Characterization of the Endocytosis of Dectin-1, an Innate Immune Receptor

by

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ABSTRACT

Fungal pathogens are recognized by Dectin-1, a pattern recognition receptor expressed on mammalian innate immune cells. Dectin-1 detects β-glucans, which are polymers of glucose that are a main component of the fungal cell wall. While purified, soluble β-glucans have been used in the clinic as immunostimulatory agents, their mechanism of action is unclear. Thus, we sought to elucidate the effects they have on the signaling and endocytosis of Dectin-1. We expressed Dectin-1 in murine macrophages, and developed immunofluorescence and reversible cell surface biotinylation assays to study its endocytosis. Three soluble β-glucans, laminarin, soluble Wellmune whole glucan particle, and phosphorylated curdlan, were used as ligands to the receptor and were found to trigger Dectin-1 signaling, albeit to various extents. We observed that the rate of Dectin-1 internalization increased upon stimulation with the ligands, and this uptake was determined to be dynamin- and likely clathrin-dependent. Upon internalization, Dectin-1 trafficked to early endosomes and the recycling compartment, but not to lysosomes, promoting the return of internalized receptor to the cell surface. Dectin-1 signaling was not required for its endocytosis, although endocytosis may regulate Dectin-1 signaling events. Our findings provide a molecular understanding to the therapeutic activity of purified, soluble β-glucans and offer considerations for the design of new vaccines.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AF	Alexa Fluor (fluorophore)
AP-1	activator protein 1 (transcription factor)
AP-2	adaptor protein 2 complex
BMDMs	bone marrow-derived macrophages
BSA	bovine serum albumin
BSA-*n*-lam	BSA-*n*-laminarin, a conjugate molecule of *n* laminarin molecules covalently-linked to BSA
CARD9	caspase recruitment domain-containing protein 9
clathrin HC	clathrin heavy chain
CLEC-2	C-Type Lectin-Like Receptor 2
CME	clathrin-mediated endocytosis
CRD	carbohydrate recognition domain
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide (vehicle for dissolving pharmacological inhibitors)
DNA	deoxyribonucleic acid
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
ERK	extracellular signal-regulated kinase
Fc receptor	receptor that interacts with the fragment crystallizable region of antibody molecules
GFP	enhanced green fluorescent protein
HA	human influenza hemagglutinin epitope tag
hDectin1	human Dectin-1
hemITAM	hemi (half)-ITAM
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HRP	horseradish peroxidase
IB	immunoblot
IF	immunofluorescence staining
IKK	IκB kinase complex
IL-	interleukin- (a cytokine; IL-2, IL-6, etc.)
ITAM	immunoreceptor tyrosine-based activation motif
IκB	inhibitor of κB
JNK	c-Jun N-terminal kinase
kDa	kiloDaltons

lam	laminarin
LAMP	Lysosome-Associated Membrane Protein
LB	Luria-Bertani medium
LPS	lipopolysaccharide
MAP Kinase	Mitogen-Activated Protein Kinase
MHCII	class II Major Histocompatibility Complex
MOPS	3-(N-morpholino)propanesulfonic acid
MβCD	methyl-β-cyclodextrin
N.S.	statistically not significant
NADPH oxidase	nicotinamide adenine dinucleotide phosphate oxidase
NF-κB	nuclear factor of κ light polypeptide gene enhancer in B cells
NFAT	nuclear factor of activated T cells
NLRP3 inflammasome	nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3 inflammasome
P-curdlan	phosphorylated curdlan, phospho-curdlan
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline solution
PBSTw	phosphate buffered saline + 0.1% Tween-20
PFA	paraformaldehyde
PKCδ	Protein Kinase Cδ
PLCγ2	Phospholipase Cγ2
PRR	pattern recognition receptor
Raf1	RAF proto-oncogene serine/ threonine protein kinase
RAW Dectin1	RAW 264.7 cells stably expressing full length, wild-type human Dectin-1
RAW Dectin1-Y15A	RAW 264.7 cells stably expressing signaling-deficient full length human Dectin-1 containing the Y15A mutation
RAW WT	wild-type RAW 264.7 cells, or “RAW Wild-Type”
RNA	ribonucleic acids
ROS	reactive oxygen species
S.E.M.	standard error measurement
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2 domain	Src homology 2 domain
siRNA	short interfering ribonucleic acids
Src	cellular sarcoma protein tyrosine kinase
Syk	Spleen Tyrosine Kinase
TCEP	tris(2-carboxyethyl)phosphine
TLR	Toll-Like Receptor

TNF-α	Tumour Necrosis Factor α
Tris	tris(hydroxymethyl)aminomethane
WGP	Wellmune whole glucan particle (Biothera)
WGPsol	Soluble WGP (Biothera)
β-glucans	β-(1,3)-glucans

Chapter 1. INTRODUCTION

1.1. Fungal Infections in Human Health

Opportunistic fungal pathogens pose a formidable challenge to human health. Superficial skin infections, causing athlete's foot, ringworm, and nail infections, affect a quarter of the population globally, while mucosal infections, such as vulvovaginal candidiasis can affect 50 – 75% of women during their childbearing years (Brown et al., 2012b). Beyond these common, curable "minor" infections, a smaller fraction of the population suffers from invasive fungal infections by mostly *Cryptococcus*, *Candida*, *Aspergillus*, and *Pneumocystis genera*. Unlike superficial infections, these invasive infections affect the central nervous system, blood, or lungs, and many exhibit surprisingly high mortality rates of greater than 50% (Brown et al., 2012b). In 1997, fungal infections were the seventh leading cause of infection related mortality in the United States (McNeil et al., 2001). Currently, an estimated 2 million or more individuals die from invasive fungal infections each year globally, on par with deaths from tuberculosis or malaria (Brown et al., 2012b), and the incidence is rising (Brown et al., 2012a; McNeil et al., 2001). The risk of contracting these infections is exacerbated in those that are immunocompromised, such as individuals with leukemia, patients undergoing organ transplantation, prolonged recipients of immunosuppressive drugs, or individuals with human immunodeficiency virus (HIV) infection (Brown et al., 2012b). In fact, a major cause of death from HIV infection worldwide is invasive fungal infection, with an estimated ~800,000 deaths per year (Armstrong-James et al., 2014). Individuals with altered normal flora from antibiotic treatment or breached anatomical barriers, from surgery and catheters, are also more susceptible to fungal infections (Spellberg, 2011). Not surprisingly, *Aspergillus* (Spellberg, 2011) and *Candida* (Brown et al., 2012b) species are the second and fourth most common cause of hospital-acquired blood infections, respectively.

Despite the devastating impact of fungal infections, several issues remain in

combating them. Firstly, their diagnosis is currently time-consuming, costly, and difficult, leading often to misdiagnoses (Armstrong-James et al., 2014; Brown et al., 2012a; 2012b). Furthermore, treatment with anti-fungal drugs produces only moderately successful if not disappointing prognoses. For example, the mortality from *Candida* infections is still 30 – 40% with drug treatment (Spellberg, 2011), despite the advent of new anti-fungals (Brown et al., 2012b). In addition, the threat of resistance to anti-fungal drugs is increasing (White et al., 1998), and the development of new drugs is complicated by the evolutionary similarity of fungi to humans, reducing the number of molecular targets available for inhibition (Brown et al., 2012b; Cowen, 2008).

A solution to these issues involves the development of vaccines against fungal antigens. Shockingly, no anti-fungal vaccines have been approved for clinical use (Armstrong-James et al., 2014; Brown et al., 2012b), and large economic barriers exist in pushing experimental vaccines into clinical trials (Spellberg, 2011). Further complicating vaccine development is the challenge of making them function in those that are immunocompromised, since the vaccines rely on the immune system for their therapeutic effects (Armstrong-James et al., 2014). Nevertheless, the development of vaccines will be paramount to counteract the rise in fungal infections. Vaccinating for components present on the surfaces of many fungi would provide protection against multiple pathogens, reducing the large burden on the hunt for new anti-fungal drugs. Indeed, a vaccine containing β-glucan, a common fungal cell wall component, induces cross-protection against *Candida*, *Aspergillus*, and *Cryptococcus* species (Pietrella et al., 2010; Rachini et al., 2007; Torosantucci et al., 2005), demonstrating that vaccines do not have to target specific virulence factors on particular species in order to provide a protective effect (Spellberg, 2011). To assist in the development of new vaccines, a better understanding of the interaction between fungal pathogens and the immune system is warranted.

1.2. β-Glucan, a Major Fungal Cell Wall Component

The fungal cell wall is composed of polysaccharides, which consist mainly of glucan and chitin, and glycoproteins, a majority of which are mannoproteins (Bowman and Free, 2006; Kapteyn et al., 1999; Stone, 2009) (**Figure 1**). Glucan is the major constituent, comprising 50 – 60% of the dry weight of the cell wall (Bowman and Free, 2006; Brown and Williams, 2009; Kapteyn et al., 1999; Stone, 2009). Most of this glucan component exists as linear chains of repeating glucose residues, connected by glycosidic bonds in the β-configuration, with carbon 1 on one glucose residue being connected to carbon 3 on the following residue (Stone, 2009; Wright et al., 2011) (**Figure 2**). Hence, these glucans are known as β-(1,3)-glucans, which we will refer to as β-glucans. β-(1,6)-linked branches also emanate from the β-(1,3)-linked main chain, serving to connect multiple main chains in a tight network (**Figure 2**), with the distribution and frequency of the branches dependent on the species of origin (Stone, 2009; Synytsya and Novák, 2013). Although β-glucans are usually hidden from the external environment in the cell wall (**Figure 1**), the budding of yeast cells can produce scars that lead to their exposure (Gantner et al., 2005), as can maturation-induced swelling of spores (Gersuk et al., 2006). Since most medically-relevant fungi contain β-glucan, its presence in the blood is a reliable indicator for fungal infection (Mennink-Kersten and Verweij, 2006).

Beyond fungi and yeast, β-glucans are also produced by some plants, bacteria and algae as secreted products or to make up cell walls (Mennink-Kersten and Verweij, 2006; Stone, 2009). Animals do not produce β-glucans, with the exception of a few invertebrates (Mennink-Kersten and Verweij, 2006). This makes β-glucans an excellent molecule to be recognized by the immune system for the activation of the immune response (Brown and Gordon, 2003).

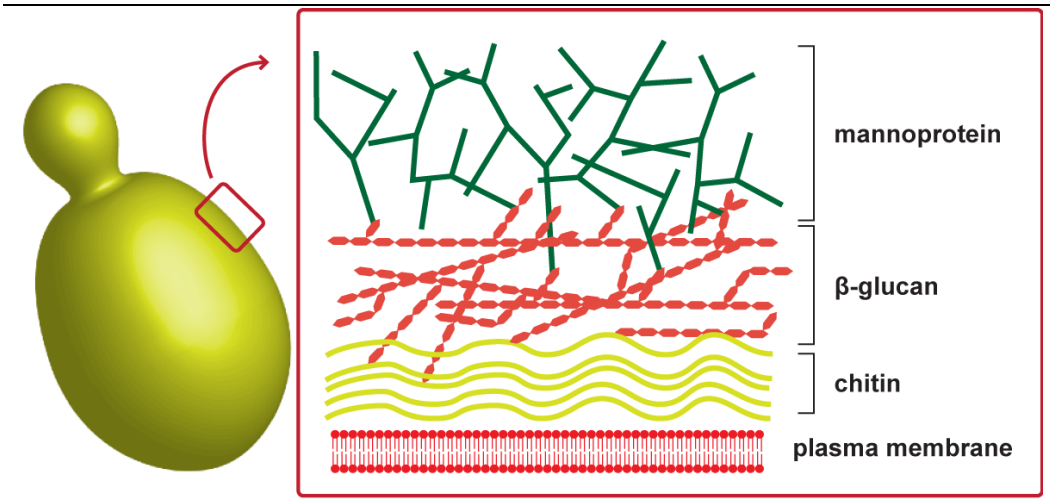

Figure 1: Structure of the Fungal Cell Wall

A dividing yeast particle is represented on the left. Shown is the layered arrangement of the three major constituents of the fungal cell wall, mannoprotein, β-glucan, and chitin (inset) (Bowman and Free, 2006; Hardison and Brown, 2012; Kapteyn et al., 1999; Stone, 2009).

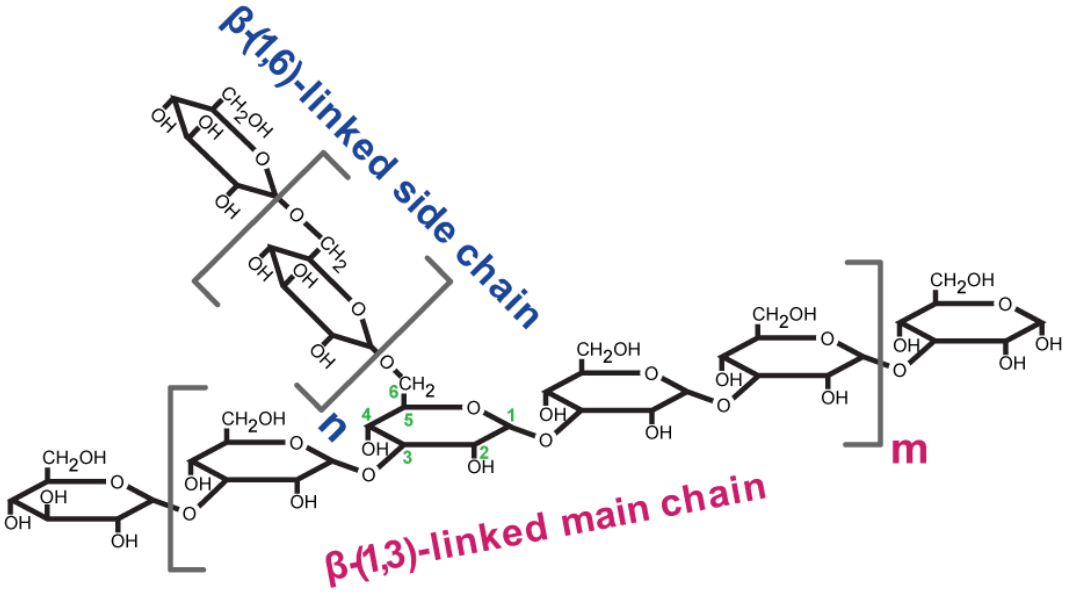

Figure 2: Structure of β-Glucans

Shown is a schematic representation of the chemical structure of β-glucan molecules. β-glucans consist of a homopolymeric main chain or backbone of glucose residues, linked by β-(1,3)-glycosidic bonds. β-(1,6)-linked side chains may also be present. The lengths of both types of chains are variable, represented by the square brackets and the coefficients *n* and *m*. The numbering of carbon atom positions in a glucose residue is denoted in green.

1.3. Soluble β-Glucans in the Bloodstream

In the clinic, upon systemic infection by pathogenic fungi, elevated β-glucan levels can be detected in patient blood plasma (Obayashi et al., 1995). The diagnostic test used involves a protein from *Limulus polyphemus*, Factor G, which upon binding to plasma β-glucan, triggers a proteolytic cascade resulting in release of a chromogenic molecule from a synthetic peptide (Wright et al., 2011). The results from the test are used to diagnose fungal infection, aid decision making to start anti-fungal therapy, and monitor the therapy's effectiveness (Reiss et al., 2000). While the fungal cell wall is insoluble due to numerous covalent associations with other components (Ishibashi et al., 2005; Kapteyn et al., 1999; Stone, 2009), the plasma β-glucans released are predicted to be water-soluble (Mennink-Kersten and Verweij, 2006; Miyazaki et al., 1995b). It is uncertain if the products arise from host defense mechanisms or as a result of fungal metabolism (Ishibashi et al., 2005), but both possibilities are plausible. Supporting the fungal metabolism perspective, soluble β-glucans are generated and released into the culture media when several fungal species are grown *in vitro* (Mennink-Kersten et al., 2006; Miyazaki et al., 1995b). This release could aid fungal infection by occupying host β-glucan receptors on immune cells, preventing the cells from binding and ingesting the live fungi (Miyazaki et al., 1995a). Alternatively, the host may generate soluble β-glucans as a result of defense against pathogens. For example, immune cells known as macrophages ingest and digest large, insoluble β-glucan particles, resulting in the release of soluble β-glucans in the culture medium, which can then re-stimulate and activate the cells (and likely other cells in the vicinity). This digestion is a reactive oxygen species (ROS)-dependent cellular process and is inhibited by ROS scavengers (Hino et al., 2012). Regardless of how they are generated, soluble β-glucans can be present in the blood during fungal infection and it could well be that host immune cells respond to them.

1.4. Purified β-Glucans for Therapy

While β-glucans are present in the bloodstream during fungal infection, purified β-glucans have been known for their immunotherapeutic benefits for decades (Brown and Gordon, 2003; Brown and Williams, 2009; Vannucci et al., 2013), particularly in China and Japan (Chan et al., 2009; Chen and Seviour, 2007; Fang et al., 2012). The β-glucans are extracted from fungal and algal cell walls, and the literature is rich with examples where parenteral administration of these isolates produces potent protective effects, whether anti-microbial, anti-fungal, anti-pyretic, or anti-tumorigenic, in mice and in humans (Albeituni and Yan, 2013; Batbayar et al., 2012; Bohn and BeMiller, 1995; Brown and Williams, 2009; Chan et al., 2009; Chen and Seviour, 2007; Fang et al., 2012; Ross et al., 1999; Vannucci et al., 2013; Williams, 1997). Interest in β-glucans is aided by the fact that their administration generally produces low toxicity profiles, at least among the water-soluble varieties (Chen et al., 2011; Lehne et al., 2006; Pretus et al., 1991; Williams et al., 1988). Because of their abilities to stimulate the immune response, β-glucans have been referred to as "biological response modifiers" (Bohn and BeMiller, 1995; Williams et al., 1991a).

Although there has been extensive study on the biological effects of β-glucan treatment, the molecular mechanisms governing how these compounds elicit their broad immunological effects have only recently come to light (Brown and Williams, 2009). For example, β-glucan isolated from *Candida* species was able to induce the maturation of immune cells known as dendritic cells, altering the surface molecules they express, upregulating their ability to ingest pathogens, and stimulating their secretion of the cytokine interleukin 12, a soluble signaling molecule utilized by the immune system for cell-cell communication (Kikuchi et al., 2002). Another β-glucan known as curdlan was found to stimulate production of the cytokine interleukin 1β in dendritic cells, as well as that of antibody molecules from another type of type of immune cell called B cells (Kumar et al., 2009). Still, a more detailed understanding of how purified β-glucans interact with immune cells is required (Fang et al., 2012; Masuda et al., 2012), with particular emphasis on how they interact with the β-glucan receptors situated on

the cells.

Further complicating β-glucan research is the fact that many preparations of therapeutic β-glucans exist. They can be isolated from a variety of organisms, and the isolates exhibit different physicochemical properties, including molecular weight, degree of polymerization, branch frequency, branch length, solubility in aqueous solution, electrostatic charge, and higher order conformation in solution. Currently, there is a lack of consensus as to how these structural characteristics (acting alone or in combination) influence the immunostimulatory capacity of β-glucans, with numerous contradictory findings in the literature (Batbayar et al., 2012; Bohn and BeMiller, 1995; Brown and Gordon, 2003). This is likely due to variation in the species the compounds were isolated from, unstandardized methods for their preparation and chemical modification, and limitations in the ability to test a wide range of molecules at once. For example, while it has been proposed that only large, water-insoluble, particulate β-glucans are immunologically active (Brown, 2006; Gallin et al., 1992; Goodridge et al., 2011; Ishibashi et al., 2001), other reports demonstrate that water-soluble β-glucans can have potent immunostimulatory effects (Batbayar et al., 2012; Bohn and BeMiller, 1995; Di Luzio et al., 1979; Masuda et al., 2012; Pretus et al., 1991; Williams et al., 1991b). Adding further confusion is the fact that some particulate glucans exhibit toxic side effects upon systemic administration (Bowers et al., 1986; RIGGI and Di Luzio, 1962; Williams et al., 1985), and converting them to soluble forms removes these effects while enhancing their immunostimulatory abilities (Williams et al., 1991a), giving benefit to the use of the soluble varieties. Among the soluble β-glucans, it is thought that those with higher molecular weight are more active than those with lower molecular weight (Batbayar et al., 2012; Brown and Gordon, 2003; Brown and Williams, 2009; Ishibashi et al., 2002; Lowe et al., 2001), although this is difficult to assess in studies where only a select few β-glucans are compared at a time. Numerous other studies comparing the effect of unbranched or branched structure, single- or triple-helical solution conformation, or branching frequency and distribution on biological activity yield similarly conflicting conclusions on the relationship between β-glucan structure

and function (Batbayar et al., 2012; Bohn and BeMiller, 1995; Falch et al., 2000; Mueller et al., 2000).

In summary, in spite of widespread research on the uses of β-glucan as immunotherapeutic agents, further work is needed to identify the precise molecular mechanisms by which they are able to activate cells of the immune system. In addition, the impact of structure on the abilities of β-glucans to function as appropriate immunostimulatory agents needs to be further assessed systematically and clarified. Perhaps a detailed understanding of the interaction of purified β-glucans with their cellular receptors will provide resolution to these urgent questions.

1.5. Innate Immune Cells and Pattern Recognition

Cells of the immune system are classified into two components, the innate arm and the adaptive arm. As the first line of defense against microbes, cells of the innate immune system are responsible for the initial recognition, ingestion, and eradication of potential pathogens. To detect potential pathogens, innate immune cells constantly sample the environment. This is done through endocytosis, a class of processes that allow extracellular fluid and molecules to be taken into the cell by the formation of membrane-bound bodies from the plasma membrane lipid bilayer (Doherty and McMahon, 2009). One endocytic mechanism is receptor-mediated endocytosis. Here, small, soluble macromolecules bind to receptors on the plasma membrane, and the ligand-receptor complexes are internalized into vesicles derived from plasma membrane invaginations. Another endocytic mechanism is phagocytosis, a receptor-driven, actin-dependent process where particles and pathogens ≥0.5 μm in size are engulfed by the cell upon their recognition by clustered surface receptors (Flannagan et al., 2012). Here, cellular protrusions known as pseudopods extend around the particles in a process known as "zippering". They eventually meet on the other side of the particle, fusing together and leaving the particle embedded in a large, membrane-bound organelle known as the phagosome. The phagosome eventually fuses with another membrane-bound organelle called the lysosome,

which contains hydrolytic enzymes that will degrade the particle into its component antigens. Molecules internalized by receptor-mediated endocytosis also can traffic to the lysosome, where they too are degraded into fragments.

Macrophages and dendritic cells are innate immune cells endowed with a special ability called antigen presentation. This is the ability to load lysosome-digested antigenic fragments from endocytosed components onto major histocompatibility complex class II (MHCII) molecules. MHCII molecules are then trafficked onto the plasma membrane, where they can then interact with a hypervariable, antigen-specific receptor on $CD4^+$ T lymphocytes (T cells) of the adaptive immune system, known as the T cell receptor. If the binding interaction between the antigen loaded onto MHCII and the T cell receptor is strong, the T cell becomes activated and can then initiate responses from the adaptive immune system. Some adaptive immune responses include the production of antibodies able to coat and neutralize the pathogens, preventing them from spreading and enabling phagocytic uptake by other innate immune cells that can recognize the antibodies, as well as the establishment of memory cells, which, upon future encounter with the same pathogen, enable the immune system to rapidly mount a similar immune response to eradicate it yet again.

The prevalence of β-glucans in fungal cells and their absence on host cells makes them a useful molecule to be recognized by the innate immune system (Brown and Gordon, 2003). Such molecules, known as pathogen-associated molecular patterns, or PAMPs, signal to the innate immune system the presence of a pathogen ("non-self") (Mogensen, 2009). The detection of PAMPs is achieved through receptors expressed on the surface of innate immune cells, aptly termed pattern recognition receptors, or PRRs (Mogensen, 2009). The first PRRs discovered were the Toll-like receptors (TLRs) and first identified in *Drosophila*. To date, eleven TLRs have been identified that are able to recognize an assortment of PAMPs. For example, lipopolysaccharide (LPS), a constituent of the Gram-negative bacterial cell wall, is a PAMP recognized by the classical PRR TLR4 (Mogensen, 2009). The binding of PAMPs to PRRs initiates signaling cascades resulting in the transcription, translation, and secretion of cytokines,

soluble molecular messengers that are recognized by and dictate the behaviour of cells in both the innate and adaptive arms (Kindt et al., 2007).

While the TLRs have been by far the most studied family of PRRs (Hardison and Brown, 2012), the C-type Lectin receptor superfamily has recently emerged as another class of PRRs that detect a variety of carbohydrate PAMPs (Drummond and Brown, 2013; Hardison and Brown, 2012; Kerrigan and Brown, 2010; 2011). Of these receptors, the transmembrane protein Dectin-1 was discovered to recognize β-glucan PAMPs (Brown and Gordon, 2001a) (**Figure 3**). Dectin-1 was the first example of a non-TLR PRR capable of activating intracellular signaling events that drive both inflammatory and adaptive immune responses upon pathogen detection (Brown, 2006; Kerrigan and Brown, 2010). It is arguably the best characterized and prototypical C-type Lectin receptor with signaling capabilities (Drummond and Brown, 2011).

Although other receptors for β-glucans have been identified (Albeituni and Yan, 2013; Brown and Williams, 2009; Spellberg, 2011), including Complement Receptor 3 (Ross et al., 1985; Thornton et al., 1996; Xia et al., 1999), the membrane sphingolipid lactosylceramide (Zimmerman et al., 1998), and certain scavenger receptors (Dushkin et al., 1996; Rice et al., 2002; Vereschagin et al., 1998), Dectin-1 is considered the predominant β-glucan receptor for the recognition of particulate β-glucans (Brown et al., 2003; 2002; Gantner et al., 2003; Pietrella et al., 2010; Qi et al., 2011; Rachini et al., 2007; Taylor et al., 2007; Torosantucci et al., 2005; Underhill et al., 2005; Willment et al., 2005). Since β-glucans are PAMPs present on fungi, the recognition of β-glucans by Dectin-1 plays a major role in mediating anti-fungal immunity (Drummond and Brown, 2011; Ferwerda et al., 2009; Taylor et al., 2007).

1.6. Dectin-1, A Receptor for β-Glucans

1.6.1. Identification, Structure, and Expression

Dectin-1 (dendritic cell-associated C-Type Lectin 1) from mouse was originally identified in dendritic cells by a subtractive cDNA screen, where the expression of mRNAs was compared between the mouse cell lines J774 (macrophage) and XS52 (dendritic cells) (Ariizumi, 2000). Sequencing revealed a polypeptide organized as a Type II integral membrane protein, with a cytoplasmic amino-terminus, a single transmembrane-spanning segment, and an extracellular domain (**Figure 3**). Two putative functional regions were found; the extracellular domain contained a globular region with substantial homology to the carbohydrate recognition domain (CRD) of the calcium-dependent (C-type) lectin protein family, and the short cytoplasmic tail contained a sequence similar to an immunoreceptor tyrosine-based activation motif (ITAM) (Ariizumi, 2000), an amino acid sequence of two stretches of YXX[I/L] residues, separated by six to twelve amino acids (YXXI/L$X_{6\text{-}12}$YXXI/L) (Reth, 1989; Underhill and Goodridge, 2007) (**Figure 3**). Separating the CRD from the transmembrane-spanning segment were 50 amino acids (Ariizumi, 2000), later termed the stalk region (Willment, 2001) or neck (Yokota et al., 2001) (**Figure 3**). Mouse Dectin-1 was later shown to also be expressed on monocytes, macrophages and neutrophils (Brown and Gordon, 2001a; Brown et al., 2002; Reid et al., 2004; Taylor et al., 2002) and act as a pattern recognition receptor for β-(1,6)-branched, β-(1,3)-glucans in a calcium-independent manner (Brown and Gordon, 2001a). This calcium independence was consistent with the lack of metal-coordinating residues in its CRD, unlike CRDs of other calcium-dependent C-type lectins (Brown and Gordon, 2001a; Willment, 2001). The receptor also mediated the β-glucan-dependent binding of the yeast cell wall extract zymosan and whole *Candida albicans* yeast particles, facilitating phagocytic uptake of the latter (Brown and Gordon, 2001a).

The human homolog of Dectin-1 was identified soon after (Grünebach et al., 2002; Hermanz-Falcón et al., 2001; Willment, 2001; Yokota et al., 2001) and

genetically mapped to a locus on chromosome 12, between 12p12.3 and 12p13.2 (Yokota et al., 2001). It exhibits ~60% sequence identity with mouse Dectin-1 (Hermanz-Falcón et al., 2001; Yokota et al., 2001) and is essentially equivalent to the mouse protein in domain structure (Willment, 2001; Yokota et al., 2001), the ability to recognize yeast particles and β-glucan (Brown and Gordon, 2001a; Brown et al., 2003; Willment, 2001; Willment et al., 2005), and the ability to stimulate production of the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α) upon activation with those ligands (Brown et al., 2003; Willment et al., 2005). The receptor is expressed on monocytes, dendritic cells, macrophages, neutrophils, eosinophils, B cells, and some T cells (Grünebach et al., 2002; Hermanz-Falcón et al., 2001; Kennedy et al., 2007; Willment, 2001; Willment et al., 2005; Yokota et al., 2001). In comparison to other receptors, the amino acid sequence of Dectin-1 exhibits substantial homology to natural killer cell lectins with the Type II membrane protein configuration (Willment, 2001), and other members of the C-type Lectin superfamily, such as C-Type Lectin-Like Receptor 2 (CLEC-2) (48% and 28.9% sequence identity for the CRD alone and entire sequence, respectively) (Hermanz-Falcón et al., 2001; Yokota et al., 2001).

1.6.2. Major Isoforms

In both mice (Yokota et al., 2001) and humans (Hermanz-Falcón et al., 2001; Willment, 2001), Dectin-1 is present as two major isoforms that are generated by alternative splicing of mRNA (**Figure 3**). Isoform A is the full-length receptor, while isoform B is missing the stalk region (Hermanz-Falcón et al., 2001; Willment, 2001; Yokota et al., 2001). Both isoforms are functional in β-glucan recognition to a similar degree (Heinsbroek et al., 2006; Willment, 2001), although in mice, the B isoform is less effective at recognition in cold temperature but has a higher capacity to induce TNF-α secretion (Heinsbroek et al., 2006). Other differences are known. For example, certain strains of mice express both isoforms at similar levels, while isoform B is predominant in other strains (Heinsbroek et al., 2006). In humans, while isoform A is present on all the cell types described by Willment *et al*. to express Dectin-1 (Willment et al., 2005), in some cell types the B isoform is predominantly expressed (Grünebach et

al., 2002; Hermanz-Falcón et al., 2001; Weck et al., 2008; Willment, 2001; Willment et al., 2005). Cell maturation and differentiation also favour expression of the B isoform in humans (Grünebach et al., 2002; Weck et al., 2008; Willment, 2001; Willment et al., 2005). Finally, human isoform A contains an *N*-linked glycosylation site in the stalk (Kato et al., 2006; Yokota et al., 2001) (**Figure 3**) which promotes receptor expression at the cell surface (Kato et al., 2006). Human Dectin-1B is not glycosylated as it does not contain the stalk (Kato et al., 2006).

