-1 is not yet understood. Also the immunomodulatory role of β-glucans is not fully understood. Although my thesis concludes that Dectin-1 is activated by clustering, and provides insights into the effect of Dectin-1 clustering on upstream and downstream signaling events, as well as on enhancing the immunogenicity of a vaccine containing multiple β-glucan (Figure 92). However, my results still do not provide deep analysis into the qualitative effect of different levels of clustering in triggering anti-fungal immune responses. Therefore it would be interesting to analyze the effect of different levels of Dectin-1 clustering on the type and strength of the immune response produced, which could provide the framework for better antifungal vaccine design especially for multicomponent vaccine conjugates. Investigating the effect of different levels of Dectin-1 clustering on the nature and type of the cytokine profile secreted by innate immune cells especially DCs is of specific importance in this regard. Identifying the nature of the Dectin-1 cytokine profile produced in response to Dectin-1 clustering by different ligands will provide insights into the type of immune response mounted against the fungal pathogen. Qualitative and quantitative analysis of the specific cytokine profile produced in response to

different-sized β-glucans could be performed on RAW Dectin-1 cells, or on primary cultures of BMDCs or BMDMs generated from mice, using larger scale multiplex ELISA kits. Large scale multiplex ELISAs could identify at least 13 cytokines at a time, and could therefore determine the precise cytokine signature produced by each ligand. This will hopefully provide insight into the type of the immune response produced by the different ligands and what properties (branching, size or number of molecules on pathogen) of the β-glucan enable it to produce an efficient and specific immune response.

5.3. Significance of Work Provided by This Thesis

Fungal infections represent a serious and widespread problem especially in immunocompromised patients. Current anti-fungal drug regimens are unable to cure patients with fungal infections Research presented in this thesis, interestingly provides important and valuable mechanistic insights into the early Dectin-1 signaling events responsible for the destruction and clearance of fungal pathogens in the host when challenged by these fungal pathogens. More specifically, results from my PhD research, which are demonstrated in this thesis, determine, at the molecular level, how Dectin-1-dependent signaling is initiated when stimulated by soluble β-glucans. Accordingly, findings from this thesis will ultimately establish a foundation for the better development of clinically approved antifungal vaccines and novel antifungal therapeutic strategies, which are hopefully more effective in treating and protecting patients susceptible to fungal infections. Furthermore, studies provided by this thesis generally offer a beneficial basis and framework for a better understanding of how innate immunity is activated by pattern recognition receptors (PRRs).

Finally, my results provide valuable insights into the molecular mechanisms of the immunostimulatory actions of medicinal soluble β-glucans, which are thought to be mainly mediated via activation of the β-glucan receptor, Dectin-1. A better understanding of the immunomodulatory effects of soluble β-glucans, which are widely used as immune boosters and nutritional supplements, would potentially lead to the development of pharmacological compounds designed to target Dectin-1 and modulate our immunity.

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