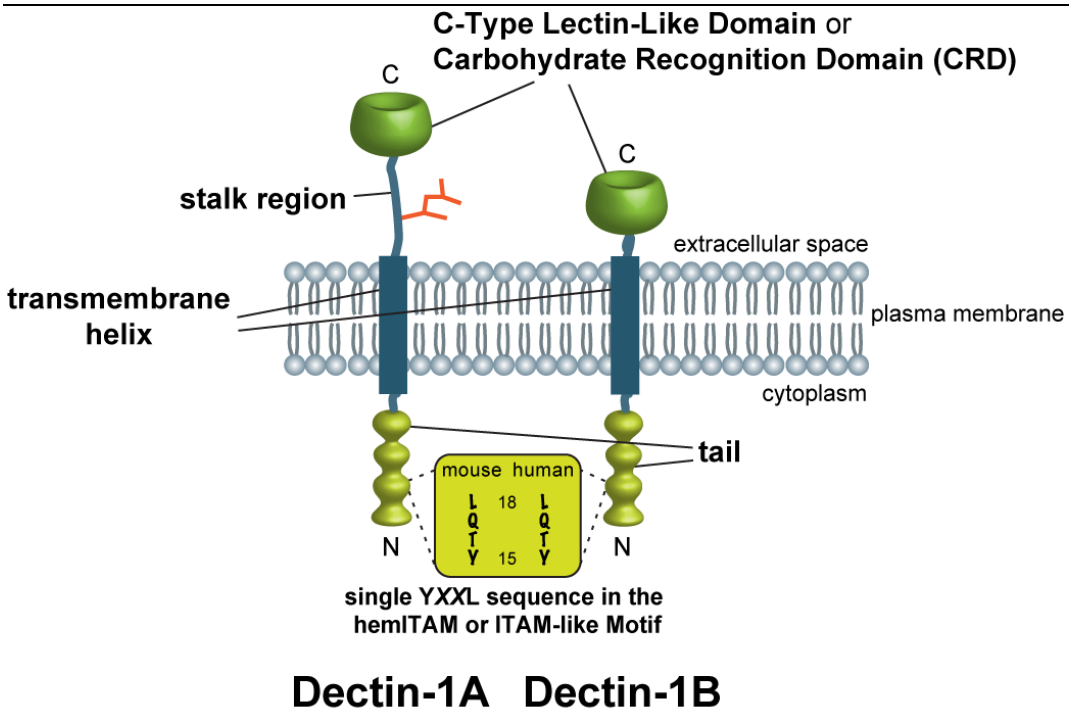

Figure 3: Structure of the Major Isoforms of Human Dectin-1

Shown are the molecular configurations of the two major isoforms of human Dectin-1. Depicted are the extracellular carbohydrate recognition domain (CRD) for β-glucan binding, the stalk region containing an N-linked glycosylation site (only in isoform A), a single transmembrane helix, and a short signaling tail in the cytoplasm. The tail contains a hemITAM or ITAM-like motif. The functional YXXL amino acid sequence of this motif, as well as its positions in the polypeptide, is highlighted. As a Type II integral membrane protein, Dectin-1 has a cytoplasmic amino-terminus (N) and an extracellular carboxy-terminus (C).

1.6.3. Ligands and Cellular Responses

Dectin-1 has been found to bind three ligands: exogenous β-(1,3)-linked glucans of more than seven glucose residues long, without specificity for any other carbohydrates (Adams et al., 2008; Brown and Gordon, 2001a; Palma et al.,

2006); a yet unidentified endogenous ligand present on T cells (Ariizumi, 2000; Brown and Gordon, 2001a; Grünebach et al., 2002; Willment, 2001), and endogenous vimentin, secreted into the extracellular environment during the inflammation of atherosclerotic lesions (Thiagarajan et al., 2013). The remainder of our discussion will focus on Dectin-1's interaction with β-glucan ligands.

Upon activation by particulate β-glucans and fungal pathogens, Dectin-1 has been shown to stimulate the following responses in innate immune cells: 1) non-opsonic phagocytosis of the β-glucan-containing particles (Brown and Gordon, 2001a; Brown et al., 2002; Herre et al., 2004; Rogers et al., 2005; Steele et al., 2003; Underhill et al., 2005), resulting in their engulfment in the phagolysosome for enzymatic degradation; 2) the respiratory burst – the production of reactive oxygen species (ROS) (Gantner et al., 2003; 2005; Underhill et al., 2005) which assist in eradicating the phagocytosed fungal pathogens, 3) synthesis of arachidonic acid metabolites (Olsson and Sundler, 2007; Suram et al., 2006), which initiate localized inflammation; 4) dendritic cell maturation (LeibundGut-Landmann et al., 2007; Qi et al., 2011; Tassi et al., 2009; Xu et al., 2009a; 2009b; Zenaro et al., 2009); and 5) production and secretion of an assortment of cytokines (Agrawal et al., 2010; Brown et al., 2003; Gantner et al., 2003; Goodridge et al., 2007; Gringhuis et al., 2009; Gross et al., 2006; Hise et al., 2009; LeibundGut-Landmann et al., 2007; Qi et al., 2011; Rogers et al., 2005) which mediate the inflammatory response, recruit other immune cells, and prime cells of the adaptive immune system (Kindt et al., 2007).

Studies have demonstrated that dendritic cells activated by Dectin-1 signaling can also direct the adaptive immune response. These dendritic cells induce the proliferation and differentiation of $CD4^+$ T cells to T helper 1 (T_H1) and T helper 17 (T_H17) cells (Agrawal et al., 2010; LeibundGut-Landmann et al., 2007; Qi et al., 2011; Tassi et al., 2009; Zenaro et al., 2009), and of $CD8^+$ T cells to cytotoxic T lymphocytes (LeibundGut-Landmann et al., 2008; Ni et al., 2010; Qi et al., 2011), instruct regulatory T (T_{reg}) cells to produce the cytokine interleukin 17 (Osorio et al., 2008), and stimulate the secretion of antibodies (Agrawal et al., 2010; LeibundGut-Landmann et al., 2007). Recently, Ma and

colleagues also observed that Dectin-1 has a direct role in antigen presentation in dendritic cells and macrophages (Ma et al., 2012). Dectin-1 signaling recruited a protein called light chain 3 (LC3) to Dectin-1^+ phagosomes containing engulged fungal particles. Here, LC3 recruited the MHCII complex, allowing fungal-derived antigens to be loaded onto the complex and presented on the cell surface more efficiently to $CD4^+$ T cells (Ma et al., 2012). Clearly, these examples illustrate how Dectin-1 activation couples the detection of β-glucans by innate immune cells to the protective responses mediated by the adaptive immune system.

1.6.4. Signaling

As mentioned earlier, the cytoplasmic tail of Dectin-1 possesses a sequence that resembles an ITAM (immunoreceptor tyrosine-based activation motif) (Ariizumi, 2000), which consists of the consensus sequence $YXXI/LX_{6-12}YXXI/L$ (Reth, 1989; Underhill and Goodridge, 2007). ITAMs are found in several immune receptors, such as T cell receptors, B cell receptors, and Fc receptors (Underhill and Goodridge, 2007). The current model for ITAM signaling is summarized as follows (Underhill and Goodridge, 2007) (**Figure 4**): ligand binding triggers clustering of immune receptors, which allows the tyrosine residues in ITAMs to become phosphorylated by Src (cellular sarcoma protein tyrosine kinase) family kinases. The phosphotyrosine residues are then recognized by members of the Syk (spleen tyrosine kinase) family of kinases, which are recruited to the activated receptors by virtue of their two tandem Src homology 2 (SH2) domains, each binding to one of the phosphotyrosine residues in the ITAM. Binding the dually-phosphorylated ITAM triggers activation of the Syk family kinase (Geahlen, 2009), and as a protein tyrosine kinase, it then phosphorylates tyrosine residues on itself and on target proteins to produce a physiological response (Underhill and Goodridge, 2007). Autophosphorylation promotes sustained activation of the Syk family kinase while also generating docking sites for target proteins, which are then recruited to the molecule and become substrates for phosphorylation (Geahlen, 2009; Mócsai et al., 2010). When phosphorylated, these target proteins undergo conformational changes that

alter their activity, or become docking sites for other proteins that recognize phosphotyrosine residues, facilitating the formation of signaling complexes assembled on protein-protein interactions (Geahlen, 2009). As Dectin-1 is an ITAM-like receptor, it is not surprising that many Dectin-1-mediated signaling and cellular responses are dependent on the Syk family member Syk itself (Dennehy et al., 2008; Drummond et al., 2011; Elsori et al., 2011; Fuller et al., 2007; Gazi et al., 2011; Gringhuis et al., 2009; Gross et al., 2006; 2009; Kankkunen et al., 2010; Kelly et al., 2010; LeibundGut-Landmann et al., 2007; 2008; Rogers et al., 2005; Slack et al., 2007; Strasser et al., 2012; Suram et al., 2006; Underhill et al., 2005; Xu et al., 2009b).

Upon the binding of particulate β-glucan ligands, the initiation of Dectin-1 signaling occurs through tyrosine phosphorylation of the receptor (Elsori et al., 2011; Gantner et al., 2003; 2005). This phosphorylation occurs on the cytoplasmic tail, which contains two tyrosine residues that are found within the ITAM-like motif (Gantner et al., 2003; Rogers et al., 2005). The phosphorylation is likely mediated by members of the Src family kinases (Brown and Williams, 2009; Geahlen, 2009; Underhill and Goodridge, 2007), since treatment with PP2, an inhibitor of the kinase activity of Src family kinases, abolishes Syk activation (Elsori et al., 2011; Olsson and Sundler, 2007; Underhill et al., 2005). Furthermore, the Src family kinase member Src was observed to associate with Dectin-1 upon stimulation of the receptor (Elsori et al., 2011). Combined with data demonstrating the cytoplasmic tail of Dectin-1 is required for Syk activation and recruitment (Rogers et al., 2005), the findings suggest Src family kinase-dependent phosphorylation of Dectin-1 precedes Syk activation. With their immediate role in Dectin-1 and Syk activation, the activation of Src family kinases is likely a prerequisite for Dectin-1-dependent cellular responses. For example, the production of ROS (Elsori et al., 2011; Underhill et al., 2005) along with activation of Raf1 (RAF proto-oncogene serine/threonine protein kinase), phospholipase Cγ2 (PLCγ2), protein kinase Cδ (PKCδ), and extracellular signal-regulated kinases (ERK), all signaling events that occur downstream of Dectin-1 activation (see **Figure 5**), are inhibited upon treatment with PP2 (Gringhuis et al.,

2009; Kelly et al., 2010; Olsson and Sundler, 2007; Strasser et al., 2012; Xu et al., 2009b). In addition, cells deficient in the Src family kinase member Lck (lymphocyte-specific protein tyrosine kinase) are unable to fulfill Dectin-1-dependent transcription (Fuller et al., 2007).

Interestingly, Dectin-1's putative ITAM differs slightly from the consensus ITAM sequence: while the membrane-proximal tyrosine residue (Y15) is situated the correct distance away from its corresponding leucine (L18), separated by two amino acids (**Figure 3**), this is not true for the membrane-distal tyrosine (Y3). Y3 is not in the correct position, since three, not two, amino acids separate it from the corresponding leucine (L7) (Underhill and Goodridge, 2007; Underhill et al., 2005). Indeed, unlike traditional ITAM signaling (Brown, 2006; Geahlen, 2009; Underhill and Goodridge, 2007), a single phosphorylation at Y15 is necessary and sufficient for Syk recruitment to the receptor tail (Rogers et al., 2005). This recruitment does not depend on the phosphorylation status of Y3 or even require a tyrosine residue at the position (Rogers et al., 2005). Furthermore, residue Y15 is necessary for Dectin-1-mediated cellular responses, such as Syk phosphorylation, phagocytosis, the activation of NF-κB (nuclear factor of κ light polypeptide gene enhancer in B cells)- and AP-1 (activator protein 1)-dependent transcription, synthesis of the cytokines TNF-α, interleukins 6, 10 and 12, and dendritic cell maturation (Brown et al., 2003; Gantner et al., 2003; Herre et al., 2004; LeibundGut-Landmann et al., 2007; Rogers et al., 2005; Slack et al., 2007; Toyotome et al., 2008; Underhill et al., 2005), while Y3 is dispensable (Herre et al., 2004; Rogers et al., 2005; Underhill et al., 2005). As only the membrane-proximal half of Dectin-1's putative ITAM is essential for activation of signaling, the sequence has been coined a hemITAM (Robinson et al., 2006). Comparably, the related receptor CLEC-2 also signals via a hemITAM, as it contains only a single YXXL motif present in its intracellular tail (Fuller et al., 2007; Suzuki-Inoue, 2006).

Although the single tyrosine phosphorylation at residue Y15 is sufficient for Syk recruitment (Rogers et al., 2005), Syk contains two SH2 domains (Geahlen, 2009), both of which together are required for transduction of Dectin-1 activation

downstream (Fuller et al., 2007). Thus, it has been suggested that Syk recruitment and activation are in fact promoted by receptor dimerization (Brown, 2006; Goodridge et al., 2009b; Kerrigan and Brown, 2010; Rogers et al., 2005) (**Figure 4**). Given the necessity of receptor dimerization for Syk activation, and the polymeric nature of β-glucan ligands, it is likely that higher-order clustering of Dectin-1 occurs upon ligand binding (Adams et al., 2008; Brown and Williams, 2009; Goodridge et al., 2011; 2012; Lowe et al., 2001; Michalek et al., 1998; Qi et al., 2011; Tapper and Sundler, 1995), similar to the receptor CLEC-2 (Hughes et al., 2010; Suzuki-Inoue, 2006; Watson et al., 2009).

To summarize, activation of Dectin-1 signaling is thought to occur as follows (Goodridge et al., 2012) (**Figure 5**): β-glucan binding to surface-localized Dectin-1 cross-links the receptor into multimers, inducing the formation of receptor clusters on the cell surface. It is in these clusters that Src family kinases become activated, phosphorylating the hemITAM tyrosine residue in the cytoplasmic tails of multiple Dectin-1 molecules. Pairs of adjacent, phosphorylated Dectin-1 hemITAMs then serve as docking sites for the dual SH2 domains in Syk, facilitating its recruitment, activation, and engagement of intracellular signaling events.

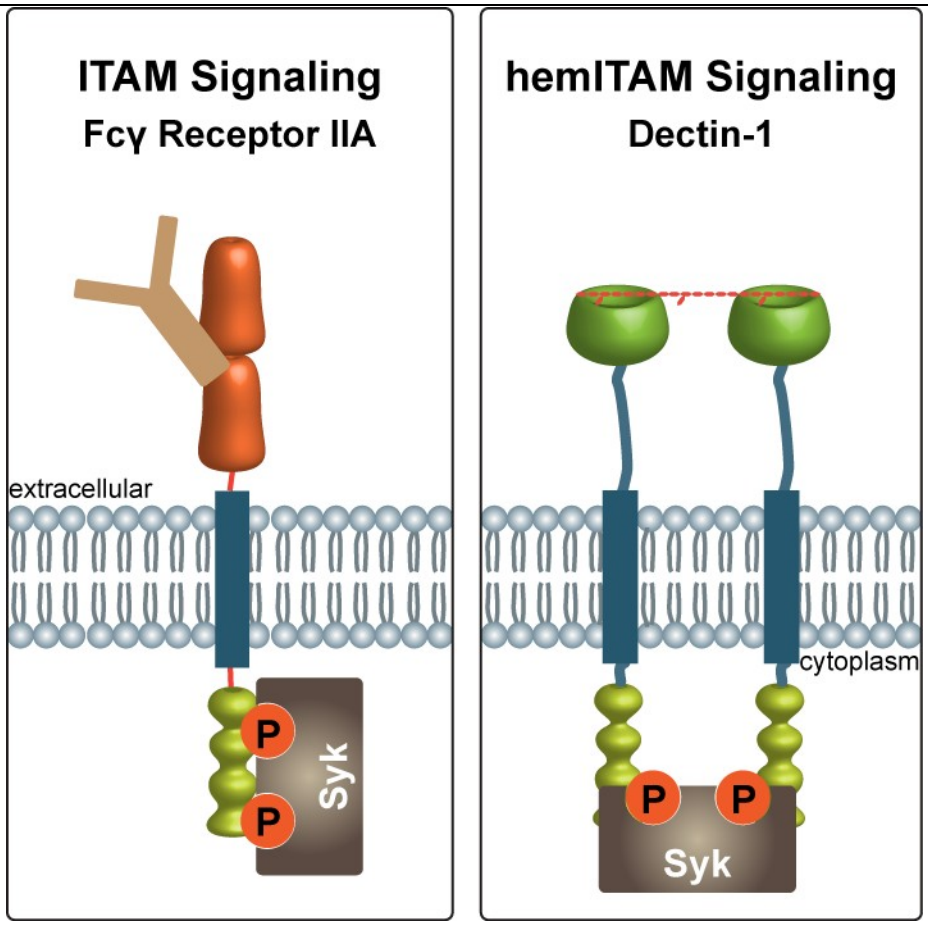

Figure 4: Activation Models of ITAM- and hemITAM-Containing Receptors
Conventional ITAM motifs contain two spaced tyrosine residues that are phosphorylated by Src family kinases upon ligand ligation and subsequent receptor activation. One receptor that undergoes conventional ITAM signaling is Fcγ Receptor IIA (Van den Herik-Oudijk et al., 1995), which binds the Fc region of Immunoglobulin G molecules (represented as the Y-shaped molecule). Members of the Syk family of non-receptor protein tyrosine kinases, such as Syk (depicted), are recruited to these phosphotyrosinyl sites by virtue of two tandem SH2 domains (represented as pockets in Syk), each binding one phosphotyrosine residue in the receptor ITAM. This results in activation of the Syk family kinase and its initiation of downstream signaling cascades by phosphorylating target proteins. On the other hand, hemITAM motifs require phosphorylation of only a single tyrosine residue for signal transduction upon ligand binding. Thus, hemITAM-containing receptors likely depend on dimerization or oligomerization to generate the appropriate docking site for recruitment of the two SH2 domains in Syk family kinases. Dectin-1 is the first and now classical example of a receptor that signals via a hemITAM. The polymeric nature of β-glucans likely promotes the receptor multimerization required for Dectin-1 to recruit Syk.

The activation of Dectin-1 results in several signaling cascades that produce the cellular responses to Dectin-1 activation. We will now highlight several signaling events that are induced when Dectin-1 is stimulated by yeast

cells or particulate β-glucan preparations (**Figure 5**) (Albeituni and Yan, 2013; Batbayar et al., 2012; Drummond et al., 2011; Goodridge et al., 2009b; Plato et al., 2013). While we have made an effort to represent key findings in the field appropriately, the cell type in which Dectin-1 is expressed (Goodridge et al., 2009a) and the chemical structure of the stimulating β-glucan likely result in variations in which molecular players and pathways are activated during receptor stimulation.

Stimulation of Dectin-1 with β-glucan particles results in their phagocytic uptake (Brown and Gordon, 2001a; Brown et al., 2002; Herre et al., 2004; Rogers et al., 2005; Steele et al., 2003; Underhill et al., 2005). (While we will not discuss the mechanisms of Dectin-1-mediated phagocytosis, the topic was reviewed recently by Goodridge and colleagues (Goodridge et al., 2012)). To aid in particle digestion, the protein complex NADPH (nicotinamide adenine dinucleotide phosphate) oxidase is activated in a Dectin-1-, Src family kinase-, and Syk-dependent manner (Gantner et al., 2003; 2005; Underhill et al., 2005), assembling on the phagosomal membrane. Once assembled, it catalyzes the production of the reactive anion superoxide from molecular oxygen, releasing superoxide into the phagosomal lumen. Superoxide is eventually converted to other reactive oxygen species (ROS), which together contribute to pathogen or particle destruction (Segal, 2008).

The Dectin-1-mediated recruitment and activation of Syk also results in the activation of phospholipase Cγ2 (PLCγ2) (Fuller et al., 2007; Olsson and Sundler, 2007; Tassi et al., 2009; Xu et al., 2009a; 2009b), probably by Syk-dependent tyrosine phosphorylation (Geahlen, 2009; Mócsai et al., 2010; Xu et al., 2009b). PLCγ2 is an enzyme that hydrolyzes the plasmalemmal lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce two second messenger molecules, one of which is the water-soluble lipid inositol 1,4,5-trisphosphate (IP_3). IP_3 diffuses to the endoplasmic reticulum and binds to IP_3 receptors there, triggering the release of the organelle's store of calcium ions into the cytoplasm, along with an ensuing entry of extracellular calcium ions into the cell (Kurosaki et al., 2000). The resulting calcium influx (Xu et al., 2009b) stimulates the protein

calcineurin (Greenblatt et al., 2010), which, as a protein phosphatase, binds to and dephosphorylates the transcription factor NFAT (nuclear factor of activated T cells), rendering it active and permitting its translocation to the nucleus (Hogan et al., 2003) where it can initiate transcription of genes (Fuller et al., 2007; Goodridge et al., 2007; Greenblatt et al., 2010; Tassi et al., 2009; Xu et al., 2009b).

The other second messenger generated by PLCγ2 activity is the lipid diacylglycerol (DAG), which is retained in the plasma membrane (Kurosaki et al., 2000). The production of DAG activates protein kinase Cδ (PKCδ) (Elsori et al., 2011; Kurosaki et al., 2000; Strasser et al., 2012), which triggers assembly of an adaptor protein complex consisting of CARD9 (caspase recruitment domain-containing protein 9), Bcl-10 (B cell lymphoma/leukemia 10), and MALT1 (mucosa-associated lymphoid tissue lymphoma translocation protein 1) by phosphorylating CARD9 (Goodridge et al., 2009a; Gross et al., 2006; Hara et al., 2007; LeibundGut-Landmann et al., 2007; Strasser et al., 2012; Xu et al., 2009b). The CARD9-Bcl-10-MALT1 complex then activates the transcription factor NF-κB (nuclear factor of κ light polypeptide gene enhancer in B cells) (Gantner et al., 2003; Goodridge et al., 2009a; Gringhuis et al., 2009; Gross et al., 2006; Hara et al., 2007; LeibundGut-Landmann et al., 2007; Strasser et al., 2012; Tassi et al., 2009; Xu et al., 2009b), which can exist as a heterodimer of the proteins p50 and p65. Typically, inactive NF-κB is localized in the cytoplasm and prevented from entering the nucleus by its association with an inhibitor protein, IκB (inhibitor of κB) (Kingeter and Lin, 2012). However, when the CARD9-Bcl-10-MALT1 complex is activated, the signal is relayed to the heterotrimeric IκB kinase complex (IKK) (Strasser et al., 2012), which phosphorylates IκB. This phosphorylation targets IκB for ubiquitinylation and subsequent proteasomal degradation, leaving NF-κB free to undergo nuclear translocation. Once in the nucleus, NF-κB is modified by phosphorylation and acetylation to become fully activated, allowing it to bind DNA tightly and more efficiently initiate transcription (Kingeter and Lin, 2012).

Another pathway activated upon Dectin-1 stimulation is the MAP kinase

(mitogen-activated protein kinase) phosphorylation cascade, which is dependent on the activation of Syk, PLCγ2 and the calcium influx (Kelly et al., 2010; Slack et al., 2007; Tassi et al., 2009; Xu et al., 2009b) but not PKCδ or the CARD9-Bcl-10-MALT1 complex (Hara et al., 2007; Slack et al., 2007; Strasser et al., 2012). The cascade ultimately results in the phosphorylation and activation of the ERK (extracellular signal-regulated kinase), p38, and JNK (c-Jun N-terminal kinase) MAP kinases (Hernanz-Falcón et al., 2009; Kelly et al., 2010; Kock et al., 2011; LeibundGut-Landmann et al., 2007; Olsson and Sundler, 2007; Slack et al., 2007; Tassi et al., 2009; Xu et al., 2009b), which stimulate the transcription factor AP-1 (activator protein 1, a heterodimer of the proteins c-Fos and c-Jun) (Tassi et al., 2009; Toyotome et al., 2008; Whitmarsh and Davis, 1996; Xu et al., 2009b).

Ultimately, the activated transcription factors NF-κB, NFAT, and AP-1 all translocate to the nucleus, activating transcription for genes encoding cytokines, which are then secreted by the cell. Dectin-1 signaling results in the secretion of TNF-α and interleukins 1β, -2, -6, -10, -12, and -23 (IL-1β, IL-2, IL-6, IL-10, IL-12, IL-23) (Agrawal et al., 2010; Brown et al., 2003; Gantner et al., 2003; Goodridge et al., 2007; Gringhuis et al., 2009; Gross et al., 2006; Hernanz-Falcón et al., 2009; Hise et al., 2009; LeibundGut-Landmann et al., 2007; Qi et al., 2011; Rogers et al., 2005; Rosas et al., 2008). An additional step exists for the production of IL-1β: prior to secretion, the inactive, full-length precursor to IL-1β, pro-IL-1β, must be processed by caspase 1-mediated proteolytic cleavage to generate the active form of the cytokine (Gross et al., 2009; Hise et al., 2009; Joly et al., 2009; Kankkunen et al., 2010; Saïd-Sadier et al., 2010). Caspase 1 (cysteine-dependent, aspartate-directed protease 1) itself is activated by proteolytic cleavage. This is facilitated by a large, multiprotein complex known as the NLRP3 (nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3) inflammasome, whose assembly is dependent on ROS production (perhaps from activation of the NADPH oxidase) and Syk activation (Franchi et al., 2012; Gross et al., 2009; Hise et al., 2009; Kankkunen et al., 2010; Kumar et al., 2009; Saïd-Sadier et al., 2010; Tschopp and Schroder, 2010). The activated transcription factors also transcribe other genes. For example, Dectin-1

stimulation also triggers NFAT-dependent transcription of the gene for cyclooxygenase-2 (COX-2) (Goodridge et al., 2007; Greenblatt et al., 2010). This gene encodes an enzyme involved in the production of arachidonic acid metabolites, the release of which is a downstream response to Dectin-1 stimulation (Olsson and Sundler, 2007; Suram et al., 2006).

In this section, we have discussed how the recognition of particulate β-glucans to Dectin-1 is transduced across the plasma membrane to Syk, and how other signaling molecules transmit the message to produce cellular responses such as cytokine release and the respiratory burst. We have, however, discussed these events from a Dectin-1- and Syk-centric perspective. In fact, while the signaling events represented in **Figure 5** are Syk-dependent, it is noteworthy that a Raf1-dependent, Syk-independent signaling pathway also exists, regulating NF-κB-dependent transcription (Gringhuis et al., 2009). The signaling events illustrated in **Figure 5** are also all dependent on Dectin-1 activation. However, it is well established that the magnitude of certain Dectin-1-stimulated responses can be amplified by collaboration with TLR2 (Toll-like receptor 2), which signals via the adaptor protein, MyD88 (myeloid differentiation primary response 88) (Dennehy et al., 2008; 2009; Gersuk et al., 2006; Goodridge et al., 2007; Rogers et al., 2005; Shin et al., 2008). Responses such as phagocytosis, arachidonic acid release, NF-κB-dependent transcription, and the production of IL-1β, IL-10, IL-12 and TNF-α are even enhanced synergistically when Dectin-1 and TLR2 are stimulated simultaneously (Brown et al., 2003; Dennehy et al., 2008; 2009; Gantner et al., 2003; Hise et al., 2009; Shin et al., 2008; Suram et al., 2006). These studies are probably better representations of anti-fungal immune responses, since fungal particles do not consist solely of β-glucan but contain a variety of other PAMPs that can be detected by other pattern recognition receptors (Bourgeois and Kuchler, 2012; Netea et al., 2006; Romani, 2011). Perhaps some of these receptors may also collaborate with Dectin-1 (Drummond et al., 2011; Hardison and Brown, 2012; Kerrigan and Brown, 2010; Plato et al., 2013).

We have now described the signaling cascades that result from engagement of Dectin-1. Next, we will introduce the various β-glucan ligands,

soluble and particulate, that are relevant to our investigation.

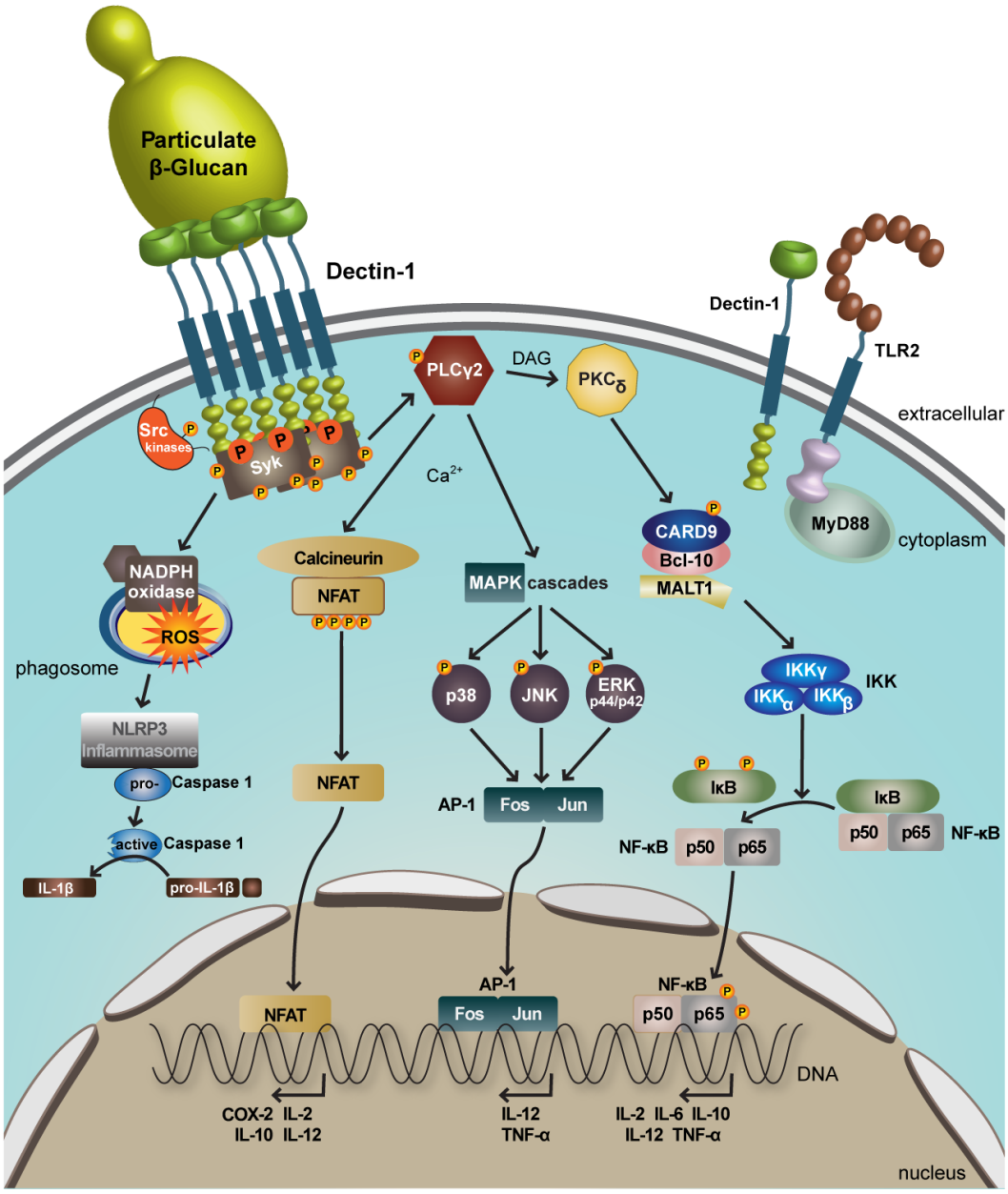

Figure 5: Selected Dectin-1-Stimulated Signaling Events and Responses

This schematic diagram illustrates selected cellular signal transduction players, pathways, and responses activated upon Dectin-1 stimulation by yeast cells or particulate β-glucan preparations such as zymosan and curdlan (see text for a detailed description). The downstream cellular responses depicted include reactive oxygen species (ROS) generation, transcription of genes encoding various cytokines and cyclooxygenase-2 (COX-2), and activation of the pro-inflammatory cytokine interleukin 1β (IL-1β) by caspase 1-dependent proteolytic cleavage. The induction of certain responses may be enhanced by simultaneous stimulation of TLR2-/MyD88-dependent signaling (top right, pathways not shown).

1.7. β-Glucan Ligands Relevant to This Study

1.7.1. A. Zymosan

Zymosan is a crude, water-insoluble, particulate yeast cell wall extract with immunostimulatory properties (Brown, 2006; Daum and Rohrbach, 1992; DI CARLO and FIORE, 1958; Pillemer and Ecker, 1941). It is derived from *Saccharomyces cerevisiae* and generated by boiling the cells prior to and after trypsin digestion (Albeituni and Yan, 2013). Resembling hollowed "ghosts" of yeast cells with an average diameter of 3 μm, it consists of approximately 55% β-glucan, 19% mannan, 15% protein, and 1% chitin (DI CARLO and FIORE, 1958). Primarily β-glucan, it has been used as a classical and prototypical ligand for Dectin-1 (Brown and Gordon, 2001a; Brown et al., 2002; 2003; Gantner et al., 2003; Rogers et al., 2005; Underhill et al., 2005). Even earlier, zymosan was a ligand for an unidentified β-glucan receptor (Daum and Rohrbach, 1992). Zymosan can also bind and stimulate another pattern recognition receptor, TLR2 (Underhill et al., 1999), although this is due to the presence of the non-β-glucan constituents, as treatment with alkali can eliminate zymosan's TLR-stimulating activities but retain its abilities to bind and activate Dectin-1 (Gantner et al., 2003). Still, out of all the components, β-glucan was found to be responsible for zymosan's biological activity (RIGGI and Di Luzio, 1961) and fittingly, cellular recognition of and response to zymosan is dependent on Dectin-1 expression (Brown et al., 2003; Gantner et al., 2003). Because of its large, particulate nature, zymosan has since been used as a ligand in many studies characterizing Dectin-1-mediated phagocytosis (Goodridge et al., 2007; Herre et al., 2004; Kennedy et al., 2007; Underhill et al., 2005).

1.7.2. Whole Glucan Particle (WGP)

Yeast cell wall glucan is composed primarily of β-(1,3)-linked backbone homopolymers with branches connected by β-(1,6)-linkages (Manners et al., 1973). An alkali-insoluble isolate of this component is known as whole glucan particle (WGP), which are particulate, water-insoluble hollow spheres ("ghosts") of purified β-glucan between 2 – 6 μm in size (US Patent #5082936A) (Albeituni

and Yan, 2013; Jamas et al., 1992). As a testament to their purity, unlike zymosan, WGP does not contain any TLR-stimulating activity (Goodridge et al., 2011). To enhance its structural, physical, and biological activation properties, WGP is prepared from genetically-modified *Saccharomyces cerevisiae*, which produce a higher frequency of β-(1,6)-linkages than wild-type cells (Ha et al., 2002; Jamas, 1990; Jamas et al., 1992; 1986). WGP has been found to be an effective anti-tumorigenic agent alone (Li et al., 2010; Qi et al., 2011) and in combination with tumour-specific monoclonal antibodies (Cheung et al., 2002; Hong et al., 2004; Li et al., 2006; Liu et al., 2009), an adjuvant or vehicle for vaccine delivery (US Patent #5607677A) (Easson et al., 1997a), and can induce production of co-stimulatory molecules and pro-inflammatory cytokines in macrophages (Li et al., 2007).

1.7.3. Soluble WGP

WGP can be solubilized to a substance known as neutral soluble β-glucan. The intent of this was to produce an therapeutic agent that activated an immune response without the unwanted effects of fever and the release of the pro-inflammatory cytokines TNF-α and interleukin 1 that occur with WGP administration (US patent #5622939A) (Easson et al., 1997b). The procedure is summarized as follows: 1) particulate WGP is solubilized by acid treatment, 2) the resulting heterogeneous molecules associate in various higher-order conformations in solution, so these associations are denatured in alkali; 3) specific molecular weight fractions of interest are purified and then 4) re-annealed to a triple-helical conformation, with 5) final purification steps to remove conformational anomalies like single helices and aggregates. The final product is a chemically unmodified, conformationally homogeneous water-soluble β-glucan of specific size (Easson et al., 1997b).

For our investigations, we purchased a water-soluble β-glucan derived from WGP known as soluble Wellmune WGP (soluble WGP or WGPsol) that is currently used as a food additive with immunostimulatory effects (Biothera). Although we do not have access to the method by which it was prepared, we are

aware that WGPsol is produced by dis-aggregating WGP by a non-enzymatic mechanism (personal communication, Dr. D. Cox, Biothera, Eagan, MN, USA, 2013). It is plausible then that WGPsol is prepared from WGP by a procedure similar, at least in some aspects, to the method for generating neutral soluble β-glucan (Easson et al., 1997b). Indeed, acid-catalyzed hydrolysis has been used to prepare soluble β-glucans of specific molecular weight from WGP (Goodridge et al., 2011), although how closely the resulting molecules resemble the WGPsol we used remains undetermined.

1.7.4. Curdlan

Curdlan is a linear, unbranched β-(1,3)-glucan found in the capsules of certain bacteria, such as the rhizobiaceae (Harada et al., 1968; Lee, 2005; Stone, 2009). As a high molecular weight β-glucan, it can be up to ~2 X 10^6 Da, which equates to a polymer containing ~12,000 glucose residues (FUTATSUYAMA et al., 1999; Stone, 2009). It is a particulate, water-insoluble β-glucan but can be dissolved in dilute alkali solution (Stone, 2009). In its particulate state, the particles are large enough at >100 μm (and sometimes >200 μm) in diameter that they cannot be phagocytosed by macrophages and dendritic cells (Hernanz-Falcón et al., 2009; Rosas et al., 2008). While commonly used as a thickening agent in the food industry (Lee, 2005), curdlan is a well-characterized Dectin-1-selective, TLR-independent ligand (Ferwerda et al., 2009; Gringhuis et al., 2009; Hernanz-Falcón et al., 2009; LeibundGut-Landmann et al., 2007; 2008; Palma et al., 2006; Rosas et al., 2008; Yoshitomi et al., 2005). It is a potent activator of molecules in the Dectin-1 signaling pathways, such as PLCγ2 (Tassi et al., 2009; Xu et al., 2009b), PKCδ (Strasser et al., 2012), NF-κB (Gringhuis et al., 2009), the NLRP3 inflammasome (Kankkunen et al., 2010; Kumar et al., 2009), and calcineurin (Greenblatt et al., 2010), and stimulates cytokine secretion (Hernanz-Falcón et al., 2009; Rosas et al., 2008) (see **Figure 5**). As a potent immunostimulant, curdlan stimulation of Dectin-1 induces TLR-independent dendritic cell maturation, resulting in the differentiation of naïve $CD4^+$ and $CD8^+$ T cells to helper and cytotoxic T lymphocytes, respectively, and antibody production in B cells (Agrawal et al., 2010; LeibundGut-Landmann et al., 2007; 2008; Tassi et al.,

2009). Furthermore, curdlan acts as an immunotherapeutic adjuvant in mice, promoting the response of cytotoxic and helper T lymphocytes challenged with foreign antigens and tumours (Agrawal et al., 2010; LeibundGut-Landmann et al., 2007; 2008).

Professor David Bundle's group (Department of Chemistry, University of Alberta, Edmonton, AB, Canada) was able to generate a water-soluble version of curdlan upon phosphorylation of the particulate material for our use. This molecule was then demonstrated to stimulate Syk phosphorylation and NF-κB nuclear translocation in bone marrow-derived macrophages (Lipinski et al., 2013) (see **Figure 5**).

1.7.5. Laminarin

Laminarin is a low molecular weight (7.7 kDa (Mueller et al., 2000)), water-soluble β-glucan isolated from chromistan brown algae (Myklestad and Granum, 2009; Stone, 2009). Its main chain is short, with a degree of polymerization of generally 20 – 30 and up to 40 glucose residues (Chizhov et al., 1998; Read et al., 1996). Occasional β-(1,6)-linked side-branches of a single glucose or mannitol residue can be found in the molecule, with approximately one branch every ten main chain residues (Chizhov et al., 1998; Read et al., 1996; Rolf et al., 1985; Williams et al., 1991a). In aqueous solution, strands of laminarin molecules assemble in a triple-helical conformation (Williams et al., 1991b). In the Dectin-1 field, laminarin is frequently used as a representative of small, low molecular weight ligands as well as an inhibitor of the cellular responses to particulate β-glucans (Brown et al., 2002; Gantner et al., 2003; 2005; Underhill et al., 2005). It antagonizes these responses by binding to Dectin-1 and blocking particulate β-glucans from interacting with the receptor, without inducing Dectin-1 signaling on its own (Fang et al., 2012; Gantner et al., 2003; 2005; Goodridge et al., 2011; Lowe et al., 2001).

1.8. Soluble β-Glucans and the Activation of Dectin-1 Signaling

In the Dectin-1 field, the ability for purified water-soluble β-glucans to

activate Dectin-1 signaling and cellular responses is currently divisive. On one hand, they are deemed as biologically inactive ligands to the receptor, unlike massive, water-insoluble, particulate ligands, which rapidly induce Dectin-1-dependent signaling (Brown, 2006; Brown et al., 2003; Goodridge et al., 2011; Kennedy et al., 2007; Michalek et al., 1998; Underhill et al., 2005), a model supported most notably by recent findings from Goodridge and coworkers (Goodridge et al., 2011). Despite being able to bind Dectin-1, soluble β-glucans have been found unable to initiate Dectin-1-mediated signaling and cytokine production *in vitro* (Brown et al., 2003; Gantner et al., 2003; 2005; Goodridge et al., 2011; Lowe et al., 2001; Michalek et al., 1998; Palma et al., 2006). For example, Goodridge and coworkers found that soluble fragments generated from acid hydrolysis of the particulate β-glucan WGP were unable to stimulate Dectin-1 signaling events, including Syk or p38 MAP Kinase activation, NFAT induction, or production of TNF-α and ROS, although they were capable of binding Dectin-1. Laminarin, too, was unable to induce production of TNF-α or ROS on its own (Goodridge et al., 2011). In another study, laminarin and β-glucan oligosaccharides of five to seven residues long were unable to stimulate the binding of NF-κB to its consensus DNA sequence in the nucleus, as detected by electrophoretic mobility shift (Lowe et al., 2001). Soluble β-glucans are even considered inhibitors or antagonists of Dectin-1 activation (Palma et al., 2006). When they are bound to the receptor before or during stimulation with particulate β-glucans, the particulate ligands are blocked from binding and thus cannot initiate Dectin-1 signaling. In fact, laminarin and glucan phosphate, a ~110 kDa triple-helical, water-soluble β-glucan preparation from the yeast *Saccharomyces cerevisiae* (Williams et al., 1991a), have been used frequently to block particulate β-glucans from binding and activating Dectin-1 (Brown and Gordon, 2001a; Brown et al., 2002; 2003; Elsori et al., 2011; Gantner et al., 2005; Goodridge et al., 2007; Kataoka et al., 2002; Kennedy et al., 2007; Olsson and Sundler, 2007; Palma et al., 2006; Rogers et al., 2005; Taylor et al., 2002; Toyotome et al., 2008; Underhill et al., 2005; Willment, 2001; Xu et al., 2009b), a practice that continues today (Fang et al., 2012). Even prior to the identification of Dectin-1, the binding

of particulate β-glucans to cells (and subsequently, signaling events and particle phagocytosis) was inhibited by treatment with soluble β-glucan preparations (Adachi et al., 1997; Czop and Austen, 1985; Daum and Rohrbach, 1992; Giaimis et al., 1993; Goldman, 1988; Hoffman et al., 1993; Ishibashi et al., 2001; Janusz et al., 1986; Kadish et al., 1986; Konopski et al., 1991; Okazaki et al., 1995; Tapper and Sundler, 1995; Vassallo et al., 2000). In summary, their inability to stimulate signal transduction events and use as receptor antagonists has likely led to the notion that soluble β-glucans are biologically inactive Dectin-1 ligands.

On the contrary, there are data demonstrating that some soluble β-glucans can be biologically active. An alternative proposal, then, is that larger, structurally complex β-glucans (for instance, with higher molecular weight and increased branching frequency) are more able to activate Dectin-1 signaling than smaller, structurally simple β-glucans (Batbayar et al., 2012; Brown and Gordon, 2003; Brown and Williams, 2009). As this model does not dictate whether the larger ligands must be soluble or particulate in nature, the findings of Goodridge *et al.* (Goodridge et al., 2011) are still compatible with the model. However, data demonstrating the size-dependent immunomodulatory effects by soluble β-glucans can also be taken into account. In one study, for example, while laminarin was unable to, a larger, 300 kDa soluble β-glucan from the mushroom *Grifola frondosa* induced Syk and NF-κB activation as well as TNF-α and interleukin 6 secretion in murine peritoneal macrophages. These signaling events were shown to be Dectin-1-dependent (Fang et al., 2012). In a different study, grifolan, another β-glucan preparation from the same mushroom, was fractionated by water solubility and size and incubated with RAW 264.7 cells. The large, particulate fraction stimulated the most TNF-α production from the cells, followed by the high molecular weight soluble fraction (≥100 kDa), which induced moderate TNF-α release. The low molecular weight soluble fraction induced only minimal TNF-α secretion (Ishibashi et al., 2001). A similar correlation between molecular weight and TNF-α secretion was found for fractions of higher molecular weight, gel-forming soluble grifolan (50 kDa to ≥450 kDa) (Okazaki et al., 1995). *In vivo*, laminarin was also unable to trigger

innate immune responses that promoted survival in a mouse model of polymicrobial infection and sepsis. The higher molecular weight soluble β-glucans scleroglucan and glucan phosphate were able to, however (Williams et al., 1999). Finally, in an early study that examined the stimulatory effects of carbohydrates on murine peritoneal macrophages, laminarin was deemed non-stimulatory compared to large, particulate yeast β-glucan. However, chemically cross-linking laminarin molecules rendered the product as stimulatory as the yeast glucan (Seijelid et al., 1981). Thus, there is evidence that some soluble β-glucans have the capacity to stimulate immune cells and that this is correlated to their size and structural complexity. While it is not entirely clear which β-glucan receptor(s) these ligands were activating, perhaps their effects could have been mediated by Dectin-1.

Regardless of the model, two molecular mechanisms may underlie these differential capacities for β-glucans to activate Dectin-1 signaling. Firstly, differences in binding affinity could result from the structural complexity of the ligands. Indeed, while β-glucans of more than seven main chain glucose residues are able to bind Dectin-1 (Adams et al., 2008; Brown and Gordon, 2001a; Palma et al., 2006), soluble β-glucans with longer main chain length or containing β-(1,6)-linked branches of single glucose residues exhibit increased binding affinity to the receptor (Adams et al., 2008). Similar effects of branching and molecular weight on ligand binding affinity have been observed for unidentified β-glucan receptors (Mueller et al., 2000; Tapper and Sundler, 1995). These effects could be due to cooperativity, with the binding of each glucose residue to Dectin-1 increasing the proximity of other residues in the polymer chain to the receptor and thereby increasing the likelihood of interaction. As such, in a complex β-glucan, the existence of branches and/or longer main chains would make it easier for more glucose residues in the molecule to interact with the receptors, increasing the binding affinity of the ligand relative to a smaller, simpler β-glucan. It could well be that a sustained, high affinity ligand-receptor interaction is required to induce the conformational changes needed in Dectin-1 to initiate signaling events. Secondly, Dectin-1 dimerization is likely required to recruit and activate Syk, an

early event in Dectin-1 signaling (Section 1.6.4). Perhaps high molecular weight, structurally complex ligands induce more clustering of the receptor than small ligands (Adams et al., 2008; Brown and Williams, 2009; Goodridge et al., 2011; 2012; Lowe et al., 2001; Michalek et al., 1998; Qi et al., 2011; Tapper and Sundler, 1995), rendering them more capable of activating Syk and initiating the downstream signaling events. There has even been speculation that β-glucans must exceed a specific size threshold to cross-link enough Dectin-1 molecules for signaling to be initiated (Brown and Williams, 2009), although this has yet to be demonstrated. The formation of receptor multimers on the plasma membrane could also serve a different purpose. Receptor clustering could further increase the binding affinity of the ligand to the receptor, with the clusters making multiple contacts throughout the length of the β-glucan, stabilizing the overall receptor-ligand interaction (Adams et al., 2008; Tapper and Sundler, 1995) and promoting conformational changes necessary for receptor activation. Therefore, both increased ligand affinity and heightened receptor cross-linking provide molecular explanations to why larger, more complex β-glucans could be more potent activators of Dectin-1 signaling.

To conclude, in the Dectin-1 field, it has been proposed that unlike particulate β-glucans, soluble β-glucans are biologically inactive Dectin-1 ligands. However, there is evidence that some soluble β-glucans, particularly those with high molecular weight, are able to stimulate immune cells. Although it is uncertain if many of these stimulatory effects were Dectin-1-dependent, it is plausible that the receptor could be involved and elicit cellular responses to larger, structurally complex soluble β-glucans, just as it responds to particulate β-glucans. The molecular mechanisms behind this could involve a combination of ligand-induced receptor clustering and high affinity ligand binding interactions. Currently, research from our laboratory seeks to verify if certain soluble β-glucans are indeed capable of activating Dectin-1 signaling and cellular responses, and to determine which molecular mechanisms may underlie the differential capacities for β-glucans to activate Dectin-1.

1.9. Rationale and Objectives

Much of the molecular research on Dectin-1 has focused on its interaction with large, particulate β-glucans, such as zymosan, WGP, and curdlan, and their internalization by phagocytosis (Goodridge et al., 2011; Heinsbroek et al., 2009; Hernanz-Falcón et al., 2009; Herre et al., 2004; Hino et al., 2012; Ma et al., 2012; Mansour et al., 2013; McCann et al., 2005; Nakamura and Watanabe, 2010; Rosas et al., 2008; Shah et al., 2009; Strijbis et al., 2013; Underhill et al., 2005; Xu et al., 2009a). In contrast, soluble β-glucans, and their internalization (likely by receptor-mediated endocytosis), are less well studied. As described above, they are sometimes deemed as biologically inactive and often considered as inhibitors of Dectin-1. Nevertheless, the effects of soluble β-glucans on Dectin-1 are an interesting avenue of investigation and warrant further study. Numerous reports, some of which were published before the identification of Dectin-1 as the β-glucan receptor, illustrate the stimulatory effects of therapeutic soluble β-glucans on the immune system (Albeituni and Yan, 2013; Batbayar et al., 2012; Bohn and BeMiller, 1995; Brown and Williams, 2009; Chan et al., 2009; Chen and Seviour, 2007; Fang et al., 2012; Ross et al., 1999; Vannucci et al., 2013; Williams, 1997), although the precise mechanisms of how they stimulate the immune system are still being determined (Fang et al., 2012; Masuda et al., 2012). It is possible that these compounds could be exerting their biological effects through the binding and activation of Dectin-1 (Brown and Gordon, 2003). The study of soluble β-glucans has other applications. As discussed previously, they are present in the blood during fungal infection, and expulsion of soluble β-glucans into the extracellular space occurs upon phagocytosis and digestion of β-glucan particles by macrophages. It is possible then that these soluble β-glucans are able to bind Dectin-1 and elicit continued biological effects. As well, soluble β-glucans can be used in the design of anti-fungal therapies. For instance, an anti-fungal vaccine consisting of laminarin, a soluble β-glucan, coupled to a carrier protein, diphtheria toxoid, was able to protect against candidiasis (Pietrella et al., 2010). Such work is critical to combat the recent rise in invasive fungal infections (Armstrong-James et al., 2014; Brown et al., 2012a; 2012b).

The aim of our study was to investigate what effect soluble β-glucans have on Dectin-1 upon binding to the receptor, with an emphasis on receptor-mediated endocytic dynamics. This would mirror previous studies examining the events of Dectin-1 phagocytosis upon binding of the particulate ligand zymosan. Study of endocytic events could provide molecular details explaining the immunostimulatory effects of therapeutic soluble β-glucans, and contribute to knowledge of what happens to soluble β-glucans released during invasive fungal infection. Our findings could also provide insight to the mechanism of action of β-glucan-containing vaccines, and contribute to the design of more efficacious therapies. Of interest to us too was if differences in endocytosis and trafficking could underlie differences for β-glucans to activate Dectin-1.

We addressed the aim using a 2-pronged approach. We first sought to develop biochemical and imaging tools to visualize Dectin-1 endocytosis and trafficking. Once the tools were established, we pursued characterization of the endocytic pathway by addressing the following questions: 1) Is Dectin-1 internalized upon ligand stimulation? If so, what are the kinetics of Dectin-1 uptake? 2) What cellular machinery does Dectin-1 use to be taken up into the cell? 3) Once in the cell, where is Dectin-1 trafficked? What is its fate? 4) Is the endocytosis of Dectin-1 related to its ability to signal, and *vice versa*? These research questions were investigated for both low and high molecular weight β-glucans to see if the difference in ligand size produced any bifurcations in the results. The following chapters discuss the methods used in the study and the results obtained, followed by their interpretation and considerations for future research.

Chapter 2. MATERIALS AND METHODS

2.1. Reagents

2.1.1. Miscellaneous Reagents

Unless stated otherwise, general laboratory reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pitstop 2 and dyngo-4A were purchased from Abcam (Cambridge, MA, USA). PP2, piceatannol, and Syk inhibitor IV (BAY 61-3606) were purchased from EMD Millipore (Billerica, MA, USA). Transferrin-AF488 and phalloidin-AF488 were obtained from Life Technologies (Carlsbad, CA, USA). Cytochalasin B and dynasore were from Sigma-Aldrich.

2.1.2. Antibodies

Antibodies raised against the following epitopes were used in this study for immunofluorescence staining: Clathrin Heavy Chain (#ab21679) from Abcam (Cambridge, MA, USA); mouse immunoglobulin G 2B negative/isotype control from AbD Serotec (Raleigh, NC, USA); (1→3)-β-glucan from Biosupplies Australia (Bundoora, VIC, Australia); P-Syk at Y352 (#2701) and Rab 7 (D95F2, #9367) from Cell Signaling Technology (Danvers, MA, USA); Giantin (#PRB-114C) from Covance (Princeton, NJ, USA); LAMP2 (#ABL-93) from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA); rat anti-mouse Dectin-1 (#MAB17561), mouse anti-human Dectin-1 (#MAB1859), and goat anti-human Dectin-1 (#AF1859) from R&D Systems (Minneapolis, MN, USA); NF-κB p65 (#sc-8008) from Santa Cruz Biotechnology (Dallas, TX, USA).

Antibodies raised against the following epitopes were used for immunoblotting: Clathrin Heavy Chain (#610500) from BD Biosciences (San Jose, CA, USA), HA epitope (HA.11 clone 16B12, #MMS-101R) from Covance (Princeton, NJ, USA); P-Src at residue Y416 (#2101), P-Syk at residues Y525/526 (#2711), P-Syk at Y352 (#2701), P-PLCγ2 at Y579 (#3874), P-PKCδ at

Y311 (#2055), P-p44/42 MAP Kinase (ERK1/ERK2) 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) from Sigma-Aldrich (St. Louis, MO, USA).

HRP-, Cy3- and DyLight 649- 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).

2.1.3. β-Glucans

Curdlan from *Alcaligenes faecalis* and laminarin from *Laminaria digitata* were from Sigma-Aldrich. Soluble Wellmune WGP (WGPsol) manufactured by Biothera (Eagan, MN, USA) was obtained from Invivogen (San Diego, CA, USA) and from Quadra Ingredients (Vaudreuil-Dorion, QC, Canada).

We are fortunate to have as our collaborators Professor David Bundle (Department of Chemistry, University of Alberta, Edmonton, AB, Canada) and members of his laboratory, Tomasz Lipinski, Adam Szpacenko, and Pui-Hang Tam, who contributed greatly to the preparation and characterization of several ligands. To solubilize curdlan, Bundle and coworkers prepared a phosphorylated derivative by reacting curdlan with phosphoric acid (Lipinski et al., 2013), based on a procedure for preparing the soluble β-glucan glucan phosphate (Williams et al., 1991a). The resultant compound was termed phospho-curdlan (P-curdlan).

Protein-β-glucan conjugates, where multiple (e.g. 17) laminarin molecules were covalently coupled to bovine serum albumin (BSA), were also prepared by Bundle and coworkers and termed BSA-*n*-laminarin (e.g. BSA-17-laminarin).

Prof. Bundle's group also assisted in the generation of fluorescently labeled BSA-laminarin conjugates. This was accomplished by reacting succinimidyl ester-AF546 (Life Technologies) with amine groups present in the ligands, as previously described (Lipinski et al., 2013).

The soluble β-Glucans P-curdlan, 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 their sterility was actively maintained. Immediately prior to use, the stock solutions were warmed to 37°C for 5-10 minutes, mixed on the vortex for 1 minute to promote maximum dissolution, and centrifuged at 18000x g for 2 minutes at 37°C to pellet aggregates or insoluble components. They were then diluted to a working concentration of 100 μg/mL in warmed media for stimulation of the cells.

2.2. Cell Culture and Stable Expression of Dectin-1 Constructs

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) CO_2 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. During RNA interference experiments, 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, DNA of 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 (neo^R). 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".

To generate signaling-deficient Dectin-1, a missense mutation was introduced in the cytoplasmic tail of the protein, where the tyrosine residue at position 15 (Ariizumi, 2000; Brown et al., 2003; Gantner et al., 2003; Rogers et al., 2005) was substituted with alanine, similar to Y15S and Y15F mutants generated previously (Brown et al., 2003; Fang et al., 2012; Gantner et al., 2003; Herre et al., 2004; LeibundGut-Landmann et al., 2007; Rogers et al., 2005; Toyotome et al., 2008; Underhill et al., 2005). This was performed by polymerase chain reaction using the Quikchange II Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions. The pFB-Neo-Dectin-1 construct served as the DNA template, and the following oligonucleotides served as primers (nucleotides varying from the original Dectin-1 sequence are underlined):

5'-gatttagaaaatttggatgaagatggagctactcaattacacttcgactctcaaag-3' (sense), and

5'-ctttgagagtcgaagtgtaattgagtagctccatcttcatccaaattttctaaatc-3' (anti-sense). After amplification, template DNA was digested with *Dpn I* endonuclease, leaving the mutation-containing synthetic DNA, which was transformed into XL1-Blue Supercompetent *E. coli* cells. Plasmidic DNAs from several resulting colonies were isolated and sequenced to confirm appropriate mutagenesis. DNA from one colony was amplified in a culture of Library Efficiency DH5α Competent Cells (Life Technologies), purified with the Plasmid Maxi Kit (Qiagen), and stably expressed in RAW 264.7 cells by the retroviral transduction procedure described

above. The resultant cells were termed “RAW Dectin-1-Y15A”.

Bone marrow-derived macrophages (BMDMs) were a generous gift from Professor Shairaz Baksh (Department of Pediatrics, University of Alberta, Edmonton, AB, Canada), and generated from bone marrow cells extracted from wild-type C57BL/6 mice.

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 were performed in HEPES-buffered RPMI media (H-RPMI, Wisent Bioproducts) or serum-free MEMα (Life Technologies). Quick rinses were performed with PBS solution.

2.3. DNA Constructs

2.3.1. Sources

mEmerald is a monomeric derivative of green fluorescent protein (GFP) with enhanced brightness and photostability, while mApple is a bright, monomeric variant of the red fluorescent protein mRFP1 with optical properties similar to the AF568 dye (Rizzo et al., 2009). A DNA construct encoding a fusion protein of mEmerald, a ten amino acid linker, and human Dectin-1 (Genbank no. NM_197947.2), at the C-terminus, was a gift from Professor Michael Davidson (Optical Microscopy Division, National High Magnetic Field Laboratory, Florida State University) and termed “mEmerald-Dectin-1”. The same construct, with mApple instead of mEmerald, was also provided by Prof. Davidson, and termed “mApple-Dectin-1”.

Plasmids encoding GFP- or HA-tagged cDNAs for various vesicular markers, endocytic players, and proteins of interest were used for fluorescent microscopy in our investigations. These included Rab4A-GFP, Rab5A-GFP, and

Rab11A-GFP, gifts from Professor John Brummell (The Hospital for Sick Children, Toronto, ON, Canada); and Syk-GFP, Rab5-GFP, Clathrin-GFP, Dynamin2-K44A-HA, and VAMP3-GFP, gifts from Professor Sergio Grinstein (The Hospital for Sick Children, Toronto, ON, Canada).

2.3.2. 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 200-250 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 concentration of DNA in solution was determined by measuring its absorbance of 260 nm light with a Nanodrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, Rockford, IL, USA) and adjusted to between 1-2 μg/mL.

2.3.3. Transient Expression of DNA Constructs by Lipofection

RAW 264.7 and HeLa cells were seeded on 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 transfection mixture was prepared, 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.

2.4. RNA Interference

A SMARTpool of four ON-TARGETplus short interfering RNAs (siRNAs) against mouse Clathrin Heavy Chain (Dharmacon, Lafayette, CO, USA) was resuspended to a stock solution of 20 μM according to the manufacturer's directions. 24 hours prior to transfection, 4 X 10^5 RAW Dectin-1 cells were plated per well in a 12-well plate.

Immediately before transfection, the cells were rinsed three times in sterilized PBS and replaced with 0.5 mL of Opti-MEM I Reduced Serum Media (Life Technologies). For each transfection, 5 μL of Lipofectamine 2000 (Life Technologies) and 100 pmol of siRNA were each resuspended in separate tubes containing 0.25 mL of Opti-MEM I. The contents of both tubes were combined, resuspended, and left for 30 minutes at room temperature, after which the entire volume was added to one well of cells for 4 hours. The transfection volume was subsequently replaced with MEMα + 10% heat inactivated fetal bovine serum for 20 hours. A second, identical transfection was performed 24 hours later. Experiments were performed on the cells 48 hours after the first transfection. The silencing of Clathrin Heavy Chain was compared to cells that were mock-transfected with Lipofectamine 2000 and grown in parallel. Depletion efficiency was confirmed by immunoblotting and immunofluorescence.

2.5. SDS-PAGE and Immunoblotting of Cell Lysates

2.5.1. 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.

2.5.2. 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, resulting 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.

2.5.3. 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, Hercules, CA, USA) 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) 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) 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.

2.5.4. Immunoblotting

Nitrocellulose membranes were blocked at room temperature for 1 hour in 3% gelatin from cold water fish skin (Sigma-Aldrich) dissolved in PBS + 0.1% Tween-20 (together, referred to as “IB Blocking Buffer”). Primary antibodies

raised 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 PBS + 0.1% Tween-20 (PBSTw) 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) 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, Basel, Switzerland) for 2 minutes, and exposing them to autoradiography film (Super RX Fuji Medical X-Ray Film, Fujifilm, Tokyo, Japan) in the dark room. Films were developed with an SRX-101A Medical Film Processor (Konika Minolta Medical & Graphic, Tokyo, Japan).

When membranes were processed for detection by fluorescence, the appropriate IRDye-coupled secondary antibodies (LI-COR Biosciences) were diluted in IB Blocking Buffer and bound to the membranes for 1 hour at room temperature. Three 5 minute washes in PBSTw, and three more 5 minute washes in PBS, were then performed. Immunoblotted proteins were detected using an Odyssey Infrared Imaging System scanner (LI-COR Biosciences), with acquisition in the 700 and 800 nm channels.

When necessary, 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 PBSTw.

2.5.5. Quantification of Immunoblots

When chemiluminescence was used for detection, the resulting films were scanned to obtain a digital image. When fluorescence was used to detect immunoblots, the images were subsequently contrast-enhanced in Photoshop CS4 (Adobe Systems) for presentation in this manuscript. For either detection method, care was taken to ensure the immunoreactive bands quantified were not above saturation. The intensities of the bands were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/).

2.5.6. Statistics

Data values were entered into Prism Version 6.0c (Graphpad Software, La Jolla, CA, USA) and are represented as mean ± standard error (SEM). For experiments performed at least three times, paired, two-tailed parametric *t* tests were performed with the software to determine the statistical significance of the differences between the means of two conditions. Statistical significance is represented by the following symbols in the figures: not significant (ns), $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****).

2.6. Deglycosylation of Glycoproteins

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 detection of Dectin-1. Electrophoretic mobility shifts in enzyme-treated Dectin-1 led to insights on its glycosylation and biosynthesis.

2.7. Chemical Cross-Linking

Cells were seeded on 6-well plates and grown for 18 hours to achieve

100% confluency. After 10 minutes 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, thiol-cleavable cross-linker DTSSP (3,3'-dithiobis(sulfosuccinimidylpropionate), Thermo Scientific) at 2 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, then separated into two halves of equal volume. Dithiothreitol was added to the first half to a concentration of 50 mM to reduce DTSSP; the second half was left untreated. Both halves were incubated at 37°C for 30 minutes. 4X Sample Buffer without 2-mercaptoethanol was then added to each to a final concentration of 1X and the mixture was heated at 65°C for 5 minutes. Non-reducing SDS-PAGE on a 7.5% polyacrylamide gel and subsequent immunoblotting for Dectin-1 were performed.

2.8. Detection of Signaling Events

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. Next, β-glucan ligands were prepared in warmed serum-free MEMα and the cells were replaced with the ligand solution for the indicated times at 37°C. After three washes in cold PBS, the cells were lysed and prepared for SDS-PAGE as per Sections 2.5.1 and 2.5.2, 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 Diagnostics) prepared according to the manufacturer's instructions, pH 7.0.). SDS-PAGE and immunoblotting were performed.

2.9. Pharmacological Inhibition

Pharmacological inhibitors were dissolved in ultrapure water or 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.

2.10. Quantification of Endocytosis and Trafficking by Reversible Cell Surface Biotinylation

2.10.1. General Procedure

The cell surface biotinylation protocol was developed based on one described by Gabriel and coworkers (Gabriel et al., 2009). Cell viability was monitored visually by light microscopy and maintained throughout the procedure to ensure cell membranes remained intact.

RAW Dectin-1 cells were seeded on 6-well plates and incubated for 16 – 24 hours to achieve 80 – 100% confluency. The cells were chilled on ice to block endocytosis and surface-exposed proteins were biotinylated with the amine-reactive, membrane-impermeant, and thiol-cleavable reagent EZ-Link Sulfo-NHS-SS-biotin (Thermo Scientific). Here, 0.75 mL of 1 mg/mL EZ-Link Sulfo-NHS-SS-biotin in cold borate buffer (154 mM sodium chloride, 7.2 mM potassium chloride, 1.8 mM calcium chloride, 10 mM boric acid, pH 9.0) were added to each well for two 10- minute incubations with agitation. The reaction was quenched by two 5-minute washes in cold 100 mM glycine, with agitation. After three washes in cold PBS, endocytosis was triggered by warming the cells to 37°C in the presence of medium alone or containing soluble β-glucan ligands. After an elapsed time, the cells were washed in cold PBS and chilled on ice to inhibit endocytosis. The biotin label was then stripped from surface-remaining biotinylated proteins by the membrane-impermeant reducing agent tris(2-carboxyethyl)phosphine, or TCEP (Thermo Scientific). Each well was incubated in 1 mL of cold 100 mM TCEP in NT Buffer (150 mM sodium chloride, 1 mM EDTA, 0.2% BSA, 20 mM Tris, pH 8.6) for 5 minutes with gentle agitation. Three quick washes and three 2-minute washes in cold NT Buffer then served to

remove TCEP. Cell lysates then were prepared in TNT Buffer (see Section 2.5.1).

For 90% of the lysate volume, biotinylated proteins were pulled-down onto Streptavidin Plus UltraLink Resin (Thermo Scientific) by an overnight incubation on an orbital mixer at 4°C. The resin was washed five times with TNT Buffer to remove unbound protein. Pulled-down proteins were eluted from the resin at 60°C for 1 – 2 hours by the addition of 2X Laemmli Sample Buffer containing 10% 2-mercaptoethanol. The remaining 10% of lysate volume was kept to represent the whole cell lysate. Resin eluates and whole cell lysates were subsequently processed via SDS-PAGE and immunoblotted for detection of Dectin-1 or a loading control (actin, β-tubulin, or Dectin-1). The intensity of immunoreactive bands was quantified by densitometry with ImageJ software (National Institutes of Health) (Section 2.5.5). Before further quantitative analysis (Section 2.10.2), to account for differences in the amount of protein loaded on the resin, the intensity for each sample of pulled down Dectin-1 was then normalized to the intensity of a loading control in the corresponding whole cell lysate fraction.

2.10.2. Quantification of Receptor Internalization

Each biotinylation experiment contained two controls performed in parallel to the experimental conditions, *0 min* and *0 min + strip*. *0 min* was the amount of Dectin-1 pulled-down by streptavidin resin after the cells were biotinylated and immediately lysed, representing the total amount of Dectin-1 present at the cell surface at the time of biotinylation. *0 min + strip* was the amount of Dectin-1 pulled-down by streptavidin resin after the cells were biotinylated, TCEP-stripped, and lysed, and was used to determine the background signal remaining after stripping. To calculate the percentage of internalized Dectin-1, the intensity of the *0 min + strip* control was subtracted from that of Dectin-1 pulled-down from each experimental condition. The resulting value was divided by the intensity of the *0 min* control and multiplied by 100.

Hence,

$$\%\ \text{internalized receptor} = \frac{(\text{intensity of condition} - \text{intensity at "0 min + strip"}) * 100}{\text{intensity at "0 min"}}$$

It was also convenient to calculate *fold internalized Dectin-1*:

$$\text{fold internalized receptor} = \frac{(\%\ \text{internalized receptor})_{\text{condition of interest}}}{(\%\ \text{internalized receptor})_{\text{control condition}}}$$

The statistical significance in comparisons of *% internalized receptor* or *fold internalized receptor* between different conditions was determined as per Section 2.5.6.

2.10.3. Dectin-1 Endocytosis in the Presence of Endocytic and Signaling Inhibitors

The cell surface biotinylation procedure was performed as above (Section 2.10.1), except that after surface biotinylation and prior to ligand stimulation, the cells were pre-treated with endocytic or signaling inhibitors or vehicle for 5 – 30 minutes at 37°C. Dectin-1 was then stimulated by media containing the β-glucan ligand in the presence of the inhibitor of interest or the vehicle control.

2.10.4. Dectin-1 Endocytosis During Lysosomal Inhibition

The cell surface biotinylation procedure was performed as above (Section 2.10.1) except for a few additions. For leupeptin inhibition, 250 μM leupeptin was added to the cell culture media 2 hours prior to cell surface biotinylation as pre-treatment. After surface biotinylation, leupeptin at 250 μM was also present throughout the stimulation of the cells at 37°C.

For chloroquine inhibition, 250 μM chloroquine was added to the media during the 37°C ligand stimulation step, but 30 minutes after the step had begun. This was to prevent the inhibitor from disrupting endosomal pH during early internalization events.

2.10.5. Recycling of Internalized Dectin-1

A modification of the cell surface biotinylation procedure above (Section 2.10.1) was used to detect recycling of previously biotinylated receptor and was based off a procedure detailed by Nishimura and Sasaki (Nishimura and Sasaki, 2008). Briefly, two wells of cells were surface-biotinylated and Dectin-1 endocytosis was stimulated by treating them with soluble β-glucan ligands for 30 minutes at 37°C. After TCEP-stripping of the biotinyl-moiety from surface-remaining proteins, the pool of internalized biotinylated membrane proteins was allowed to traffic for another 30 minutes by an additional incubation at 37°C in medium without any ligands. After chilling the cells on ice to inhibit endocytosis, the two wells were processed in parallel, one being lysed immediately ("single strip") while the other was stripped for a second time with TCEP ("double strip"). Subsequent streptavidin pull-down and elution of biotinylated proteins, SDS-PAGE, and immunoblotting for Dectin-1 were performed as described in the general procedure (Section 2.10.1). A decrease in pulled-down Dectin-1 in the "double strip" condition compared to "single strip" suggests that the pool of internalized Dectin-1 receptor becomes accessible to TCEP stripping once again, implying that it is recycling to the plasma membrane. Care was taken to monitor and maintain cell viability throughout the procedure to ensure cell membranes remained intact.

2.11. Immunofluorescence Staining

2.11.1. General Procedure

The following describes general aspects of the immunofluorescence staining performed in this study. Modifications of this procedure to suit various experiments are detailed in the sections following. All solutions for the procedure were prepared in PBS.

2.11.1.1. Cells

For all immunofluorescence staining experiments, RAW 264.7 cells were seeded on 18 mm glass coverslips and grown for 16 – 24 hours.

2.11.1.2. Fixation

Prior to fixation, cells were washed three times in PBS. Fixation was performed in cold 4% paraformaldehyde (PFA) for 20 minutes, then quenched in cold 0.1 M ammonium chloride. Three post-fixation PBS washes ensured removal of unreacted fixative.

On occasion, a 10 minute treatment in 3% PFA + 0.1% glutaraldehyde at room temperature, followed by a 7 minute reduction of unreacted aldehyde moieties with 0.1% sodium borohydride at room temperature, was substituted for the fixation step mentioned above.

2.11.1.3. 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.

Membrane permeabilization was accomplished by incubating the cells in room temperature 0.1% Triton X-100 or 0.1% saponin for 20 – 30 minutes. When permeabilization was required, it was often performed simultaneously with blocking. When saponin was used, antibody binding and rinsing steps after permeabilization still included 0.1% saponin to maintain membrane permeability, except when anti-β-glucan antibodies were bound, as the detergent interfered with recognition of the epitope.

2.11.1.4. 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 the appropriate fluorescent dye-conjugated secondary antibodies for 30 minutes at room temperature. Unbound secondary antibodies were removed with six washes in PBS.

2.11.1.5. Preparation for Fluorescence Imaging

When immunostaining was complete, the cover slips 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, Burlington, ON, Canada). After hardening of the media, the slides were visualized on the confocal fluorescence microscope (Section 2.14).

2.11.2. Surface Targeting of Exogenously Expressed Dectin-1

RAW Dectin-1 cells were fixed in 4% PFA and blocked. 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 secondary antibodies were used. Confocal microscopy (Section 2.14) was used to visualize the samples. Puncta that overlapped in both fluorescent channels revealed surface-localized Dectin-1; puncta labeled only in AF488 identified intracellular populations.

2.11.3. Internalization of Surface-Localized Dectin-1

An antibody uptake assay was developed to label surface-localized Dectin-1, follow its endocytosis over a period of time, and distinguish between internalized and surface-remaining receptor. RAW Dectin-1 cells were chilled on ice, blocked in cold 5% donkey serum for 20 minutes, and bound with 0.4 μg/mL goat anti-hDectin-1 antibody for another 20 minutes in the same blocking solution. Six washes in cold PBS served to remove unbound antibody. The cells were incubated at 37°C in H-RPMI media lacking or containing soluble β-glucan ligands for various timepoints. Next, they were chilled on ice and washed three times in cold PBS to inhibit endocytosis, then fixed in 4% PFA and blocked. To label the surface-remaining fraction of antibody, a Cy3-coupled anti-goat secondary antibody was bound to the cells. After permeabilization in 0.1% Triton X-100 and blocking, the cells were incubated with an AF488-coupled anti-goat secondary antibody to label the entire population of anti-hDectin-1 antibodies. On the confocal microscope, internalized anti-hDectin-1 antibodies would be detected in only the AF488 channel, whereas surface-remaining antibodies would be detected in both AF488 and Cy3 channels. An increase in intensity of solely AF488-labeled features, with a concomitant decrease in dually-labeled features,

would suggest uptake of the antibody and thus internalization of Dectin-1.

2.11.4. Co-Detection of Surface-Localized Dectin-1, Total Dectin-1, and β-Glucan

RAW Dectin-1 cells were incubated at 37°C in MEMα lacking or containing soluble β-glucan ligands for various timepoints. Next, they were chilled on ice and washed three times in cold PBS to inhibit endocytosis, then fixed in 4% PFA. To label surface Dectin-1, cells were blocked and incubated with goat anti-hDectin-1 antibody, followed by DyLight 649-conjugated anti-goat secondary antibodies. To label total Dectin-1, the cells were permeabilized with 0.1% saponin, then incubated again with goat anti-human Dectin-1 antibody. To detect β-glucans, mouse anti-β-glucan antibody was bound to the cells. Co-incubation of AF488-coupled anti-goat and Cy3-coupled anti-mouse secondary antibodies completed the staining procedure.

2.11.5. Dectin-1 and Soluble β-Glucan Trafficking

RAW Dectin-1 cells were pulse-stimulated in MEMα containing soluble β-glucan ligands for 15 minutes at 37°C. After three PBS washes, the bound ligands were chased in MEMα for various timepoints, also at 37°C. The cells were then chilled on ice and washed three times in cold PBS to inhibit endocytosis, fixed in 4% PFA, and permeabilized with 0.1% saponin and blocked. Following were consecutive incubations with goat anti-hDectin-1 antibody, mouse anti-β-glucan antibody, and/or an antibody recognizing a marker of an intracellular compartment. A mixture of the appropriate AF488-, Cy3-, and/or DyLight 649-conjugated secondary antibodies was then bound.

2.11.6. Nuclear Translocation of NF-κB

RAW 264.7 cells were incubated for 20 minutes at 37°C with the appropriate soluble β-glucan ligands. Unbound ligand was removed with three washes of PBS. The cells were fixed in 4% PFA, permeabilized in 0.1% Triton X-100, and blocked. Incubation with antibodies raised against the p65 subunit of NF-κB, then AF488-conjugated secondary antibodies, followed. A 30-minute, room temperature staining with DAPI at 2 μg/mL was performed to label cellular

nuclei. The localization of p65 relative to the nuclei was examined under the confocal microscope at Z-sections passing through the nuclei.

2.12. Potassium Depletion to Inhibit Clathrin-Mediated Endocytosis

Experiments were performed with the potassium-depleted and potassium-supplemented conditions in parallel, according to Hansen and colleagues (Hansen et al., 1993a). First, the cells were washed twice in Depletion Buffer (140 mM sodium chloride, 20 mM HEPES, 1 mM calcium chloride, 1 mM magnesium chloride, 1 g/L D-glucose, pH 7.4) and replaced with Shock Buffer (1:1 Depletion Buffer : ultrapure water, pH 7.4) for 5 minutes, at 37°C, to induce a hypotonic shock. After 3 washes in Depletion Buffer, the cells were bathed in either Depletion Buffer or Supplementation Buffer (Depletion Buffer with the addition of 10 mM potassium chloride) for 30 min at 37°C. Dectin-1 was then stimulated with the β-glucan of interest, diluted in the same solutions.

2.13. Transferrin Uptake

Uptake of 5 μg/mL AF488-labeled transferrin served to visualize the internalization dynamics of the transferrin receptor when examined by confocal microscopy. Prior to stimulation with transferrin, the cells were serum starved for 30 minutes. After uptake, to improve visualization of transferrin in intracellular compartments, surface-remaining transferrin was dissociated from transferrin receptor by acid wash. This was done by placing the cells on ice and replacing the media with cold Acid Wash Buffer (150 mM sodium chloride, 200 mM acetic acid, pH 2.0) for 5 minutes, then replacing it with cold PBS for 1 minute to allow for recovery. Three acid washes were performed prior to fixation of the cells.

2.14. Confocal Microscopy and Digital 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, Tokyo, 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., Hamamatsu, 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, San Jose, CA, USA) for presentation.

Chapter 3. RESULTS

3.1. Expression of Functional Dectin-1 in RAW 264.7 Macrophages

3.1.1. Exogenous Expression of Dectin-1 in RAW 264.7 Macrophages

Dectin-1 is expressed on innate immune cells such as macrophages (**Figure 6C**), dendritic cells, and neutrophils (Ariizumi, 2000; Brown and Gordon, 2001a; Brown et al., 2002; Grünebach et al., 2002; Hermanz-Falcón et al., 2001; Kennedy et al., 2007; Reid et al., 2004; Taylor et al., 2002; Willment, 2001; Willment et al., 2005; Yokota et al., 2001). To study the endocytosis of Dectin-1 in a relevant system, we chose to work with the murine monocyte/macrophage cell line RAW 264.7. Since RAW 264.7 cells express endogenous Dectin-1 at low levels (Brown et al., 2003; Goodridge et al., 2011), we expressed full-length human Dectin-1 (isoform A) in the cells via retroviral transduction (**Figure 6**). Despite cell type-specific differences in expression of the B and A isoforms (Grünebach et al., 2002; Hermanz-Falcón et al., 2001; Weck et al., 2008; Willment, 2001; Willment et al., 2005), as many key studies in Dectin-1 biology have exogenously expressed full-length Dectin-1 in cells (Brown and Gordon, 2001a; Brown et al., 2002; 2003; Gantner et al., 2003; Goodridge et al., 2011; Hernanz-Falcón et al., 2009; Herre et al., 2004; Rogers et al., 2005; Underhill et al., 2005), we performed our investigations with the A isoform. Furthermore, the A isoform of human Dectin-1 is more efficiently targeted to the plasma membrane than isoform B (Kato et al., 2006). Immunofluorescence staining and immunoblotting detected expression of Dectin-1 in the transduced cells ("RAW Dectin-1" cells) but not the wild-type cells ("RAW WT" cells) (**Figure 6A**, **B**). The immunofluorescence images also revealed that exogenous Dectin-1 was appropriately targeted to the plasma membrane (**Figure 6A**). Upon SDS-PAGE, human Dectin-1 migrated to a similar position as endogenous mouse Dectin-1

expressed in bone marrow-derived macrophages (BMDMs) (**Figure 6B**, **C**), consistent with the mouse homologue containing a similar number of amino acids as the human receptor (244 amino acids versus 247, respectively) (Ariizumi, 2000; Hermanz-Falcón et al., 2001).

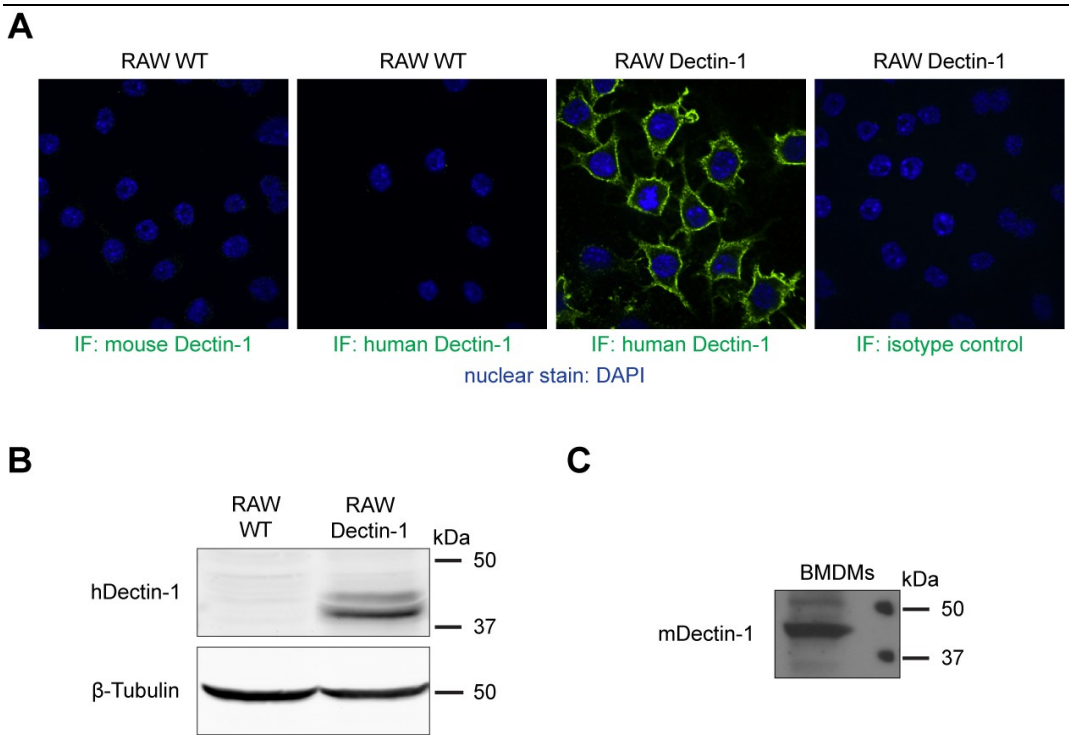

Figure 6: Expression of Dectin-1 in RAW 264.7 Cells and BMDMs

(**A**) The surface expression of endogenous mouse Dectin-1 and exogenous human Dectin-1 in RAW 264.7 cells (RAW WT) or RAW 264.7 cells transduced to express human Dectin-1 (RAW Dectin-1) was probed by immunofluorescence (IF). The cells were fixed and stained with rat anti-mouse Dectin-1, mouse anti-human Dectin-1, or mouse IgG_{2B} isotype control antibodies, followed by detection with the appropriate Cy3-coupled secondary antibodies (shown in green). Cell nuclei were stained with DAPI (blue). The same acquisition parameters were used for all images, and it was ensured the anti-human Dectin-1 antibody chosen did not cross-react with mouse Dectin-1. The lack of labeling of RAW Dectin-1 cells with the isotype control demonstrates the specificity of the anti-human Dectin-1 antibody to its antigen. (**B** and **C**) After SDS-PAGE of cellular lysates, immunoblotting was performed to detect expression of human (h) Dectin-1 in RAW WT and RAW Dectin-1 cells (**B**) and endogenous mouse (m) Dectin-1 in bone marrow-derived macrophages (BMDMs) (**C**). The positions of molecular weight standards, in kiloDaltons (kDa), are indicated to the right of the immunoblots. β-Tubulin was used as the loading control in (**B**).

3.1.2. Exogenously Expressed Dectin-1 is Glycosylated

When the Dectin-1 was expressed in RAW 264.7 cells, the mature form of the protein migrated as a smear of bands between 37 and 50 kDa upon SDS-

PAGE (**Figure 6B**). Although the predicted molecular weight of full-length human Dectin-1 is 27.6 kDa, these higher positions could represent mature, glycosylated forms of the receptor, which has an N-linked glycosylation site in its stalk region (Kato et al., 2006). Interestingly, this molecular weight range is inconsistent with two other publications, which report a molecular weight for mature human Dectin-1 at 33 kDa (Yokota et al., 2001) and between 30 – 40 kDa (Kato et al., 2006). However, the first report was unable to detect Dectin-1-specific immunoreactivity from ~40 – 53 kDa due to a non-specific band at that position (Yokota et al., 2001), and the other did not display immunoblots above the 40 kDa position (Kato et al., 2006). Regardless, to test if the higher position of immunoreactivity was due to protein glycosylation, we deglycosylated the lysates with the enzymes peptide-*N*-glycosidase F (PNGase F) and endoglycosidase H (Endo H) (**Figure 7**). PNGase F removes all N-linked carbohydrates from glycoproteins, hydrolyzing the side-chain amide linkage of an asparagine residue bonded to the innermost N-acetylglucosamine residue of the glycan chain (Plummer et al., 1984; Tarentino et al., 1985). On the other hand, Endo H specifically hydrolyzes high-mannose-containing structures (Tarentino and Maley, 1974; Tarentino et al., 1974), which are prevalent on glycoproteins during processing in the endoplasmic reticulum and early progression through the *cis*-Golgi apparatus (Kornfeld and Kornfeld, 1985). Thus, sensitivity to Endo H is usually restricted to intracellular, immature forms of a glycoprotein (Kornfeld and Kornfeld, 1985). Treatment of RAW Dectin-1 lysates with PNGase F collapsed the Dectin-1-reactive bands to a smaller precursor ~37 kDa in size (**Figure 7B**). 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 membrane. The corresponding immunoreactive band for HA-tagged Dectin-1, expressed in HeLa cells, exhibited a similar response to the deglycosylation assay (**Figure 7A**). However, immunoblotting also detected a second, lower band of HA-Dectin-1 between 25 – 37 kDa, suggestive of an immature form of the receptor. In support of this, the lower band was both PNGase F- and Endo H-sensitive, and both treatments were able to reduce its

molecular weight to a position near the predicted size of 27.6 kDa. A band corresponding to immature Dectin-1 was not detected in the RAW Dectin-1 cells, probably due to highly efficient processing of Dectin-1 such that immature forms were present at very low amounts. Another reason for the discrepancy could be the inability of the anti-Dectin-1 antibody to recognize less mature forms of the glycoprotein, unlike the anti-HA antibody which was simply detecting the presence of the HA epitope.

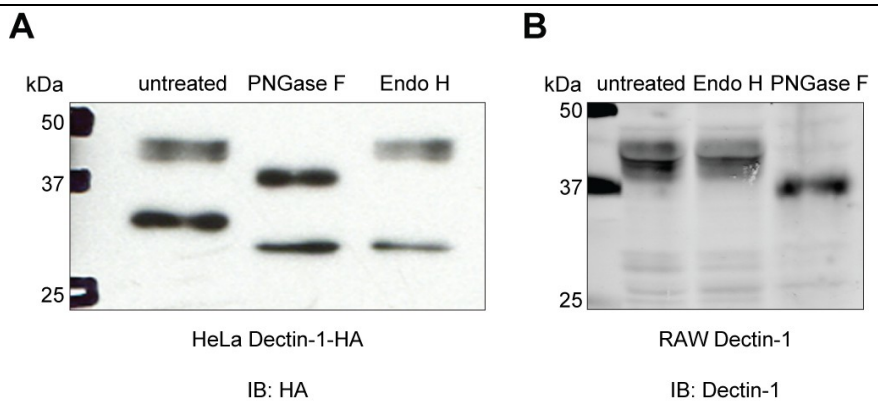

Figure 7: Full-Length Dectin-1 is Glycosylated

Lysates from HeLa cells expressing HA-tagged Dectin-1 (**A**) and RAW Dectin-1 cells (**B**) were denatured and treated with the enzymes PNGase F, Endo H, or neither according to the manufacturer's instructions. They were then processed by SDS-PAGE and immunoblotted (IB) to detect Dectin-1 using anti-HA or anti-Dectin-1 antibodies. Results shown are representative of three independent experiments. The markings on the left indicate the position of molecular weight standards of 25, 37, and 50 kDa.

Consistent with work on mouse Dectin-1 (Esteban et al., 2011), in both cell lines, we were unable to completely collapse the mature form of the receptor to a size consistent with the immature form with PNGase F treatment, despite denaturation of the receptor prior to deglycosylation. Perhaps more extensive treatment with the chosen glycosidases and/or the use of other anti-Dectin-1 antibodies is required. In addition, treatment of the cells with tunicamycin, a compound that inhibits the first enzymatic step of protein glycosylation (Elbein, 1987), could help determine the molecular weight of nascent Dectin-1 in our system. Nevertheless, our data validate the exogenous expression of mature, glycosylated forms of Dectin-1 in HeLa and RAW 264.7 cells.

3.1.3. Exogenously Expressed Dectin-1 is Surface-Localized and Binds Soluble β-Glucans

With Dectin-1 suitably expressed, we moved to evaluate if it was able to bind its cognate ligands (**Figure 8**). A well-established ligand of Dectin-1 is laminarin, a low molecular weight, water-soluble β-glucan with a molecular weight of 7.7 kDa (Mueller et al., 2000), commonly used as an inhibitor of Dectin-1 binding and signaling for particulate glucans (Brown et al., 2002; Gantner et al., 2003; 2005; Underhill et al., 2005). RAW WT cells were transiently transfected to express mEmerald-tagged Dectin-1. The cells were incubated on ice with a fluorescently-labeled conjugate of laminarin and bovine serum albumin (BSA) protein (Alexa Fluor 546—BSA-17-laminarin) and fixed for visualization by confocal microscopy. Remarkably, the laminarin conjugate localized to the surface of RAW WT cells that had expressed mEmerald-Dectin-1 and colocalized with the primarily plasmalemmal Dectin-1 signal (**Figure 8A**). We did not detect conjugate fluorescence on untransfected RAW WT cells, in accordance with their lack of exogenous and endogenous Dectin-1 (**Figure 6**). Thus, the expression of Dectin-1 in RAW WT cells was sufficient to mediate laminarin binding to the cells.

Since it is thought that higher molecular weight soluble β-glucans stimulate Dectin-1 more than low molecular weight ones (Batbayar et al., 2012; Brown and Gordon, 2003; Brown and Williams, 2009; Lowe et al., 2001), we were interested in comparing the cellular responses elicited by both high and low molecular weight ligands. As laminarin is a low molecular weight ligand, we sought to obtain other soluble β-glucans of high molecular weight. One of our high molecular weight ligands was soluble Wellmune whole glucan particle (abbreviated as soluble WGP or WGPsol), from Biothera (Eagan, MN, USA). Wellmune whole glucan particle (WGP, also from Biothera) is a particulate, β-glucan-rich extract of yeast cell wall from a proprietary strain of *Saccharomyces cerevisiae*, and is used as a food additive for its ability to induce immunomodulatory effects (Biothera, Eagan, MN, USA). Soluble WGP (WGPsol) is derived from WGP and dissolves readily in aqueous solution. By gel

permeation chromatography, WGPsol was found to be polydisperse, with three fractions at varying molecular weights of ~30,000, 80,000, and 800,000 – 1,000,000 Da (personal communication, Dr. A. Szpacenko, Department of Chemistry, University of Alberta, 2013).

To determine if WGPsol also bound to Dectin-1, we developed an immunofluorescent approach to label β-glucan bound to cells. When WGPsol, laminarin, or the fluorescent laminarin conjugate were incubated with RAW Dectin-1 cells on ice, immunofluorescence signal corresponding to β-glucan was observed on the cell surface and colocalized with surface Dectin-1 (**Figure 8B**). Predictably, RAW WT cells treated identically exhibited minimal β-glucan immunofluorescence, demonstrating that RAW 264.7 cells do not express significant levels of other β-glucan receptors to mediate the binding of our soluble β-glucans. We have thus demonstrated successful surface expression of Dectin-1 in RAW 264.7 cells, which confers an ability to bind soluble β-glucans such as laminarin and WGPsol.

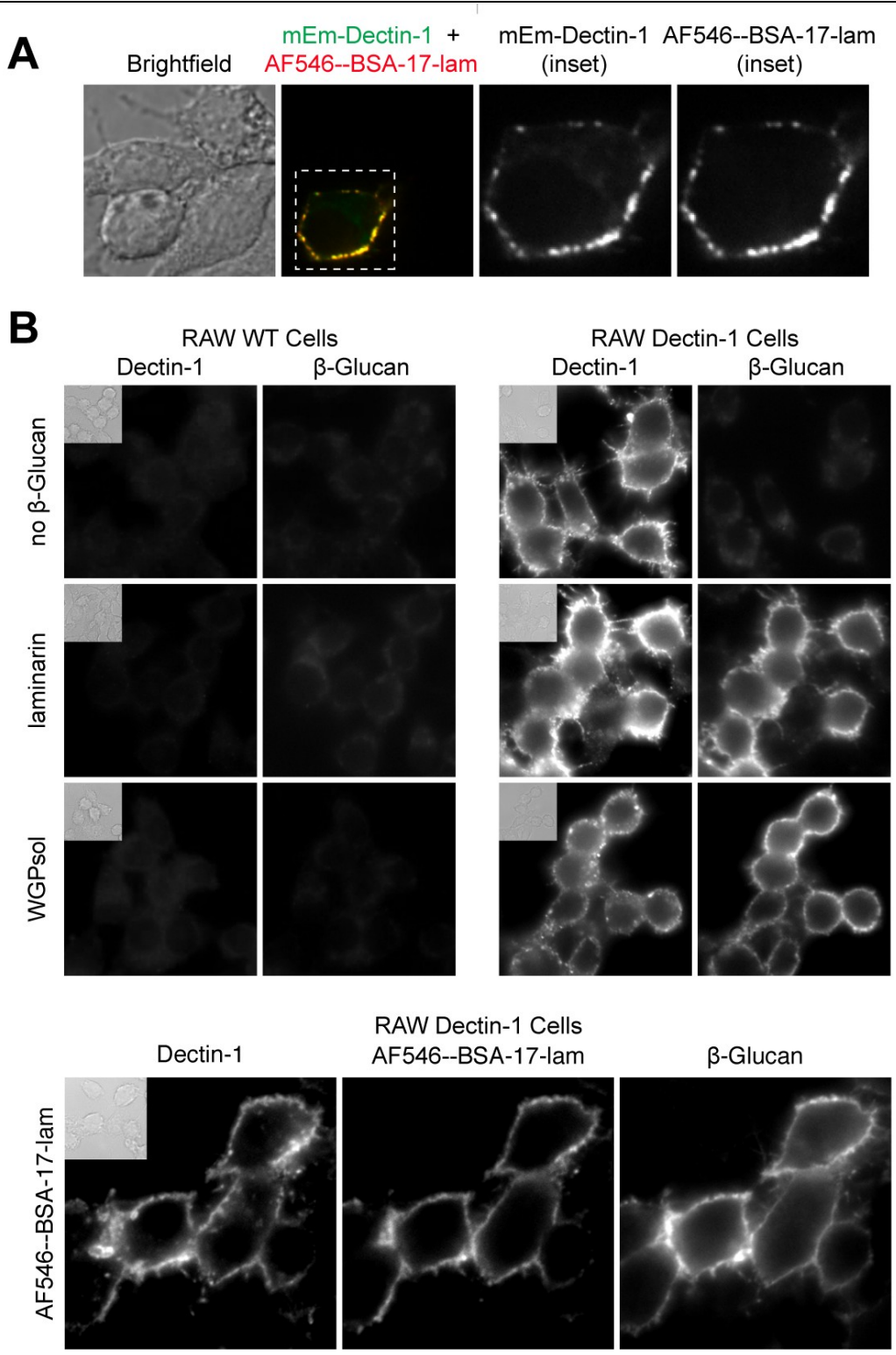

Figure 8: Soluble β-Glucans Bind to Dectin-1-Expressing RAW 264.7 Cells

(**A**) RAW WT cells were transfected with mEmerald-Dectin-1 (mEmerald is a variant of the fluorescent protein enhanced GFP) and incubated with the AF546-labeled BSA-17-laminarin conjugate (AF546—BSA-17-lam) at 100 μg/mL on ice for 20 minutes, after

which they were rinsed in PBS and fixed. Images of the cells were then acquired using brightfield and confocal fluorescence microscopy. The inset highlights an mEmerald-Dectin-1-transfected cell. (**B**) laminarin, WGPsol, the AF546-linked BSA-17-laminarin conjugate, all at 100 μg/mL, or PBS only were incubated with RAW WT or RAW Dectin-1 cells on ice for 20 minutes, rinsed in PBS and fixed. Non-permeabilized cells were then probed for Dectin-1 expression and β-glucan binding with anti-β-glucan and anti-Dectin-1 antibodies and the appropriate secondary antibodies coupled to fluorescent dyes. Confocal imaging was then performed on the cells. Insets show the same field of view acquired under brightfield illumination. All images shown are representative results from three independent experiments.

3.2. Soluble β-Glucans Stimulate Dectin-1 Signaling Events

3.2.1. Binding of Soluble β-Glucans May Induce Dectin-1 Clustering

Having determined that laminarin and WGPsol were able to bind Dectin-1, we were curious to see if this binding could trigger events that are consistent with the current knowledge of Dectin-1 activation. It is highly speculated that ligand binding induces clustering of the receptor, and that the capacity to cluster the receptor is correlated to the magnitude of signaling elicited by the β-glucan ligand (Adams et al., 2008; Brown and Williams, 2009; Goodridge et al., 2011; 2012; Lowe et al., 2001; Michalek et al., 1998; Qi et al., 2011; Tapper and Sundler, 1995). To determine if Dectin-1 might cluster after ligand stimulation, we chemically cross-linked cell surface proteins with the membrane-impermeant, thiol-cleavable cross-linker DTSSP after binding soluble β-glucans to the RAW Dectin-1 cells (**Figure 9**). The cells were lysed, processed by SDS-PAGE, and immunoblotted for Dectin-1. Remarkably, in the presence of DTSSP, the binding of all of the soluble β-glucans induced the formation of a population of apparent Dectin-1 dimers (molecular weight of ~80 kDa, compared to the monomers at ~40 kDa) that was not present when the cells were not bound to soluble β-glucan. Importantly, the addition of a reducing agent was able to disrupt these dimers, indicating that the event could only be observed in the presence of intact cross-linker. Of note, higher-order oligomers were not detected, and the differently-sized ligands did not produce differences in the receptor dimer:monomer distribution, as would be expected if larger ligands induced more Dectin-1

clustering. However, the length of the cross-linker, along with the precise molecular conformations needed for the occurrence of cross-linking between neighbouring receptors, may have precluded detection of these events. 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 Dectin-1 to neighbouring Dectin-1 molecules is increased upon ligand stimulation, suggesting that ligand binding may induce clustering of the receptor.

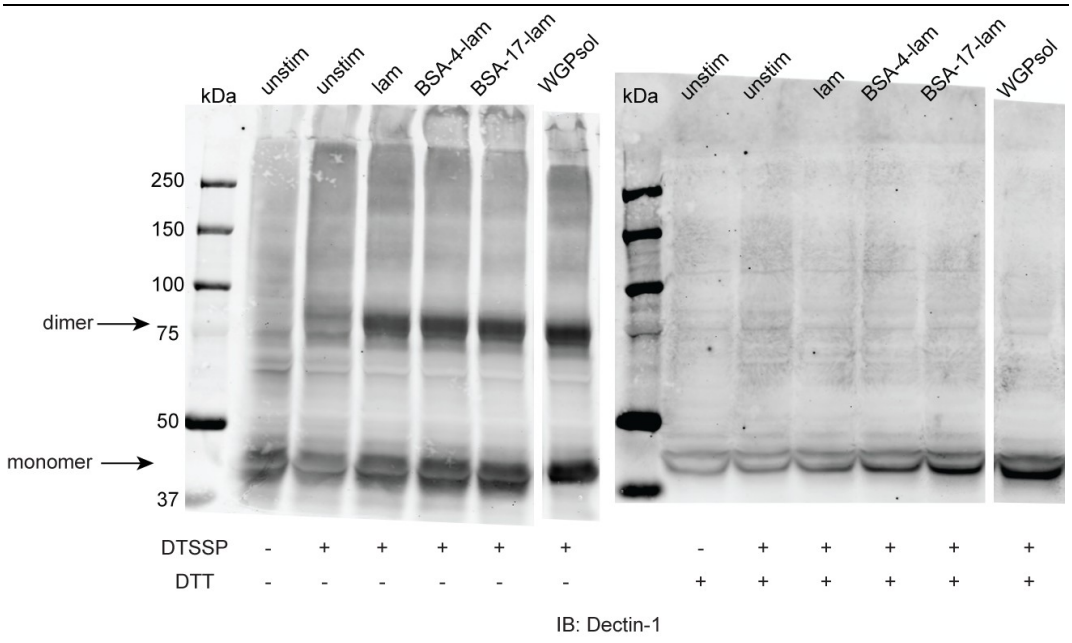

Figure 9: Soluble β-Glucan Binding Increases the Proximity of Dectin-1 to Other Dectin-1 Molecules

Monolayers of RAW Dectin-1 cells at equivalent density were stimulated at 37°C for 10 minutes with 100 μg/mL of laminarin (lam), WGPsol, or the conjugates of BSA covalently linked to four or seventeen laminarin molecules (BSA-4-lam, BSA-17-lam) or left unstimulated (unstim). The cells were then treated with 2 mM DTSSP at 4°C for 2 hours to cross-link surface proteins; the reaction was later quenched with 20 mM Tris. Lysates were prepared and separated into halves of equal volume, one of which was treated with 50 mM of the reducing agent dithiothreitol (DTT). Non-reducing SDS-PAGE was then performed on the entire volumes of both halves, followed by subsequent immunoblotting (IB) for detection of Dectin-1. Immunoreactive bands suggestive of receptor monomers (~40 kDa) and dimers formed by cross-linking (~80 kDa) are indicated by the arrows.

3.2.2. Soluble β-Glucans Induce Src Family Kinase and Syk Activation

The clustering of Dectin-1 by β-glucan binding is predicted to induce Dectin-1 signaling (Goodridge et al., 2012), initial events of which include the activation of the Src family kinases and Syk (Elsori et al., 2011; Olsson and Sundler, 2007; Rogers et al., 2005; Underhill et al., 2005). To determine if the binding of soluble β-glucan to Dectin-1 activated Src family kinases and Syk, lysates were prepared from RAW WT and RAW Dectin-1 cells treated with laminarin and WGPsol (**Figure 10B**). SDS-PAGE was performed and the lysates were immunoblotted for phosphorylated tyrosine residues in Src family kinases and Syk kinase that are sites for autophosphorylation when the kinases are active. Autophosphorylation of the activation loop residue Y416 (or equivalent) in the Src kinases is associated with maintaining the kinases in their active form (Young et al., 2001). In Syk, autophosphorylation of Y352 plays a positive role in the regulation of Syk activity, while that of Y323 plays a negative role. When phosphorylated, both Y352 and Y323 also function as docking sites for certain proteins that interact with Syk. Residues Y525 and Y526 (numbered according to their position in the human protein) are located in Syk's activation loop and are also phosphorylated by the kinase when it is active (Geahlen, 2009). Interestingly, the addition of laminarin and WGPsol to RAW Dectin-1 cells induced Src family kinase and Syk activation, as detected by the phosphorylation at Y525/Y526, Y352 and Y323 on Syk and Y416 (or equivalent) in one of the Src family kinases (the prominent immunoreactive band, migrating furthest during SDS-PAGE) (**Figure 10A**). Phosphorylation at these sites was not detected when the cells were not stimulated with soluble β-glucan.

To determine if activation of Src kinases and Syk was sustained during ligand stimulation, we treated RAW Dectin-1 cells for various times with WGPsol, lysed the cells immediately after, and employed similar immunoblot analysis (**Figure 10B**). Upon stimulation, the phosphorylation of Y525/Y526 in Syk and Y416 in the Src family kinase member increased up until 30 minutes, where it began to decrease gradually to the last timepoint examined, at 120

minutes. Importantly, RAW WT cells did not exhibit any response to WGPsol stimulation, demonstrating that these signaling events are Dectin-1-dependent and occur due to interaction of the β-glucans with the receptor. Hence, sustained stimulation by WGPsol activates a member of the Src kinases and Syk, which appear to exhibit kinetics of activation and repression over 2 hours of observation.

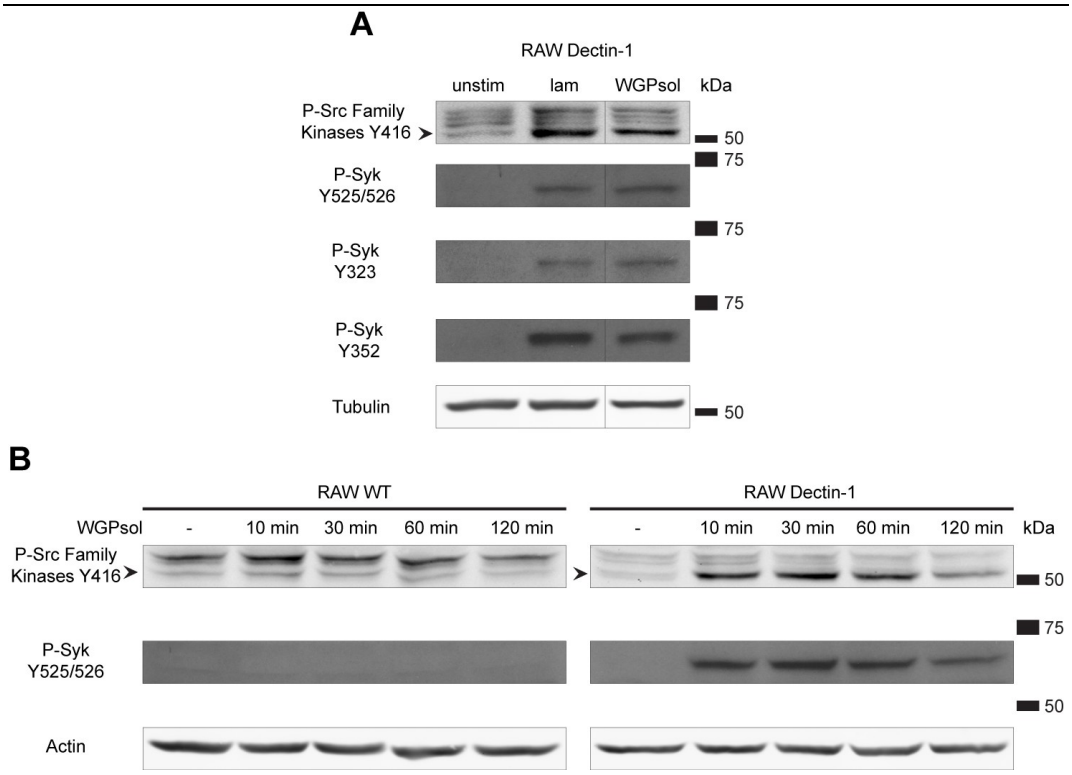

Figure 10: Laminarin and WGPsol Stimulate Src Family Kinase and Syk Activation in RAW Dectin-1 Cells

(**A**) RAW Dectin-1 cells were stimulated with 100 μg/mL laminarin, WGPsol, or neither for 20 minutes at 37°C, after which the cells were promptly lysed, processed by SDS-PAGE, and immunoblotted for the presence of phosphorylated Src family kinases and Syk at tyrosine residues that are sites of autophosphorylation, a readout of activation of the kinases. (**B**) RAW WT and RAW Dectin-1 cells were stimulated continuously in the presence of 100 μg/mL WGPsol for the indicated times at 37°C and processed according to (**A**). The arrow indicates the immunoreactive band corresponding to the particular Src family kinase member activated upon stimulation of Dectin-1 in RAW 264.7 cells. Tubulin and actin were used as loading controls. Immunoblots presented are representative of two (**A**) or three (**B**) independent experiments.

According to the current model of activation of ITAM-containing receptors and Dectin-1, Dectin-1-dependent activation of Src family kinases precedes the activation of Syk, since Src kinases phosphorylate the hemITAM tail of Dectin-1 to result in Syk recruitment and activation (Goodridge et al., 2012;

Underhill and Goodridge, 2007). To confirm if the activation of Syk was dependent on the Src family kinases, we stimulated RAW Dectin-1 cells with WGPsol in the absence or presence of the Src family kinase inhibitor PP2 and performed immunoblots to detect the autophosphorylation statuses of both Src kinases and Syk. In separate samples, we also inhibited Syk activation during WGPsol stimulation using either piceatannol or Syk inhibitor IV (BAY 61-3606) to see if perhaps Syk could regulate the activity of Src family kinases (**Figure 11**). Consistent with the model of Dectin-1 activation, inhibition of Src kinases with PP2 completely abolished the capacity of WGPsol to activate Syk, as detected by phosphorylation of residues Y525/Y526 and Y352 on Syk. However, inhibiting Syk activity with piceatannol and Syk inhibitor IV also abolished WGPsol-dependent Src family kinase activation, as detected by the levels of phosphorylated-Y416 on the Src kinases. This suggests Syk also regulates the activity of the Src family kinase members, recapitulating a finding made recently (Elsori et al., 2011). It might be that the activation states of Src family kinases and Syk are intimately linked, perhaps in a positive feedback loop where the activation of either kinase further activates the other one. Clearly, the early events of Dectin-1 signaling are not as unidirectional as previously thought. With regards to autophosphorylation, piceatannol treatment inhibited phosphorylation of Syk at Y525/Y526 and Y352, as did PP2 treatment on the Src kinase members at Y416, confirming that those residues are in fact autophosphorylated and good readouts for Src family kinase and Syk activation. We are not yet able to explain how inhibiting Syk activity with Syk inhibitor IV enhances Syk autophosphorylation, however, but it is clear that the compound is active as an inhibitor since it was able to block Src family kinase autophosphorylation, similar to piceatannol. Regardless, we have shown that the kinase activities of both Src family members and Syk ensure the event of soluble β-glucan recognition is transduced appropriately into the cell and amplified as a molecular signal.

To summarize this section, the binding of soluble glucans to Dectin-1 is able to induce the activation of Src family kinases and Syk, consistent with their established roles in Dectin-1 signaling. Intriguingly, the activation of either Src

family kinases or Syk appears to promote activation of the other, offering new insights into the early events that occur in Dectin-1 signaling.

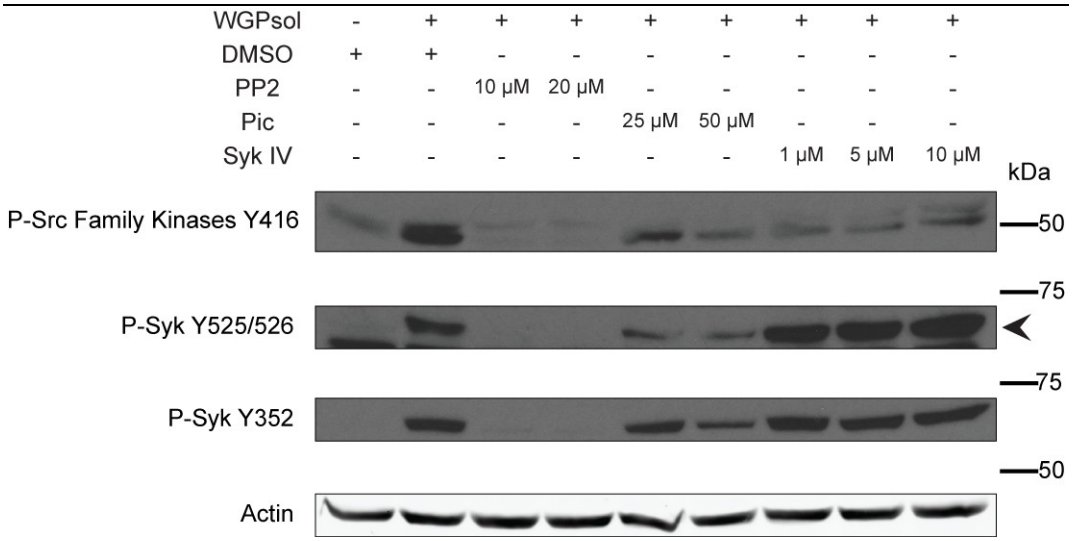

Figure 11: Src Family Kinases and Syk Reciprocally Activate Each Other During WGPsol Stimulation of Dectin-1

RAW Dectin-1 cells were pre-treated with PP2, piceatannol (Pic), Syk inhibitor IV (Syk IV), or vehicle control (0.1% DMSO) at the indicated concentrations for 30 minutes at 37°C. They were then stimulated with 100 µg/mL WGPsol in the same solutions for 20 minutes. 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 or Y352), or actin (as a loading control). Immunoblots presented are results from one experiment.

3.2.3. Soluble β-Glucans Differentially Activate Downstream Dectin-1 Signaling Events

Soluble β-glucans are deemed by some as biologically inactive ligands to the receptor, unlike particulate ligands (Brown, 2006; Brown et al., 2003; Goodridge et al., 2011; Kennedy et al., 2007; Underhill et al., 2005). Having observed that the soluble β-glucans laminarin and WGPsol were capable of binding to Dectin-1, might induce its clustering, and could stimulate the early signaling events of Src family kinase and Syk activation, we were interested to see if these ligands could indeed activate more components of the Dectin-1 signaling pathway (**Figure 5**).

We first stimulated RAW WT and RAW Dectin-1 cells again with

WGPsol, and detected if other signaling events downstream of Syk activation were activated by immunoblot (**Figure 12**). Remarkably, WGPsol binding was able to stimulate phosphorylation events in PLCγ2, PKCδ, and the MAP kinases ERK1 and ERK2, although the response at the ERK level was less pronounced compared to PLCγ and PKCδ. Still, these responses did not occur in RAW WT cells, demonstrating that the signals induced were Dectin-1-dependent.

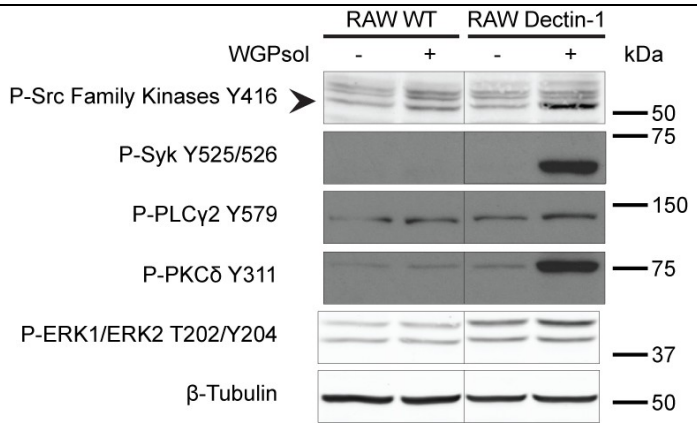

Figure 12: WGPsol Stimulates Dectin-1-Dependent Activation of PLCγ2, PKCδ, and the MAP Kinases ERK1 and ERK2

RAW WT and RAW Dectin-1 cells were stimulated with media with or without 100 μg/mL WGPsol for 20 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) or β-tubulin (as a loading control). Immunoblots presented are representative of three independent experiments.

We continued to see if WGPsol, laminarin, and the laminarin-protein conjugate BSA-17-laminarin were capable of activating the same signaling events, and some that were even further downstream. These later signaling events were related to the activation of NF-κB, and included the degradation of IκBα and the phosphorylations of the canonical NF-κB subunit p65 and the non-canonical NF-κB subunit RelB (reticuloendotheliosis viral oncogene homologue B). Since it has been proposed that larger soluble β-glucans are more able to activate Dectin-1 signaling than smaller ones (Batbayar et al., 2012; Brown and Gordon, 2003; Brown and Williams, 2009), we were also interested in seeing if the size of the β-glucans correlated with the magnitude of the resulting signaling events.

This was convenient due to the differences in size of our soluble ligands. Laminarin was measured to have a molecular weight of 7.7 kDa previously (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 a mixture of molecules at 30, 80, and 800 – 1,000 kDa in size.

We stimulated RAW Dectin-1 cells with the ligands and detected if the signaling events of interest were activated by immunoblot (**Figure 13**). Particulate curdlan was used as a positive control of Dectin-1 activation as it a potent activator of PLCγ2, PKCδ, and NF-κB (Gringhuis et al., 2009; Strasser et al., 2012; Tassi et al., 2009; Xu et al., 2009b). Lipopolysaccharide (LPS) was used as an additional positive control for the induction of NF-κB activation. We observed that WGPsol, laminarin, and BSA-17-laminarin were all capable of strongly activating Src family kinases, Syk, PLCγ2, and PKCδ, similar to curdlan. However, differences were observed between ligands. BSA-17-laminarin was the only ligand that strongly induced ERK and NF-κB activation, whereas WGPsol only induced slight ERK activation but could not activate NF-κB. Laminarin stimulation was unable to induce ERK or NF-κB activation. These results were intriguing since we predicted that of all the ligands, WGPsol would fully activate the Dectin-1 signaling pathway to the level of the transcription factors, based on it containing the largest β-glucans of up to 1000 kDa. There were also differences in the magnitude of upstream signaling events activated by the ligands. Both WGPsol and BSA-17-laminarin induced higher levels of Src family kinase, Syk, PLCγ2, and PKCδ phosphorylation than laminarin, consistent with their larger size. BSA-17-laminarin, though, was able to induce more activation of Syk and PLCγ2 than WGPsol, a factor that may have contributed to its ability to further activate ERK1, ERK 2 and NF-κB.

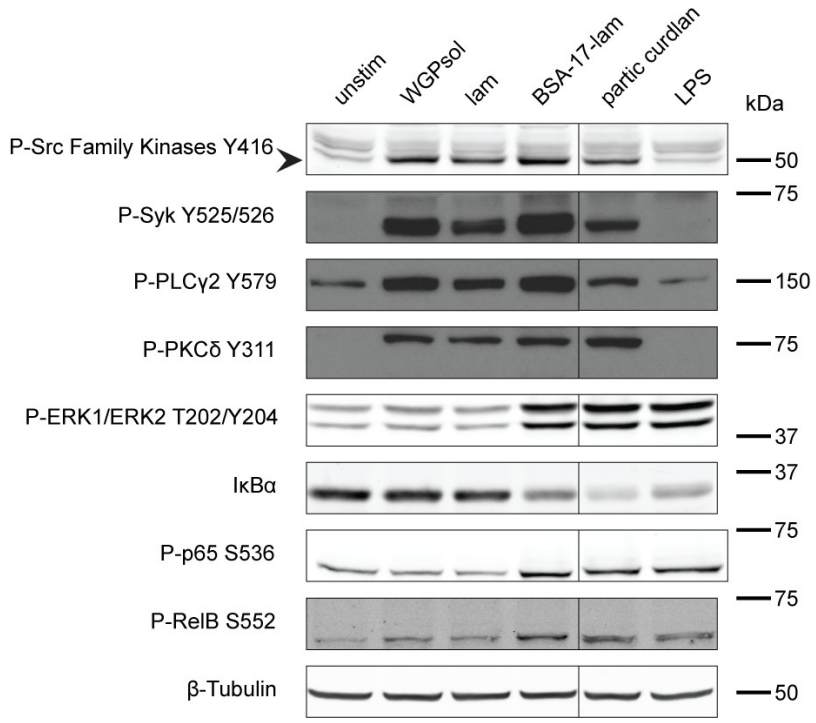

Figure 13: Laminarin, the BSA-17-Laminarin Conjugate, and WGPSol Differentially Activate Downstream Dectin-1 Signaling

RAW Dectin-1 cells were stimulated with media with or without 100 μg/mL laminarin (lam), BSA-17-laminarin (BSA-17-lam), WGPsol, particulate curdlan (partic curdlan), or 100 ng/mL lipopolysaccharide (LPS) for 20 minutes at 37°C. The cells were promptly lysed, processed by SDS-PAGE, and immunoblotted for the presence of phosphorylated Src family kinases, Syk, PLCγ2, PKCδ, ERK1 and ERK2, p65 (on residue S536), and RelB (on residue S552). Immunoblots were also performed for the presence of total IκBα (to assess IκBα degradation) and β-tubulin (as a loading control). The immunoblots presented are representative of two independent experiments.

In summary, WGPsol was able to activate Dectin-1 signaling up to the level of the ERK MAP kinases but was not able to transduce the signal further to the transcriptional level at NF-κB. Like WGPsol, BSA-17-laminarin was able to activate the upstream components of the Dectin-1 signaling pathway (Src family kinases, Syk, PLCγ2, and PKCδ) but also produced strong ERK and NF-κB activation. Laminarin was only able to activate signaling in the upstream components. The different cellular responses triggered by laminarin and the conjugate molecule, with multiple laminarin molecules anchored to BSA, suggest that differences in the capacity for soluble β-glucans to activate complete Dectin-1 signaling may stem at least in part from their different molecular weights. However, the inability for WGPsol to activate NF-κB suggests other factors may

be at play.

Since WGPsol was unable to activate Dectin-1 signaling down to the transcription factor level, we sought to obtain a purified soluble β-glucan that was able to without the necessity of being conjugated to a protein anchor. The group of Professor David Bundle (Department of Chemistry, University of Alberta) was able to generate a water-soluble version of curdlan upon phosphorylation of the particulate material. This molecule was previously demonstrated to stimulate Syk phosphorylation and NF-κB nuclear translocation in bone marrow-derived macrophages (Lipinski et al., 2013). While we did not determine the molecular weight of P-curdlan, particulate curdlan can be up to 12,000 glucose residues in length (FUTATSUYAMA et al., 1999; Stone, 2009), which corresponds to a molecular weight of ~2,000 kDa. Thus phosphorylated curdlan should also represent a high molecular weight soluble β-glucan. We then tested if this phosphorylated curdlan (abbreviated phospho-curdlan, or P-curdlan) could induce activation of Dectin-1 signaling down to the level of NF-κB by stimulating RAW Dectin-1 cells for various times up to 30 minutes and conducting immunoblot analysis of the various signaling molecules in the pathway. The effects of P-curdlan stimulation were also compared to that of laminarin to see if differences in molecular weight alter the ability to activate Dectin-1 signaling cascades (**Figure 14**). Indeed, stimulation with both laminarin and P-curdlan induced strong activation of upstream signaling events, including that of Src family kinases, Syk, and even Raf1. However, P-curdlan triggered slightly more activation of these events, as detected by the increased intensities of the immunoreactive signal for the phosphoproteins compared to in laminarin stimulation. Perhaps this heightened activation translated to potent stimulation of NF-κB, as curdlan strongly activated IKK, the phosphorylation and degradation of IκBα (**Figure 5**), as well as phosphorylation of the NF-κB subunits p105 (the full-length precursor to the p50 subunit seen in **Figure 5**), p65, and RelB. Laminarin, perhaps as an effect of its small size, was unable to trigger any of those events, confirming its ability to activate NF-κB (**Figure 13**).

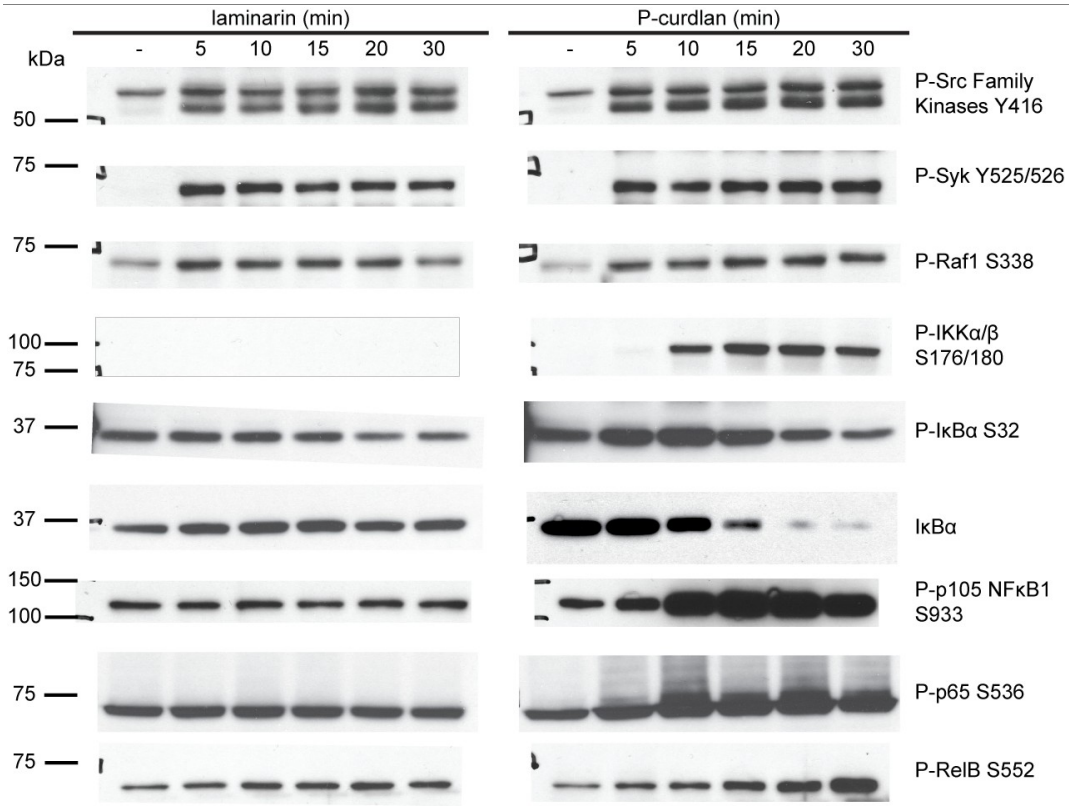

Figure 14: P-Curdlan, but not Laminarin, Activates NF-κB

RAW Dectin-1 cells were stimulated continuously in the presence of 100 μg/mL laminarin or P-curdlan 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, Raf1 (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, and RelB. The presence of total IκBα was also detected by immunoblot to assess IκBα degradation. 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 for each timepoint were loaded onto the polyacrylamide gels. The experiment was performed by Ms. Amira Fitieh, Ms. Sandra Ungarian and Dr. Nicolas Touret, and the immunoblots presented are representative of five independent experiments.

Thus, we have identified P-curdlan as a high molecular weight soluble β-glucan with a strong capacity to stimulate Dectin-1 signaling down to the level of the transcription factor NF-κB. This effect is likely a result of its size as a β-glucan. On the other hand, laminarin is a small soluble β-glucan with abilities to stimulate upstream events in Dectin-1 signaling, but this signal is not carried through to the downstream molecules in the pathway such as the ERK MAP kinases and NF-κB.

We eventually depleted the initial stocks of P-curdlan that could stimulate Dectin-1 signaling down to NF-κB activation (**Figure 14**) and replaced them with a newer batch. However, after numerous experiments, we became suspicious that this newer batch was not activating Dectin-1. To determine if the new batch of P-curdlan was able to induce Dectin-1-mediated signaling, we stimulated RAW WT and RAW Dectin-1 cells with the compound, prepared cellular extracts, and performed SDS-PAGE and immunoblotting for detection of various active signaling molecules (**Figure 15**). While the new P-curdlan was able to induce strong ERK phosphorylation and NF-κB activation, it was unable to activate molecules that are hallmarks of Dectin-1 activation, including the Src family kinases, Syk, PLCγ2, and PKCδ. As positive controls, treatment of RAW Dectin-1 cells with the ligands WGPsol and curdlan solubilized in alkali was able to activate those molecules. In addition, the ability for the new P-curdlan to activate the ERKs and NF-κB was not blocked upon treatment with the Src Family kinase inhibitor PP2 nor the Syk inhibitors piceatannol and Syk inhibitor IV. Furthermore, the new P-curdlan could also activate the ERKs and NF-κB in RAW WT cells, which do not express detectable levels of Dectin-1. Taken together, the results suggest that stimulation of the ERKs and NF-κB by the new P-curdlan was probably due to activation of signaling pathways separate from Dectin-1.

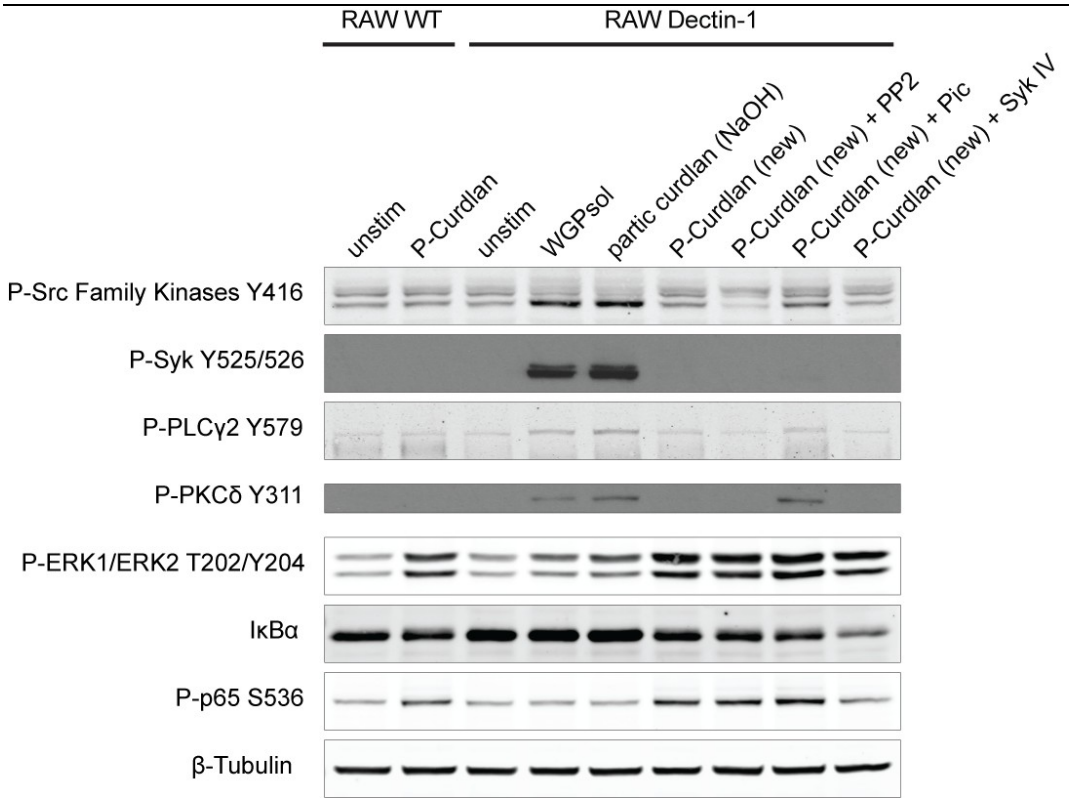

Figure 15: A New Batch of P-Curdlan was Unable to Stimulate Dectin-1-Dependent Signaling

RAW WT and RAW Dectin-1 cells were stimulated with media with or without 100 μg/mL of a new batch of P-curdlan or curdlan (solubilized first in 0.15 M sodium hydroxide solution) for 20 minutes at 37°C. Some samples were simultaneous treated with the pharmacological inhibitors PP2 (at 10 μM), piceatannol (Pic, at 25 μM), or Syk inhibitor IV (Syk IV, at 5 μM). Prior to stimulation with the new batch of P-curdlan, the inhibitor-treated samples were also pre-treated with the inhibitors for 30 minutes at 37°C. All cells were promptly lysed after ligand stimulation, processed by SDS-PAGE, and immunoblotted for the presence of phosphorylated Src family kinases, Syk, PLCγ2, PKCδ, ERK1 and ERK2, and p65. Immunoblots were also performed for the presence of total IκBα (to assess IκBα degradation) and β-tubulin (as a loading control). The immunoblots presented are representative of three independent experiments.

We are unsure of why the new batch of P-curdlan behaved this way, since it was prepared in a similar manner to the original P-curdlan. Despite this setback, many experiments had already been performed using the original P-curdlan as a ligand, and any further data presented in this manuscript utilized P-curdlan that had exhibited the potent Dectin-1 stimulating activity shown in **Figure 14**.

Nevertheless, in this section, we have presented evidence that the soluble β-glucans laminarin, WGPsol, and P-curdlan are all capable of activating Dectin-1

signaling, albeit to different extents down the established signaling pathways (**Figure 5**). Our results also add further proof that the Dectin-1 we exogenously expressed in the RAW 264.7 cells is a functional receptor, as it mediated these signaling events.

3.3. Dectin-1 is Endocytosed

3.3.1. Soluble β-Glucans Stimulate Dectin-1 Endocytosis, as Detected by Fluorescence Microscopy

Having observed that laminarin, WGPsol, and P-curdlan could active Dectin-1 signaling, we continued our investigation to determine if these soluble β-glucans could trigger Dectin-1 endocytosis when bound to the receptor. To determine if Dectin-1 is internalized at the steady state and during ligand stimulation, RAW Dectin-1 cells were incubated at 37°C in the absence or presence of laminarin and WGPsol for various timepoints and fixed (**Figure 16**).

An immunofluorescence staining protocol for detection of both surface-localized and total populations of Dectin-1 was then performed, containing a series of antibody incubations performed prior to and after membrane permeabilization. After confocal imaging, overlap analysis of both populations would reveal if Dectin-1 was present at the cell surface or was intracellular.

At timepoint 0 minutes (no stimulation), an intense, surface distribution was observed. Although faint puncta of total Dectin-1 were detected in intracellular compartments (likely representative of newly synthesized receptor), much of the total Dectin-1 signal colocalized with the surface Dectin-1 labeling, indicating much of the receptor was localized to the plasma membrane at the steady state. However, at 15 minutes, cells incubated with media containing laminarin or WGPsol had a large number of intracellular Dectin-1 puncta, likely representative of Dectin-1 that had internalized into endocytic vesicles and organelles (**Figure 16**). The Dectin-1 puncta also colocalized with the punctate signal for β-glucan, suggesting that the β-glucan ligands were internalized in Dectin-1-enriched endosomes after binding the receptor.

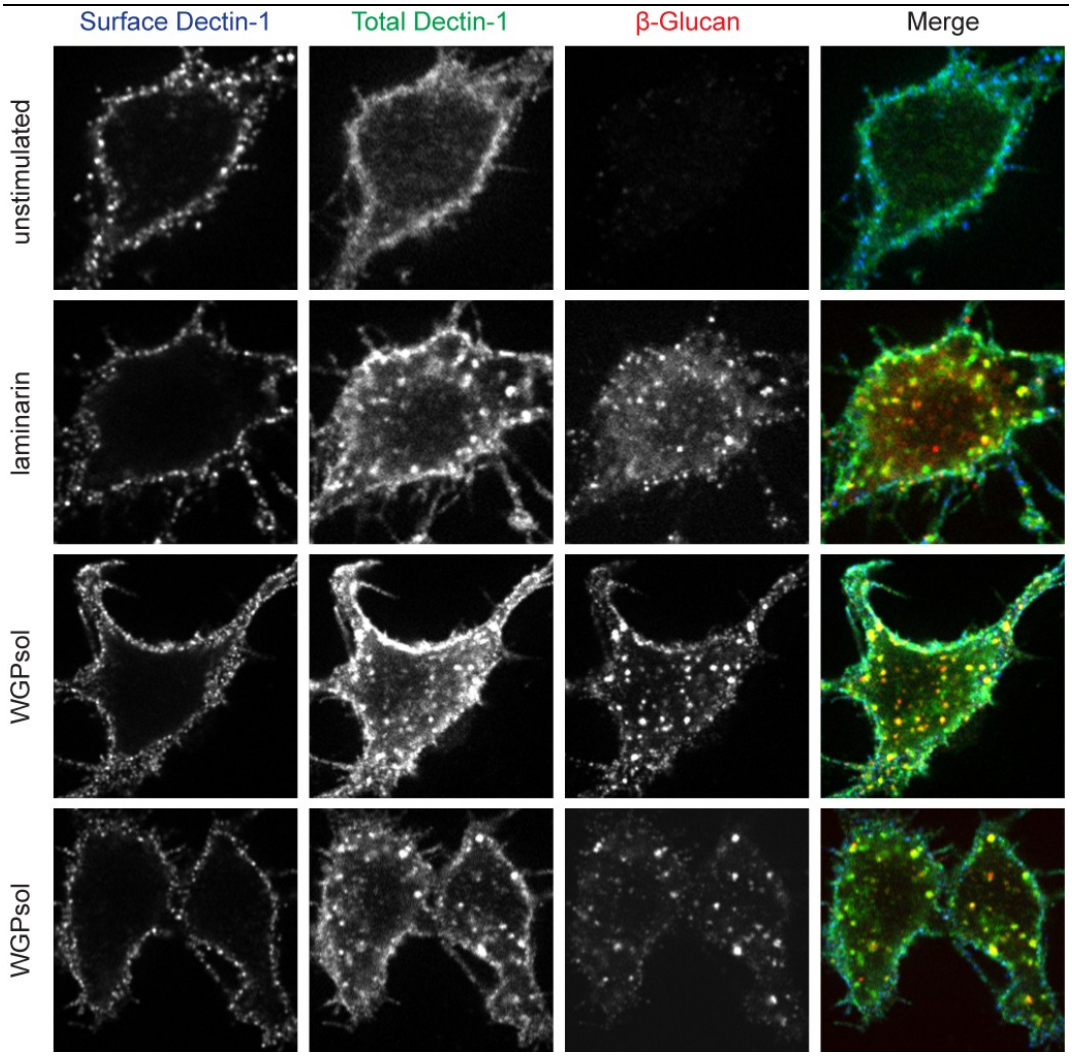

Figure 16: Laminarin and WGPsol Stimulate Dectin-1 Internalization

RAW Dectin-1 cells were treated with laminarin, WGPsol, or neither for 15 minutes at 37°C. The cells were then fixed and labeled with an anti-Dectin-1 antibody and an AF649-coupled secondary antibody to identify surface Dectin-1. After permeabilization in 0.1% saponin, the cells were labeled with anti-β-glucan and anti-Dectin-1 antibodies followed by the appropriate Cy3- and AF488-conjugated secondary antibodies to detect total Dectin-1 and the localization of the soluble β-glucan ligands. Detection was performed on the confocal microscope. Images are representative of two independent experiments.

We also were curious if stimulation of Dectin-1 with P-curdlan could induce internalization of the receptor. Here, RAW Dectin-1 cells were labeled with an anti-Dectin-1 antibody on ice, prior to ligand stimulation for 60 minutes at 37°C (**Figure 17**). The cells were fixed and labeled with Cy3-coupled secondary antibodies to detect surface localized anti-Dectin-1 antibody, then permeabilized and labeled with AF488-coupled secondary antibodies to detect the total

distribution of the anti-Dectin-1 antibodies. Clearly, stimulation with P-curdlan (and laminarin) was able to induce internalization of the receptor, as indicated by the presence of a punctate, intracellular signal that was only detected by the AF488-coupled secondary antibodies. Leaving the cells unstimulated for 60 minutes at 37°C resulted in a primarily surface staining for the total population of Dectin-1 antibodies.

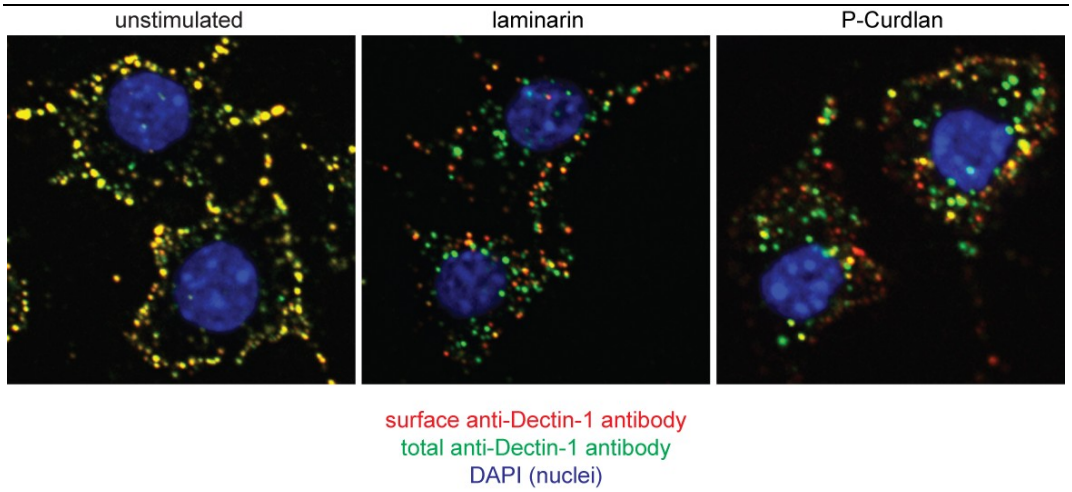

Figure 17: P-Curdlan and Laminarin Stimulate the Internalization of Surface-Bound Anti-Dectin-1 Antibodies

RAW Dectin-1 cells were chilled and labeled with anti-Dectin-1 antibodies for 20 minutes. The cells were next warmed to 37°C in the presence of 100 μg/mL laminarin, P-curdlan, or neither for 60 minutes. After fixation, surface-localized anti-Dectin-1 antibodies were labeled with a Cy3-conjugated secondary antibody (in the red channel). Subsequent membrane permeabilization in 0.1% Triton X-100 then allowed detection of the distribution of all the anti-Dectin-1 antibodies using a secondary antibody 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.

Together, our results suggest Dectin-1 is localized to the plasma membrane at the steady state, but upon binding of the soluble β-glucans laminarin, WGPsol, and P-curdlan, the endocytosis of the receptor is stimulated.

3.3.2. Reversible Cell Surface Biotinylation of Dectin-1

To confirm our findings using a more quantitative approach, we resorted to reversible cell surface biotinylation, which could be used to measure the internalization rates of a specific pool of Dectin-1. In brief, surface-localized membrane proteins on RAW Dectin-1 cells were biotinylated with the amine-

reactive, membrane-impermeant, thiol-cleavable reagent sulfo-NHS-SS-biotin. The cells were then stimulated with soluble β-glucan ligands at 37°C. After an elapsed time, the biotin label was stripped off surface-remaining membrane proteins with the membrane-impermeant reducing agent TCEP. Any remaining biotinylated proteins would have had to be endocytosed in order to be protected from TCEP stripping. After cell lysis, streptavidin pull-down was used to isolate the biotinylated proteins. Subsequent SDS-PAGE and immunoblotting were then performed to detect surface-biotinylated Dectin-1, whose amounts were quantified by densitometry.

The abilities of the technique to biotinylate and remove the biotin label off Dectin-1 were validated (**Figure 18**). After surface biotinylation, a majority of the Dectin-1 present in the whole cell lysate appeared in the eluate fraction from the streptavidin beads, corresponding to its disappearance from the flow-through fraction (**Figure 18C**). This result is consistent with the immunofluorescence images showing that Dectin-1 is primarily localized to the plasma membrane at the steady state (**Figure 6**). In contrast, treatment of the cells with TCEP after biotinylation caused Dectin-1 to disappear from the eluate fraction and reappear in the flow-through fraction (**Figure 18C**). We also confirmed the specificity of the technique for cell surface proteins by immunoblotting the same samples for the presence of actin, a cytoplasmic marker (**Figure 18C**). Minimal levels of actin were pulled-down relative to the amount present in whole cell lysate. Thus, we confirmed that the surface biotinylation procedure was able to efficiently biotinylate membrane-localized Dectin-1, and the conditions for TCEP stripping were sufficient to remove the biotin tag from the receptor.

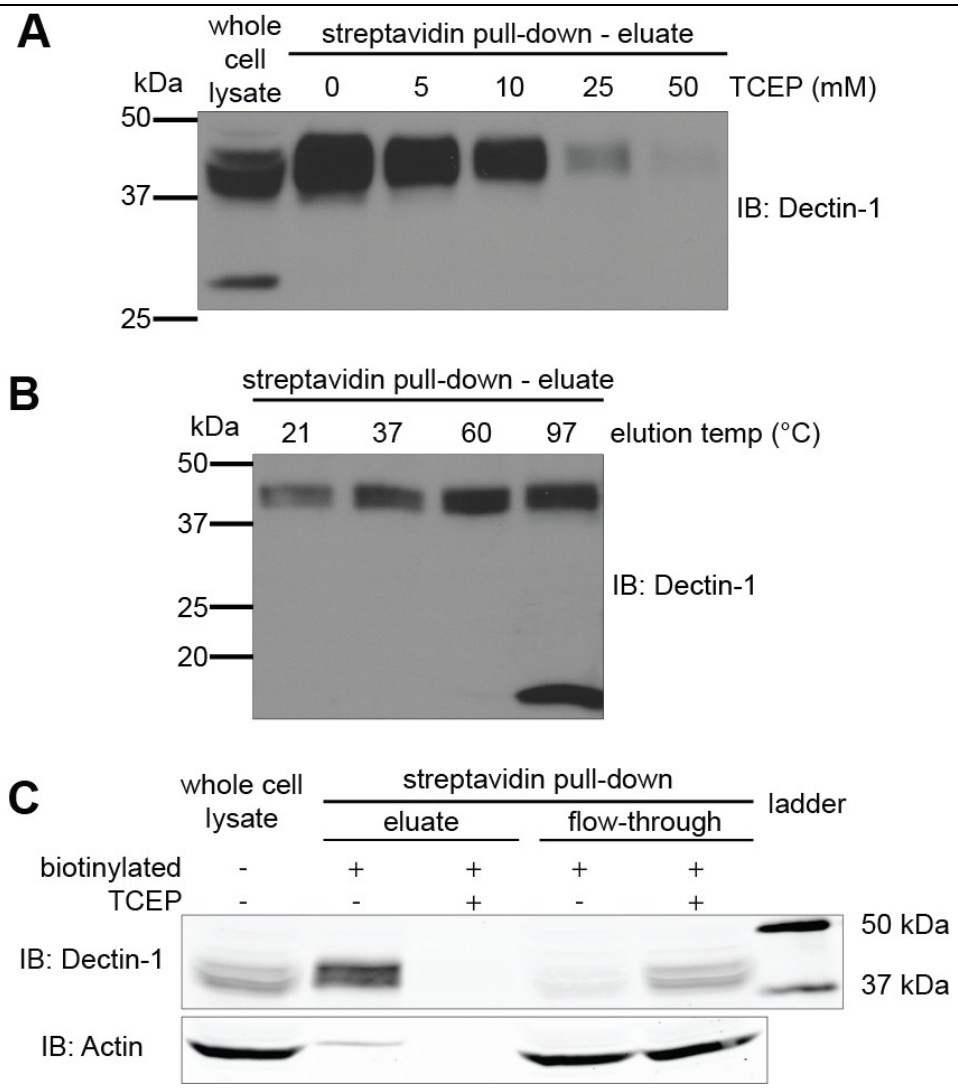

Figure 18: Reversible Cell Surface Biotinylation of Dectin-1

(**A**) Optimization of the TCEP concentration needed for effective stripping of the biotin tag from Dectin-1. RAW Dectin-1 cells were biotinylated with sulfo-NHS-SS-biotin. To test the ability of the membrane-impermeant reducing agent TCEP to cleave the biotin tag off Dectin-1, the cells were incubated with various concentrations of TCEP. Cell lysates were prepared, followed by pull-down of biotinylated proteins with streptavidin resin and their elution from the resin in 10% 2-mercaptoethanol at 37°C. SDS-PAGE was performed, and immunoblots were performed to detect Dectin-1.

(**B**) Optimization of the ideal temperature for elution of biotinylated Dectin-1 from streptavidin resin. Surface-biotinylated RAW Dectin-1 cells were lysed and biotinylated proteins were isolated by streptavidin affinity. Elution for 30 minutes with 10% 2-mercaptoethanol was attempted at various temperatures prior to SDS-PAGE and immunoblotting for Dectin-1. 60°C was selected as the optimal elution temperature.

(**C**) Validation of the procedure to effectively biotinylate and strip the biotin label off Dectin-1. Surface-biotinylated RAW Dectin-1 cells were either treated or not treated with 100 mM TCEP and lysed. Biotinylated proteins were isolated by streptavidin pull-down, and the remaining flow-through was also collected. The streptavidin resin was

then eluted with 10% 2-mercaptoethanol. The eluate and flow-through fractions, along with whole cell lysate from non-biotinylated cells, were processed for SDS-PAGE and immunoblotted for Dectin-1 and actin (a marker of the cytoplasm). Each lane represents the same volume of starting lysate from a monolayer of cells at identical confluency. Shown is a representative blot from three independent experiments.

3.3.3. Stimulation with Soluble β-Glucans Enhances the Rate of Dectin-1 Endocytosis, as Measured by Cell Surface Biotinylation

To measure the rates of Dectin-1 internalization, RAW Dectin-1 cells were surface-biotinylated and treated with media containing laminarin, WGPsol, or neither for various times before stripping with TCEP (**Figure 19**). We observed a modest constitutive uptake of Dectin-1 under unstimulated conditions, with a peak of 17.9% internalized receptor at 30 minutes (**Figure 19B**). However, addition of laminarin or WGPsol led to a quick rise in internalized receptor, with peaks at 41.9% and 47.3% internalized Dectin-1, respectively, at 30 minutes of uptake (**Figure 19B**). This corresponded to an average 2.48- or 2.97-fold increase, respectively, of internalized receptor relative to that of the basal, unstimulated condition (**Figure 19C**). While the amount of internalized Dectin-1 was slightly higher for WGPsol at all timepoints, both WGPsol and laminarin produced similar uptake kinetics, with a rapid increase in internalized receptor between 15 and 30 minutes, and a gradual decrease over 60 and 90 minutes.

In a separate experiment, P-curdlan, too, was able to stimulate increased Dectin-1 internalization, producing similar receptor uptake amounts and kinetics as laminarin (**Figure 20**). Similar to the previous experiment, the peak of the amount of internalized Dectin-1 occurred at 30 minutes, with 15.3%, 41.3%, and 43.4% internalized receptor for the unstimulated, laminarin, and P-curdlan conditions, respectively (**Figure 20B**). Treatment of the cells with the non-specific glucan dextran produced similar receptor uptake quantities and kinetics as the unstimulated condition, indicating that increased Dectin-1 uptake could only be triggered by β-glucans (**Figure 20B**). Clearly, as observed visually through fluorescent microscopic imaging (**Figure 16**, **Figure 17**), and biochemically, via reversible cell surface biotinylation (**Figure 19**, **Figure 20**), the uptake of Dectin-1 is stimulated upon binding by soluble β-glucan ligands. Interestingly, the

stimulation of Dectin-1 with various soluble β-glucans did not appear to produce differences in the kinetics of receptor uptake.

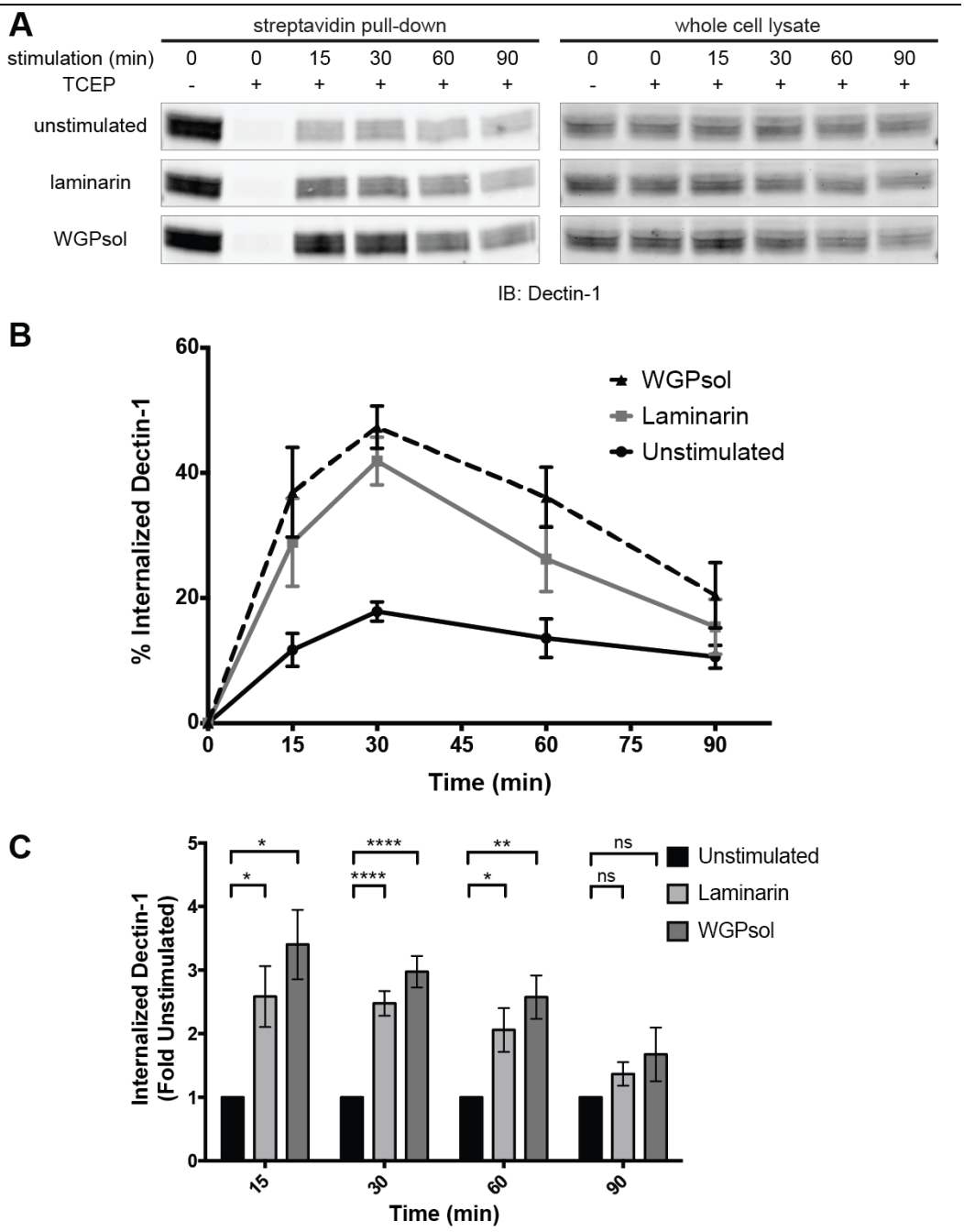

Figure 19: Kinetics of WGPsol- and Laminarin-Stimulated Dectin-1 Uptake

RAW Dectin-1 cells were surface-biotinylated on ice and either lysed immediately with or without prior TCEP stripping, or warmed to 37°C for various times in the presence or absence of 100 µg/mL laminarin or WGPsol before TCEP stripping. Biotinylated proteins were isolated by streptavidin pull-down, SDS-PAGE was performed on both pull-down and whole cell lysate fractions, and the presence of Dectin-1 was probed by

immunoblot (IB). (**A**) shows representative immunoblots from one of four independent experiments. (**B**) is a quantification of the average percentage of internalized receptor relative to total surface receptor levels (measured when the cells were surface-biotinylated and immediately lysed) over time. (**C**) is a quantification of the average ratios of the amount of internalized receptor during ligand stimulation to that of the unstimulated condition. (**B**) and (**C**) were arrived at from densitometry measurements of between nine and nineteen (**B**) or fourteen (**C**) independent experiments performed similarly to (**A**).

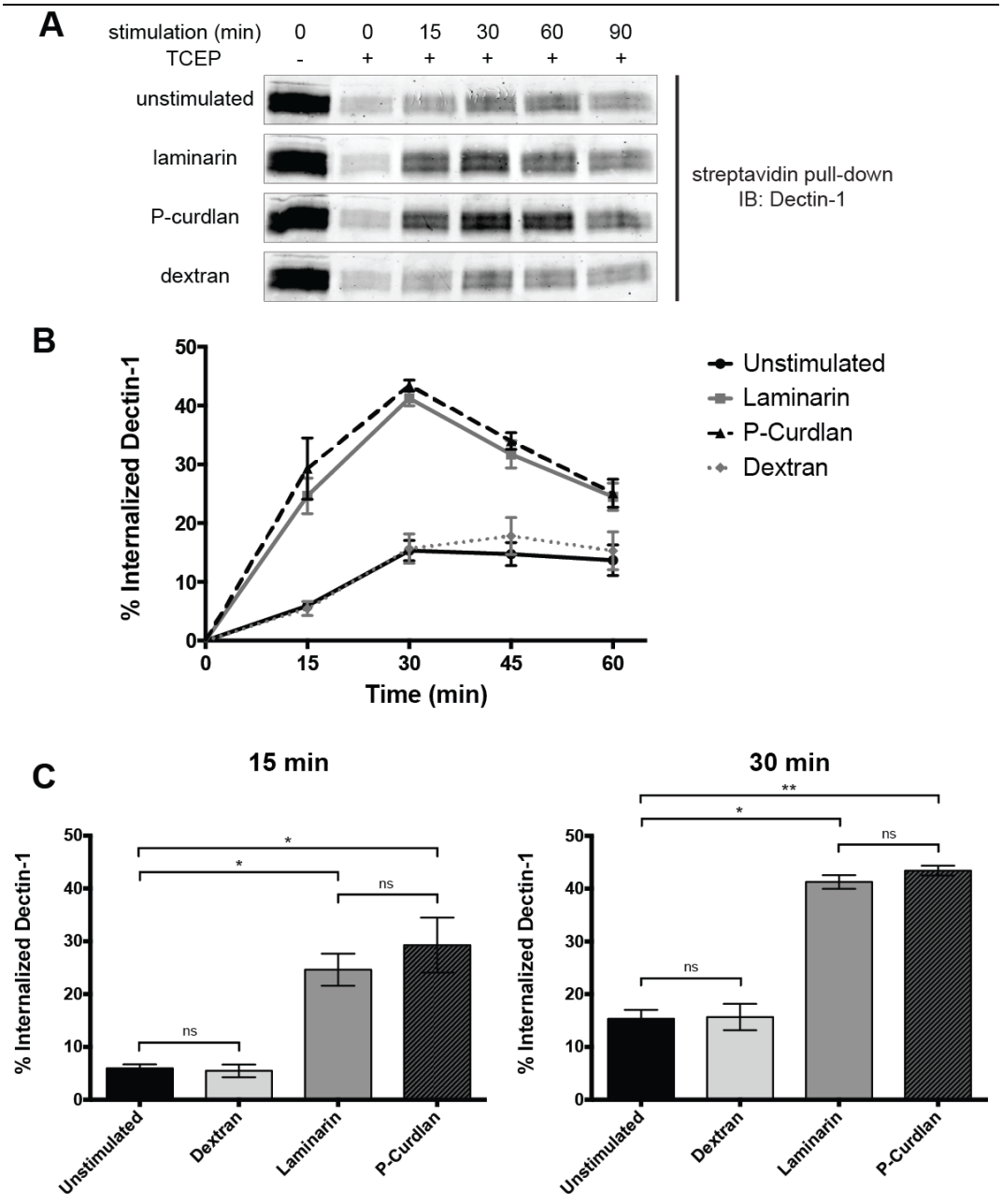

Figure 20: Kinetics of Dectin-1 Internalization Upon P-Curdlan, Laminarin, and Dextran Stimulation

RAW Dectin-1 cells were surface-biotinylated on ice and either lysed immediately with

or without prior TCEP stripping, or warmed to 37°C for various times in the presence or absence of 100 μg/mL laminarin, P-curdlan, or dextran before TCEP stripping. Biotinylated proteins were isolated by streptavidin pull-down, SDS-PAGE was performed on the streptavidin pull-down fraction, and the presence of Dectin-1 was probed by immunoblot (IB). (**A**) shows representative immunoblots from one of three independent experiments. (**B**) and (**C**) are identical quantifications of the average percentage of internalized receptor relative to total surface receptor levels (measured when the cells were surface-biotinylated and immediately lysed), but are displayed in different graphical representations. They were arrived at from densitometry measurements of the experiments in (**A**).

3.3.4. Antibody Binding to Dectin-1 Stimulates Uptake of the Receptor

Interestingly, we also observed that binding anti-Dectin-1 antibodies to the cell surface was sufficient to induce endocytosis of the receptor, independently of ligand binding. This was observed when the cells were fixed using 3% paraformaldehyde and 0.1% glutaraldehyde and permeabilized by 0.5% saponin, conditions that we find favour finer preservation of intracellular structures than our usual treatment with 4% paraformaldehyde (data not shown). When anti-Dectin-1 antibodies were bound to RAW Dectin-1 cells on ice and replaced with media at 37°C, Dectin-1 was observed in intracellular vesicular structures (**Figure 21A**). On the contrary, the receptor remained distributed on the cell surface when the cells were labeled with the antibody after fixation and permeabilization (**Figure 21B**). As a positive control, the addition of laminarin to unlabeled Dectin-1 was able to induce receptor internalization (**Figure 21C**).

The results demonstrate clearly that antibody labeling of Dectin-1 can stimulate its uptake. This could be due to the induction of a conformational change in the receptor by antibody binding. Alternatively, the ability to cluster the receptor may contribute, as the antibody used was a divalent immunoglobulin G molecule, likely promoting dimerization of neighbouring Dectin-1 molecules. Higher order cross-linking may have also occurred since the antibody was actually a polyclonal mixture of antibodies. The ability for the various antibodies to bind multiple epitopes on the extracellular domain of Dectin-1 may have allowed individual receptors to be cross-linked to multiple neighbouring receptors. In summary, like the soluble β-glucans, antibody binding alone can

trigger Dectin-1 endocytosis, perhaps by inducing a conformational change, receptor clustering, or a combination of both events.

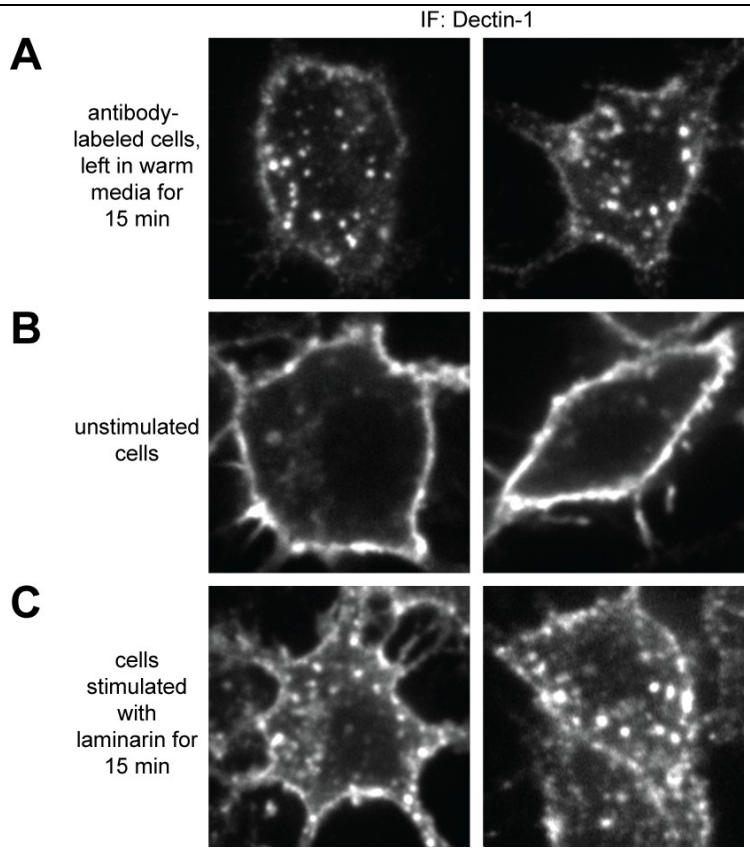

Figure 21: Antibody Binding Stimulates Dectin-1 Uptake

In (**A**), goat anti-Dectin-1 antibodies were bound to RAW Dectin-1 cells on ice for 20 minutes, after which they were removed with several PBS washes, and replaced with media at 37°C for 15 minutes. The cells were then fixed in 3% PFA and 0.1% glutaraldehyde, permeabilized in 0.5% saponin, and labeled with a fluorescent secondary antibody. In (**B**) and (**C**), the cells were incubated at 37°C for 15 minutes with media alone (**B**) or containing 100 μg/mL laminarin (**C**) before fixation and permeabilization. Goat anti-Dectin-1 antibodies and a corresponding fluorescent secondary antibody were then sequentially bound to the cells. All images were acquired on the confocal microscope, and are representative results from one experiment. Two images from the same condition are represented in side-by-side panels.

3.4. Dectin-1 Receptor-Mediated Endocytosis is Dynamin-, Clathrin-, and Actin-Dependent

3.4.1. Dectin-1-Mediated Endocytosis is Dynamin-Dependent

Having observed accelerated Dectin-1 entry upon soluble β-glucan binding, we sought to identify the cellular machinery that could be mediating this. There

are many pathways that cells utilize for endocytic uptake (Doherty and McMahon, 2009; Kumari et al., 2010; Mayor and Pagano, 2007). Several pathways utilize the protein dynamin (Doherty and McMahon, 2009), which is involved in the assembly and scission of plasma membrane invaginations during endocytosis (Ferguson and De Camilli, 2012; Sun and Tien, 2013). Dynamin polymerizes into a helix around growing invaginations, hydrolyzing the nucleotide GTP to constrict the necks of the invaginations and catalyze membrane fission, releasing intracellular vesicles off the plasma membrane (Ferguson and De Camilli, 2012; Morlot and Roux, 2013). To determine if dynamin was involved in Dectin-1 endocytosis, we expressed dynamin-K44A, a dominant-negative GTPase-dead dynamin mutant (Damke et al., 1994; van der Bliek et al., 1993), in RAW Dectin-1 cells. The cells were then stimulated with WGPsol and processed by immunofluorescence staining to detect Dectin-1. Interestingly, expression of the mutant completely abrogated the uptake of Dectin-1 induced by WGPsol binding, resulting in an intense labeling of surface Dectin-1 and essentially no intracellular staining. This occurred only in cells expressing the construct, as untransfected cells exhibited a large number of intracellular vesicles with WGPsol stimulation (**Figure 22A**).

To further verify the role of dynamin in Dectin-1 uptake, we acutely inhibited its GTP hydrolysis activity pharmacologically with the compound dynasore (Macia et al., 2006) while stimulating endocytosis with WGPsol. Immunofluorescence was used to examine the surface and intracellular Dectin-1 populations and soluble β-glucan (**Figure 22B**). As expected, for vehicle-treated cells, WGPsol and intracellular Dectin-1 colocalized and were distributed in a vesicular fashion throughout the cell (**Figure 22B**). Intriguingly, treatment with dynasore at 80 μM revealed Dectin-1 signal not only at the cell surface but also in the cell. However, the intracellular Dectin-1 appeared as a few large tubules that were consistently adjacent to the plasma membrane and that also contained WGPsol, suggesting that they were in fact plasma membrane invaginations. Increasing the dynasore concentration to 120 μM was then able to completely block Dectin-1 endocytosis, where both Dectin-1 and WGPsol remained localized

on the cell surface (**Figure 22B**). Treatment with the dynasore analogue dyngo-4A (McCluskey et al., 2013) also produced what appeared to be arrested, "un-pinched" membrane invaginations that contained WGPsol, similar to treatment with 80 μM dynasore (**Figure 23**). While somewhat unexpected, considering the dynamin-K44A mutant completely blocks Dectin-1 internalization (**Figure 22A**), the morphology of these membrane-proximal tubules is consistent with an inhibition of dynamin's role in the scission of membrane invaginations. Thus, their formation upon dynasore and dyngo-4A treatment is strong evidence that WGPsol-stimulated Dectin-1 uptake is dynamin-dependent.

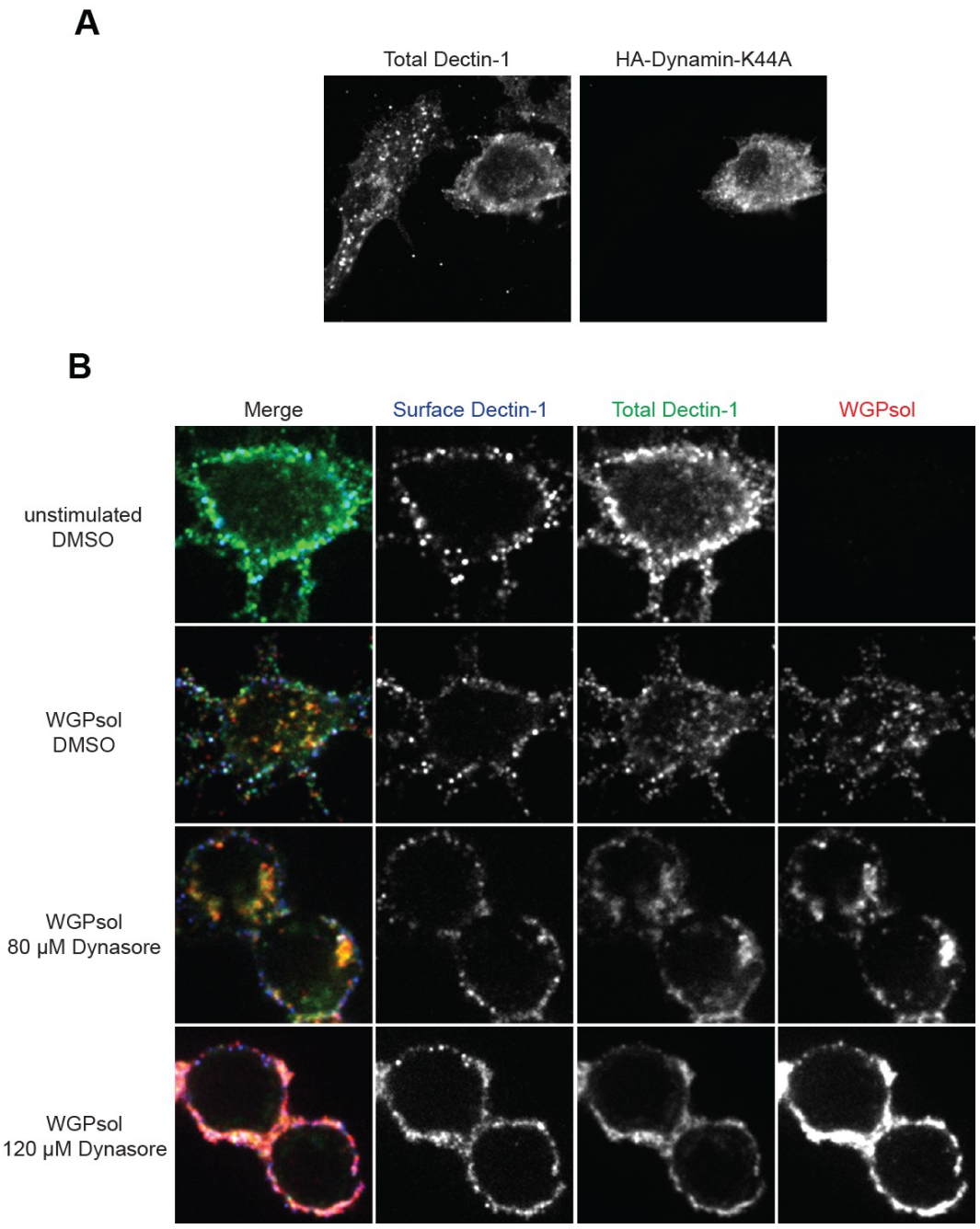

Figure 22: WGPsol-Induced Dectin-1 Internalization is Dynamin-Dependent

(**A**) RAW Dectin-1 cells were transiently transfected with an HA epitope tagged dominant-negative dynamin mutant with the substitution mutation K44A. 100 µg/mL WGPsol was added to the cells for 30 minutes at 37°C to stimulate uptake of the receptor. After fixation and permeabilization, Dectin-1 and the presence of the mutant (by virtue of the HA epitope tag) were detected by immunofluorescence staining. (**B**) RAW Dectin-1 cells were pre-treated for 30 minutes at 37°C in media containing 0.1% DMSO (vehicle control) or dynasore at 80 or 120 µM. The cells were then stimulated for 30 minutes in the same solutions with or without 100 µg/mL WGPsol. After fixation, surface Dectin-1 was labeled by immunofluorescence. WGPsol (by detecting β-glucan) and total Dectin-1

were labeled also by immunofluorescence after cell permeabilization. All images were acquired on the confocal microscope and are representative of the results from three independent experiments.

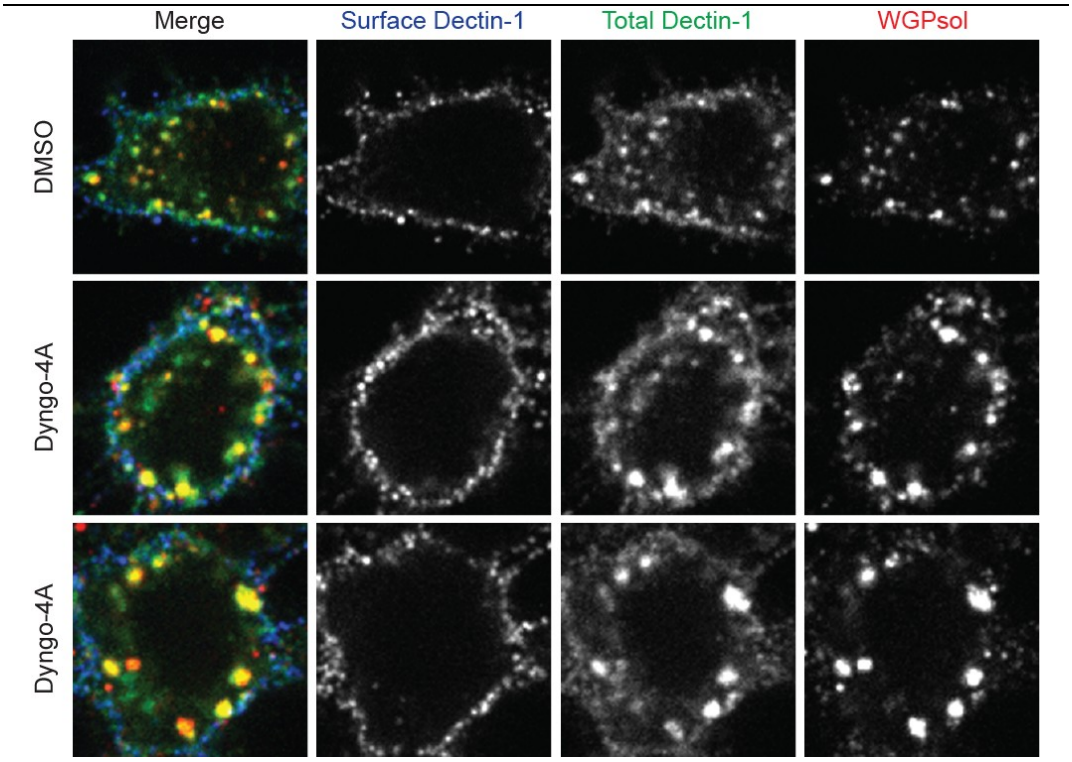

Figure 23: Dyngo-4A Treatment Inhibits the Dectin-1 Mediated Uptake of WGPsol
RAW Dectin-1 cells were pre-treated for 30 minutes at 37°C in media containing 0.1% DMSO (vehicle control) or 30 μM dyngo-4A. They were then stimulated in the same solutions supplemented with 100 μg/mL WGPsol for 30 minutes. After fixation, surface Dectin-1 was labeled by immunofluorescence. WGPsol and total Dectin-1 were labeled also by immunofluorescence, after permeabilization. Images were acquired on the confocal microscope. They are representative of the results from three independent experiments.

We next asked if P-curdlan-induced Dectin-1 uptake was also dynamin-dependent. Indeed, like for WGPsol stimulation, cells that expressed the dynamin-K44A mutant were unable to internalize Dectin-1 upon P-curdlan stimulation (**Figure 24A**). Furthermore, cells that were treated with 80 μM dynasore also produced tubules that appeared to be internalized but unreleased plasmalemmal invaginations (**Figure 24B**). Taken together, our results with dynasore and dyngo-4A treatment along with the expression of dominant-negative dynamin demonstrate clearly that dynamin is involved in the uptake of Dectin-1 and soluble β-glucans.

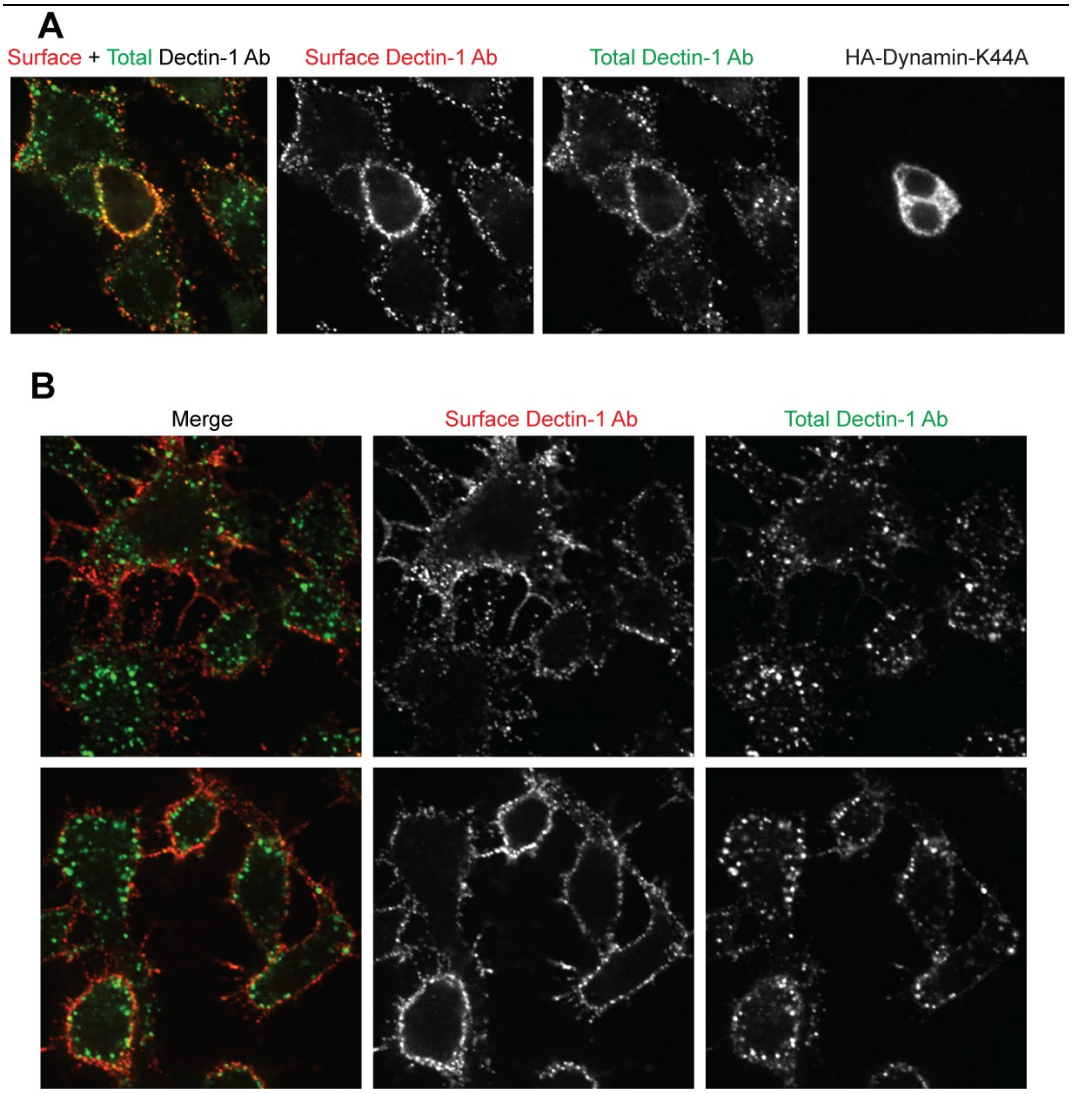

Figure 24: P-Curdlan-Induced Dectin-1 Internalization is Dynamin-Dependent

(**A**) RAW Dectin-1 cells were transiently transfected with the HA-tagged dynamin-K44A mutant. Prior to stimulation, they were chilled and labeled with anti-Dectin-1 antibodies for 20 minutes. The cells were then stimulated with 100 µg/mL P-curdlan for 30 minutes at 37°C. After their fixation, surface-localized anti-Dectin-1 antibodies were labeled by a Cy3-conjugated secondary antibody (in red). Subsequent permeabilization then allowed labeling of all the anti-Dectin-1 antibodies with a secondary antibody coupled to AF488 (in green). Finally, the dynamin mutant was detected by immunostaining for HA.

(**B**) RAW Dectin-1 cells were pre-treated for 30 minutes at 37°C in media containing 0.1% DMSO (vehicle control) or dynasore at 80 or 120 µM. The cells were then chilled in the same solutions and labeled with anti-Dectin-1 antibodies for 20 minutes. 100 µg/mL P-curdlan was then added to the cells in media containing 0.1% DMSO or 80 µM dynasore. After their fixation, surface-localized anti-Dectin-1 antibodies were labeled by a Cy3-conjugated secondary antibody (in red). Permeabilization then allowed labeling of all the anti-Dectin-1 antibodies using a secondary antibody coupled to AF488 (in green).

All images were acquired on the confocal microscope and are representative of the results

from three independent experiments.

3.4.2. Dectin-1 Colocalizes with Clathrin During its Endocytosis

A well-characterized endocytic process that is dynamin-dependent is clathrin-mediated endocytosis (CME) (Doherty and McMahon, 2009; McMahon and Boucrot, 2011). In CME, the assembly of a cage-like lattice of multiple clathrin molecules beneath the plasma membrane induces membrane invaginations (known as clathrin-coated pits) that deepen and are eventually budded off as endocytic vesicles (McMahon and Boucrot, 2011). As clathrin cannot interact directly with the receptor cargoes that utilize CME for uptake, the initiation of clathrin cage assembly and packaging of cargoes into these pits is assisted by many adaptor and accessory proteins who recruit each other through a network of protein-protein interactions (McMahon and Boucrot, 2011). Eventually, the dynamin-dependent scission of clathrin-coated pits occurs, releasing clathrin-coated vesicles containing the receptor cargoes into the cytoplasm. Soon after, the clathrin coat is taken apart and recycled to the plasma membrane where it can facilitate the formation of other pits (McMahon and Boucrot, 2011).

To determine if Dectin-1 could be internalized by CME, we first examined if the receptor co-internalized with established cargoes for CME. The transferrin receptor is a well-characterized CME cargo (Hopkins, 1985; Hopkins and Trowbridge, 1983; Jing et al., 1990; Motley et al., 2003; Nesterov et al., 1999), and is constitutively endocytosed, continuously internalizing whether bound by its ligand or not (Hopkins et al., 1985). Its ligand is the protein transferrin, which binds to and shuttles extracellular iron into the cell by virtue of its interaction with the transferrin receptor. Once in the cell, iron is released, whereas transferrin and the transferrin receptor are recycled to the plasma membrane (Dautry-Varsat et al., 1983; Klausner et al., 1983; van Renswoude et al., 1982). Interestingly, we observed that even without stimulation with soluble β-glucan ligands, incubating RAW Dectin-1 cells with AF488-labeled transferrin resulted in Dectin-1 internalization (**Figure 24**). Dectin-1 was found in numerous intracellular puncta, likely endocytic vesicles and endosomes, that all colocalized with transferrin

puncta, suggesting that Dectin-1 had co-internalized with the transferrin receptor. In fact, it has been observed that clustering the transferrin receptor induces the formation of clathrin-coated pits (Liu et al., 2010). Perhaps the binding of transferrin to its receptor upregulated the formation of clathrin-coated pits, and Dectin-1 molecules within the vicinity were taken into the pits and internalized. Regardless, the fact that Dectin-1 internalizes with transferrin suggested that it might utilize the same endocytic pathway as the transferrin receptor, which is clathrin-mediated endocytosis.

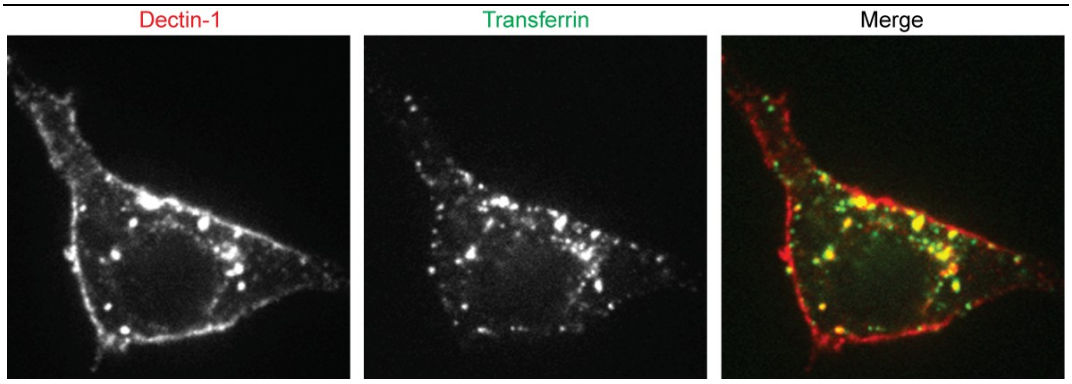

Figure 25: Dectin-1 Co-Internalizes with Transferrin
RAW Dectin-1 cells were incubated with 5 μg/mL AF488-labeled transferrin for 15 minutes, after which they were acid washed to remove surface-localized AF488-transferrin. Fixation, permeabilization and immunofluorescent detection of Dectin-1 followed. The image was acquired on the confocal microscope and is representative of the results from one experiment.

To visualize if Dectin-1 ligands actually moved into clathrin-coated pits or vesicles, we stimulated RAW Dectin-1 cells transfected with clathrin-GFP with AF546-labeled BSA-18-laminarin (**Figure 26**). Colocalization between clathrin-GFP and the conjugate ligand was observed at several puncta within 5 minutes of uptake, suggesting that the ligand was internalizing via clathrin-coated pits and vesicles (**Figure 26**).

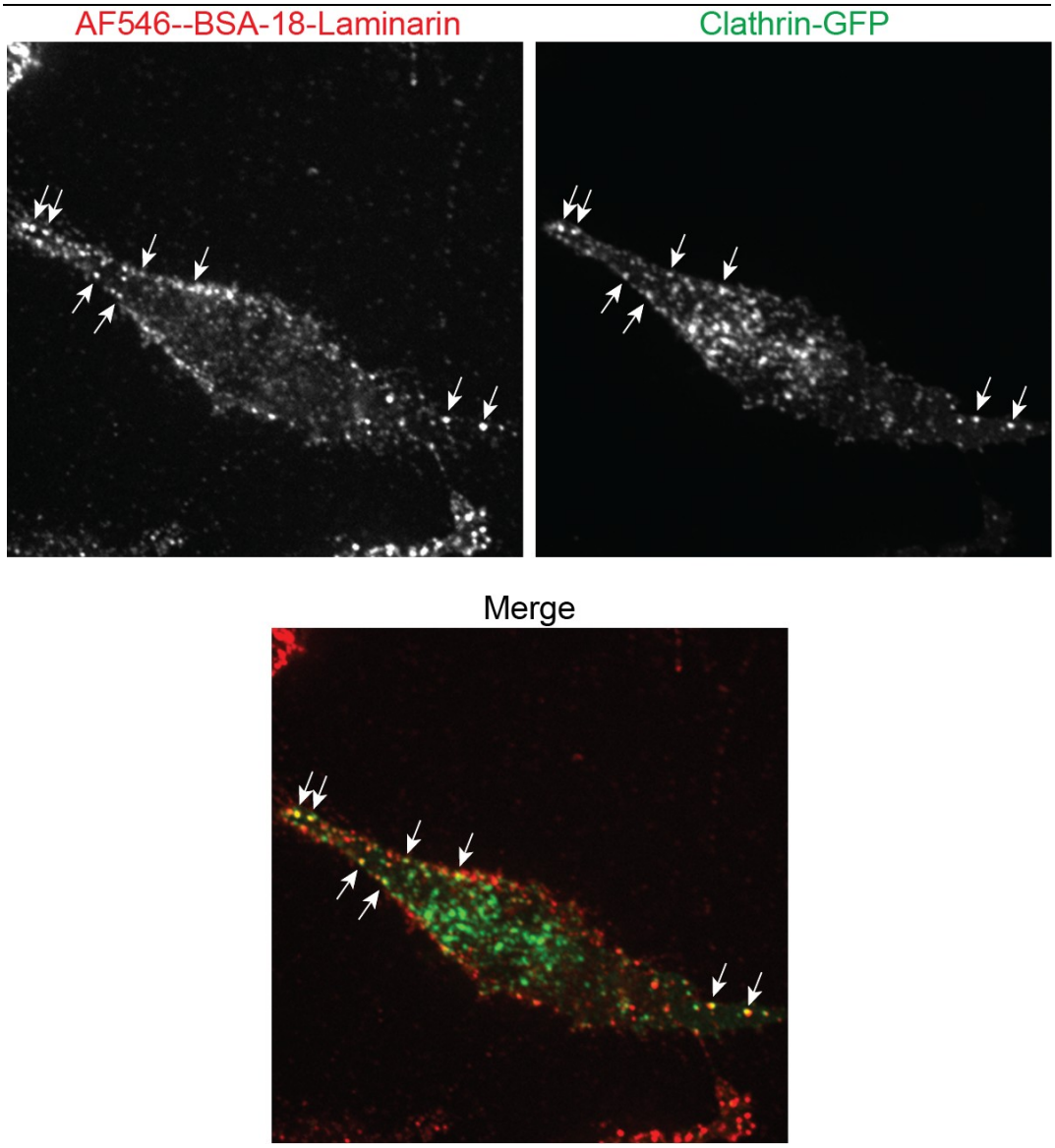

Figure 26: Internalized BSA-18-Laminarin Conjugate Ligand Colocalizes with Clathrin-GFP

RAW Dectin-1 cells were transfected with clathrin-GFP (green) and stimulated with AF546-tagged BSA-18-laminarin (red) for 5 minutes at 37°C. The cells were fixed and visualized by confocal microscopy. Arrows point to BSA-18-laminarin puncta that colocalize with clathrin-GFP. The image is representative of the results from two independent experiments.

To see if this colocalization could be reproduced when endogenous clathrin was labeled by immunofluorescence, RAW Dectin-1 cells were treated for 5 or 15 minutes with WGPsol. This time, the cells were fixed and labeled for Dectin-1, clathrin heavy chain (HC) and β-glucan by immunofluorescence (**Figure 27**). When WGPsol was present in the media, Dectin-1 that had been primarily on the

plasma membrane localized to numerous intracellular puncta. Many of these puncta colocalized with clathrin HC, suggesting we were seeing clathrin-coated vesicles of Dectin-1 loaded with Dectin-1 and WGPsol (**Figure 27**). Similar observations of a substantial colocalization between clathrin and Dectin-1 were made when the receptor was stimulated with P-curdlan (**Figure 28**). Therefore, upon ligand stimulation, Dectin-1 internalizes via endocytic pits and vesicles that are coated by clathrin.

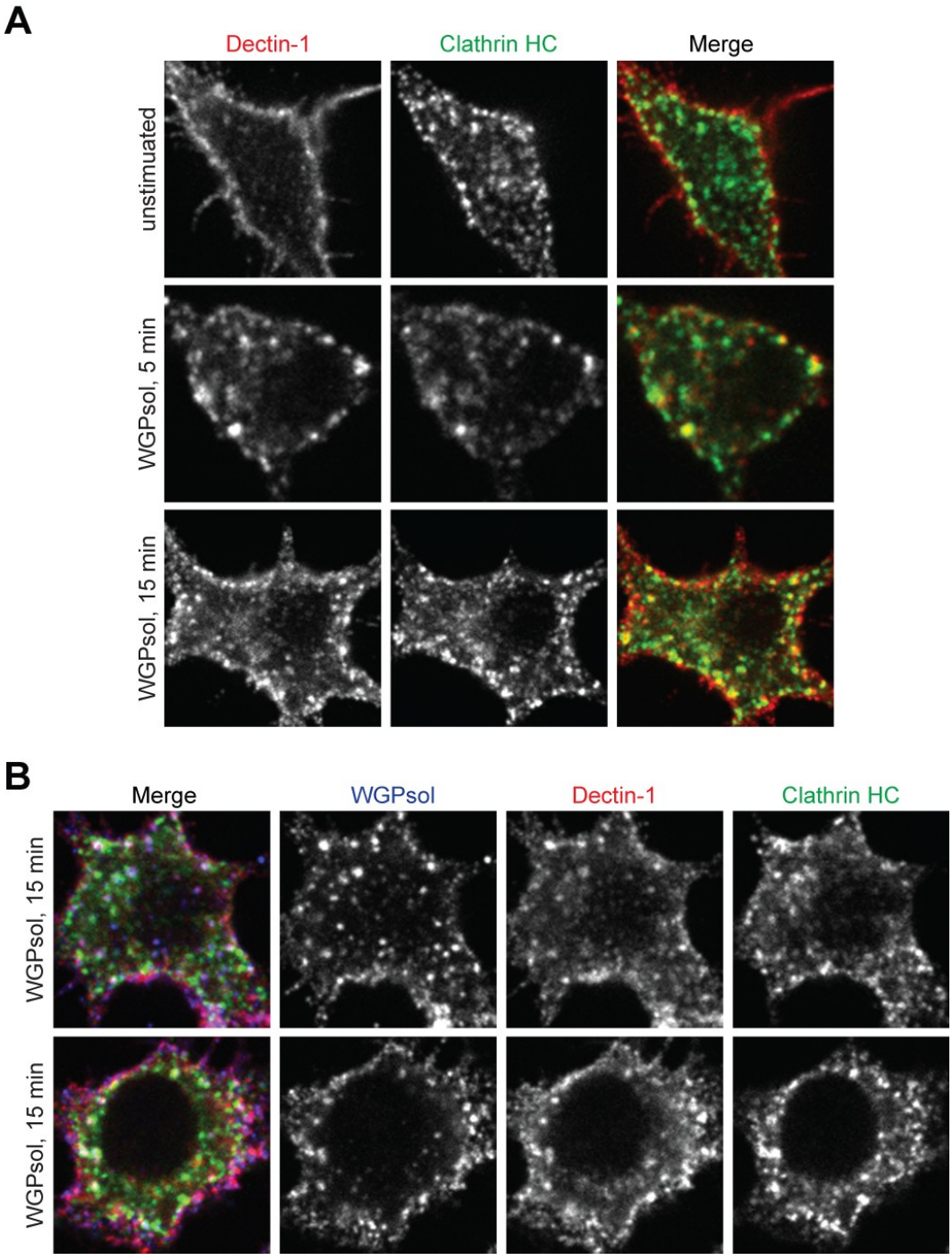

Figure 27: WGPsol-Stimulated Dectin-1 is Internalized into Puncta that Colocalize with Clathrin

RAW Dectin-1 cells were stimulated with 100 μg/mL of WGPsol for 5 or 15 minutes or left unstimulated. The cells were then fixed, permeabilized, and immunostained for clathrin heavy chain (HC) and Dectin-1 in (**A**), and clathrin HC, Dectin-1, and β-glucan in (**B**). Images were acquired on the confocal microscope and are representative of the results from three independent experiments.

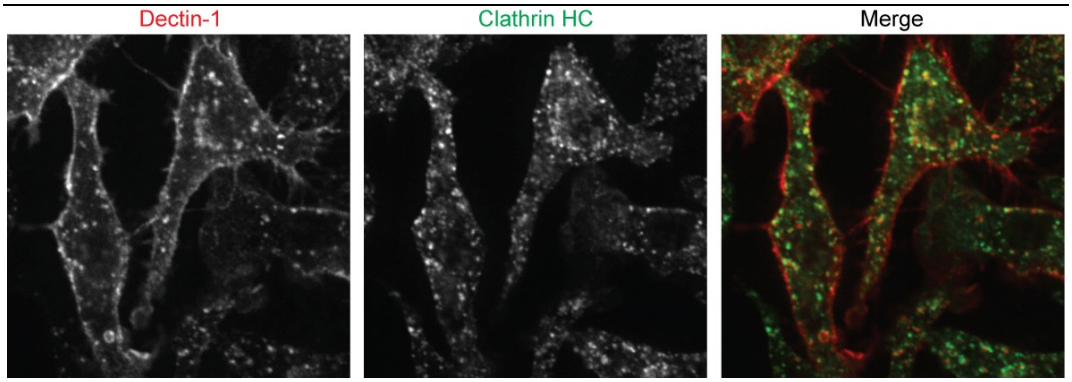

Figure 28: P-Curdlan-Stimulated Dectin-1 is Internalized into Puncta that Colocalize with Clathrin

RAW Dectin-1 cells were stimulated with 100 µg/mL of P-curdlan for 8 minutes. The cells were then fixed with in 3% PFA and 0.1% glutaraldehyde, permeabilized, and immunostained for clathrin heavy chain (HC) and Dectin-1. Images were acquired on the confocal microscope and are representative of the results from one experiment.

3.4.3. Inhibition of Clathrin-Mediated Endocytosis Blocks Dectin-1 Internalization

To determine if clathrin might actually play a role in the internalization of Dectin-1, we inhibited clathrin-mediated endocytosis pharmacologically by two means. The first, potassium depletion, is a classical method of inhibiting CME reversibly, resulting in the disappearance of clathrin coated-pits from the plasma membrane (Larkin et al., 1983; 1985; 1986). It involves incubating the cells in hypotonic buffer containing no potassium; the control buffer contains an isotonic concentration of potassium (Hansen et al., 1993b). As proof of concept, we observed that potassium depletion was able to completely inhibit the uptake of the CME cargo transferrin, as well as alter the intracellular distribution of clathrin (**Figure 29**). In the potassium-supplemented condition, transferrin was internalized appropriately and colocalized with clathrin. Thus, we were able to confirm the treatment was acting on clathrin and could block clathrin-mediated uptake. Next, we labeled RAW Dectin-1 cells with anti-Dectin-1 antibodies on ice and warmed them to 37°C in the presence of P-curdlan in potassium-depleted or potassium-supplemented media (**Figure 30**). After fixation, the surface-localized and total Dectin-1 antibodies were labeled by separate secondary antibodies coupled to different fluorescent dyes. Complete inhibition of Dectin-1 uptake was observed in the potassium-depleted condition, with Dectin-1

antibodies exhibiting an intense surface staining (yellow) and no internalized puncta (green), appearing as if the cells had been kept on ice, a classical technique used to block endocytic events in general. On the other hand, cells kept in potassium-supplemented buffer were able to internalize Dectin-1 upon P-curdlan stimulation as expected.

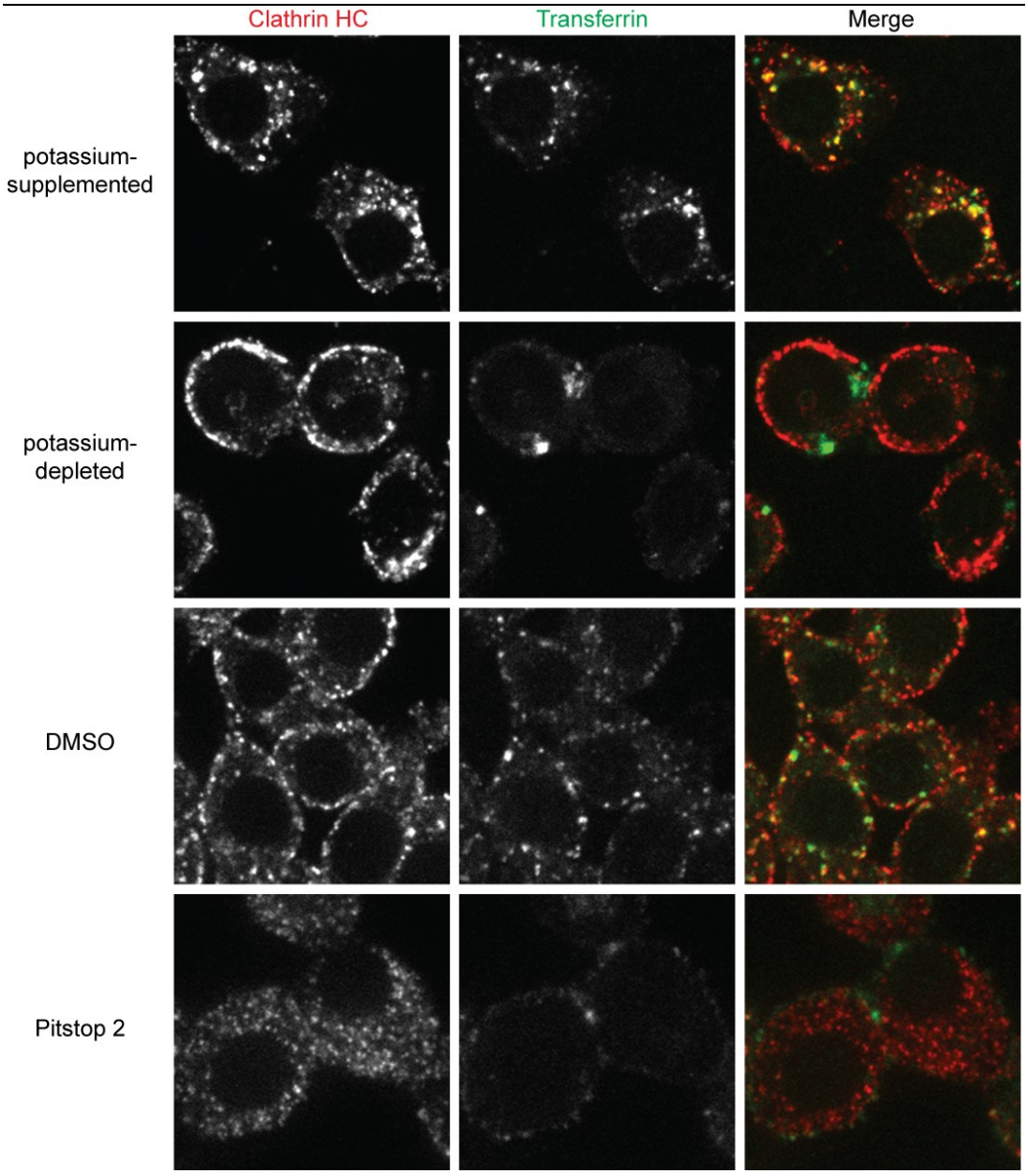

Figure 29: Potassium Depletion and Pitstop 2 Treatment Inhibit Transferrin Uptake and Alter Clathrin Localization

RAW Dectin-1 cells were incubated for 20 minutes at 37°C in potassium-supplemented or potassium-depleted media, or plain MEMα media containing 25 μM pitstop 2 or 0.1% DMSO as a vehicle control. They were then replaced with the same solutions containing

5 μg/mL AF488-labeled transferrin for 20 minutes, after which they were acid washed to remove cell surface-localized AF488-transferrin and then fixed. Permeabilization and immunofluorescent detection for clathrin heavy chain (HC) soon followed. Images were acquired on the confocal microscope and are representative results from two independent experiments.

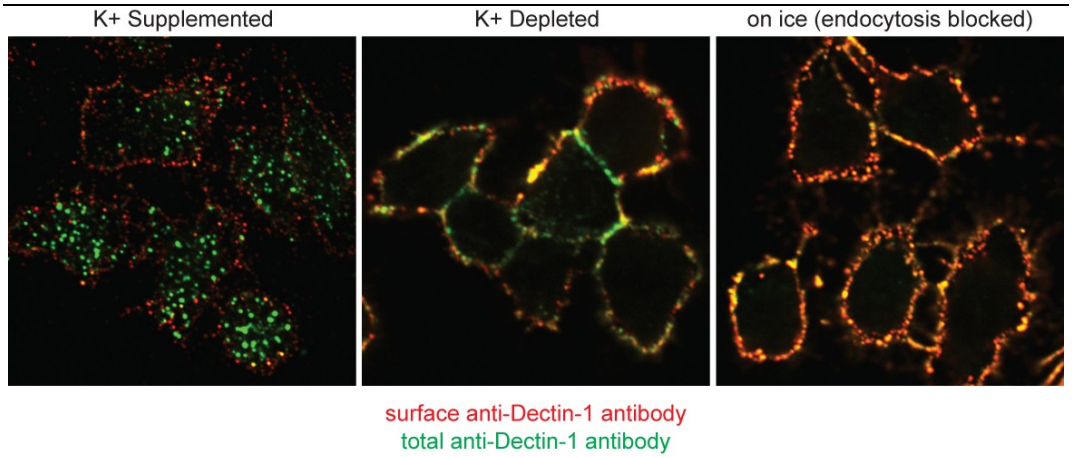

Figure 30: Potassium Depletion Blocks P-Curdlan-Stimulated Dectin-1 Uptake
RAW Dectin-1 cells were pre-treated with potassium-containing or potassium-depleted media for 30 minutes at 37°C. They were then chilled in the same solutions and labeled with anti-Dectin-1 antibodies for 20 minutes. The cells were next warmed to 37°C in potassium-containing or potassium-depleted media in the presence of 100 μg/mL P-curdlan for 30 minutes. After fixation, surface-localized anti-Dectin-1 antibodies were labeled by a Cy3-conjugated secondary antibody (in the red channel). Subsequent permeabilization then allowed detection of the distribution of all the anti-Dectin-1 antibodies with a secondary antibody coupled to AF488 (in the green channel). As a control for the absence of receptor internalization, RAW Dectin-1 cells were kept on ice and labeled with anti-Dectin-1 antibodies for 20 minutes. The cells were then fixed, labeled by a Cy3-conjugated secondary antibody, permeabilized, and labeled by an AF488-coupled secondary antibody. All images were acquired on the confocal microscope, and are representative results from four independent experiments.

To evaluate the effects of potassium depletion on Dectin-1 endocytosis using a different technique, we performed the cell surface biotinylation assay to measure P-curdlan induced Dectin-1 uptake in the presence of potassium-containing and depleted media (**Figure 31**). Indeed, potassium depletion noticeably inhibited both the ligand-stimulated and even constitutive (unstimulated) uptake of the receptor.

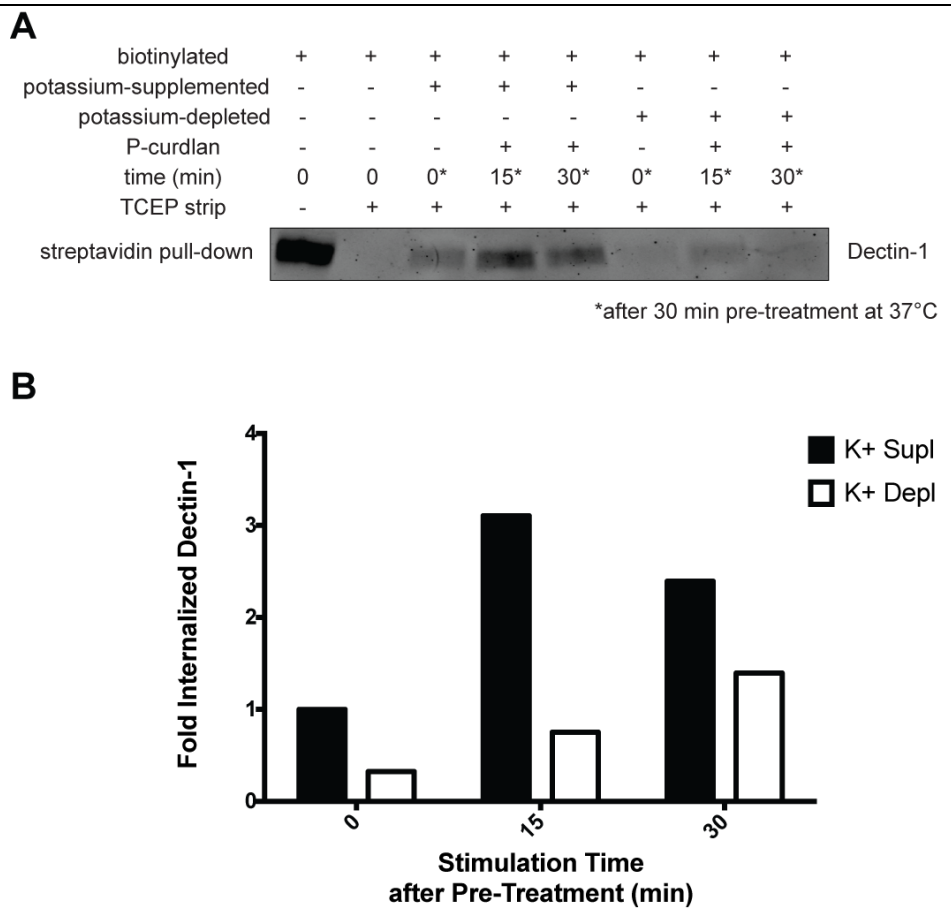

Figure 31: Potassium Depletion Inhibits P-Curdlan-Stimulated Dectin-1 Uptake, as Measured by Cell Surface Biotinylation

RAW Dectin-1 cells were surface-biotinylated and pre-treated for 30 minutes at 37°C in potassium-supplemented or -depleted media. The cells were then incubated in the same solutions containing P-curdlan at 100 μg/mL or not for 15 or 30 minutes. Subsequent preparation of cell lysates, streptavidin pull-down, SDS-PAGE, immunoblotting for Dectin-1, and quantification were performed according to the cell surface biotinylation protocol. An immunoblot of Dectin-1 in the streptavidin pull-down fraction is presented in (**A**), whereas the quantification of relative uptake is presented in (**B**). The data presented here are from one experiment.

In case potassium depletion may have produced non-specific effects on other routes of endocytosis, we treated cells with the clathrin inhibitor, pitstop 2, as a second and alternative mode of CME inhibition. Pitstop 2 binds to clathrin and inhibits the recruitment of accessory proteins necessary for CME, essentially blocking the entire process (Kleist et al., 2011). Similar to potassium depletion, treatment of RAW Dectin-1 cells with pitstop 2 noticeably abolished the uptake of transferrin and strikingly altered the intracellular localization of clathrin, which was redistributed from large vesicular structures to dim, cytoplasmic puncta

(**Figure 29**), confirming the inhibitor was active. RAW Dectin-1 cells were then stimulated with WGPsol in the presence of pitstop 2 or a vehicle control, fixed, and processed for surface/total Dectin-1 immunofluorescence with co-staining for β-glucan (**Figure 32A**), or co-immunstaining for total Dectin-1, clathrin, and β-glucan (**Figure 32B**). Treatment with pitstop 2 completely abolished uptake of Dectin-1, as staining for total Dectin-1 revealed the receptor was localized to the cell surface (**Figure 32**). Predictably, as the ligand for Dectin-1, the uptake of WGPsol was also inhibited upon Pitstop 2 treatment. On the contrary, cells that were not treated with pitstop 2 showed WGPsol colocalizing with clathrin and Dectin-1 in intracellular puncta. Thus, treatment with both potassium depleted media and the inhibitor pitstop 2 were able to inhibit Dectin-1 internalization.

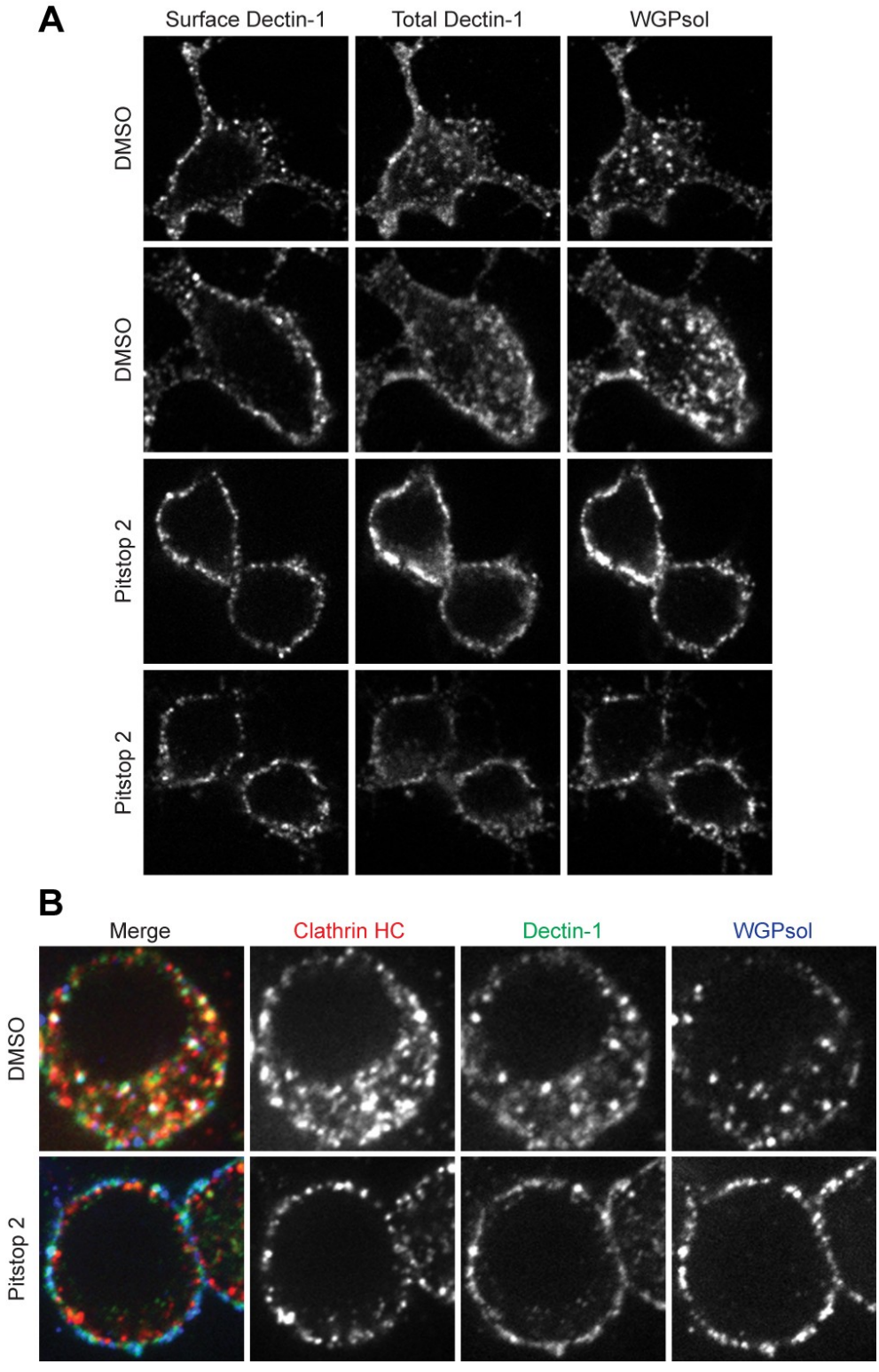

Figure 32: Pitstop 2 Treatment Inhibits the WGPsol-Stimulated Uptake of Dectin-1

RAW Dectin-1 cells were pre-treated for 15 minutes at 37°C with media containing 25 µM pitstop 2 or 0.1% DMSO (vehicle control). The cells were then stimulated with the same solutions containing 100 µg/mL WGPsol for 20 (**A**) or 15 (**B**) minutes at 37°C. The

cells were fixed and immunostained as follows. In (**A**), surface Dectin-1 was first labeled sequentially with a primary then fluorophore-coupled secondary antibody. The cells were then permeabilized and stained for total Dectin-1 using the same primary antibody but a secondary antibody coupled to a different fluorophore. Immunostaining for β-glucan was then performed. In (**B**), the cells were permeabilized and co-immunostained for clathrin heavy chain (HC), Dectin-1, and β-glucan. All images were acquired on the confocal microscope and are representative results from two independent experiments.

To verify true clathrin-dependence of Dectin-1 uptake without the use of acute chemical inhibitors, we depleted the heavy chain (HC) subunit of clathrin by RNA interference. Clathrin depletion was detectable by immunoblotting and immunostaining (**Figure 33A**, **B**), and was able to inhibit the uptake of transferrin in transfected cells (**Figure 33C**, **D**). By confocal imaging, compared to mock-transfected cells, it was clear clathrin depletion completely inhibited the uptake of antibody-labeled surface Dectin-1 that was stimulated by P-curdlan (**Figure 34**). More replicates of this experiment are needed to confirm the dependence of Dectin-1 uptake on clathrin-mediated endocytosis. However, combined with previous results where CME was inhibited pharmacologically, it is highly likely that Dectin-1 uptake is clathrin-dependent.

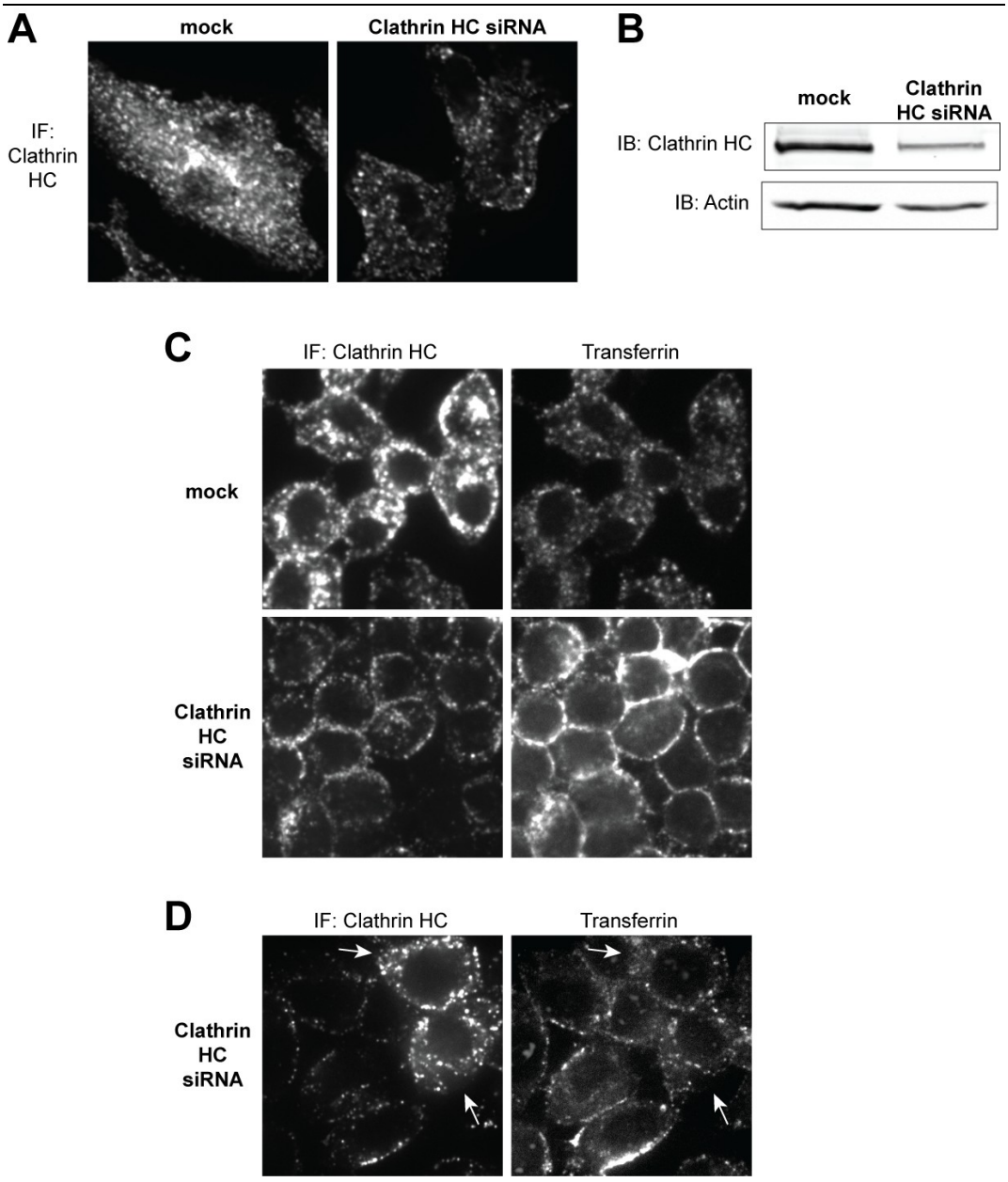

Figure 33: RAW Dectin-1 Cells Depleted of Clathrin Heavy Chain by RNA Interference are Unable to Internalize Transferrin

(**A**) and (**B**): RAW Dectin-1 cells were transfected with a pool of siRNAs targeting clathrin heavy chain (HC) at 100 nM or mock transfected with Lipofectamine 2000 (mock). To assess knockdown efficiency of clathrin HC, 48 hours post-transfection, the cells were fixed, stained by immunofluorescence (IF) for clathrin HC, and visualized by confocal microscopy (**A**), or lysed, processed by SDS-PAGE, and immunoblotted (IB) for the presence of clathrin HC and actin (used as a loading control) (**B**).

(**C**) and (**D**): Clathrin HC siRNA- or mock-transfected cells were incubated in 5 μg/mL transferrin-AF488 at 37°C for 30 minutes 48 hours post-transfection. No acid wash was

performed to allow visualization of surface-remaining transferrin. The cells were fixed, immunostained for clathrin HC and visualized under the confocal microscope. In (**C**), the bottom panel of images shows a field of view where all cells are depleted in clathrin HC. (**D**) depicts a field comparing less clathrin HC-depleted or untransfected cells (arrows) to the surrounding depleted cells.

The data shown are representative results from three (**A**), six (**B**), and two (**C**, **D**) independent experiments. Images of both the mock- and Clathrin HC siRNA-transfected conditions in (**A**) and (**C**) were captured with the same acquisition settings.

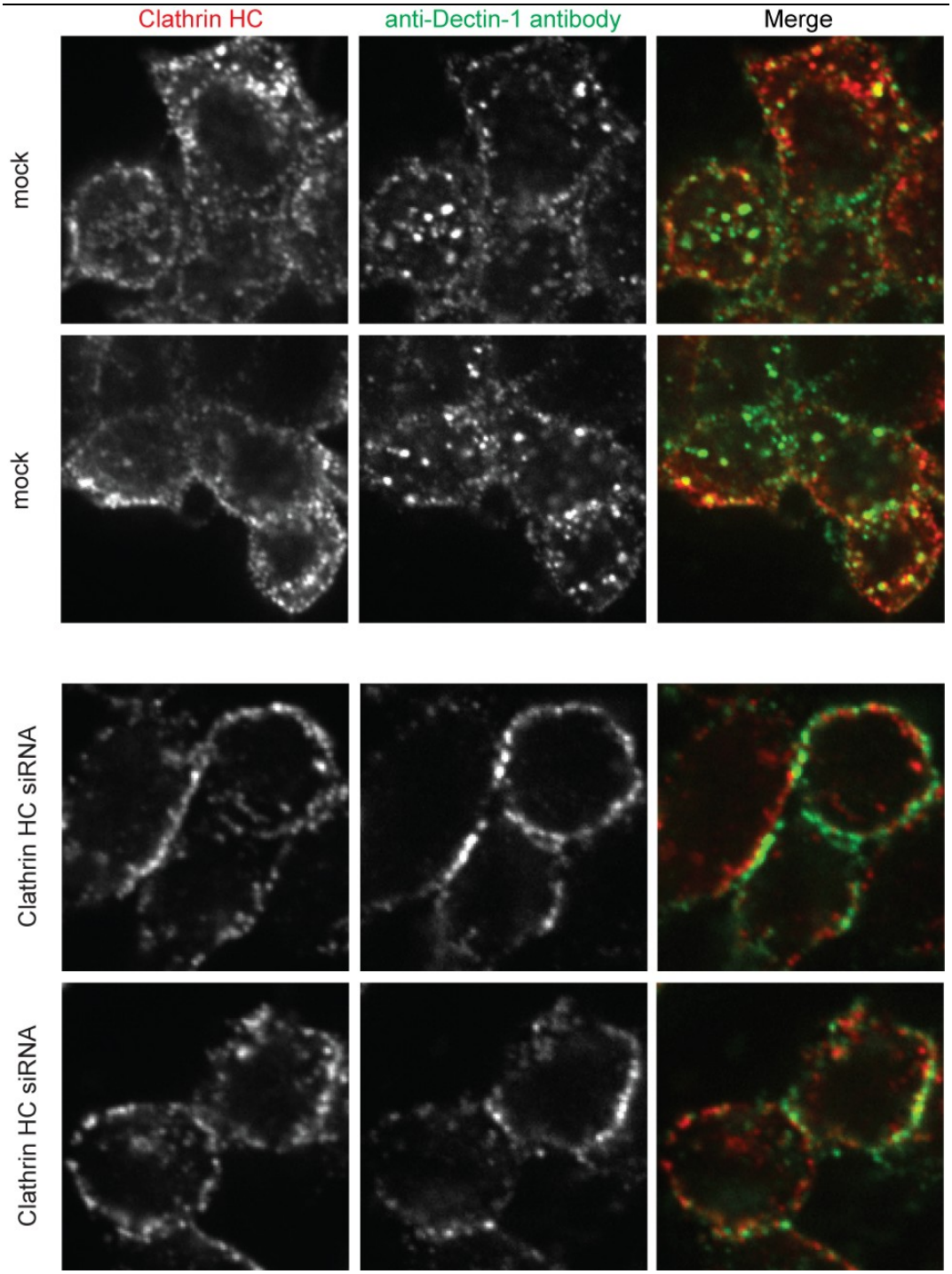

Figure 34: P-Curdlan-Induced Dectin-1 Uptake is Inhibited in Cells Depleted of Clathrin Heavy Chain by RNA Interference

48 hours post-transfection, clathrin HC siRNA- or mock-transfected RAW Dectin-1 cells were labeled on ice with anti-Dectin-1 antibodies for 20 minutes, washed in PBS, and incubated in media containing 100 µg/mL P-curdlan at 37°C for 30 minutes. After fixation and permeabilization, the cells were immunolabeled for clathrin HC and the bound Dectin-1 antibody, then visualized under the confocal microscope. Shown are

representative images from one experiment.

3.4.4. Dectin-1 Receptor-Mediated Endocytosis is Potentially Actin-Dependent

Inhibition of actin polymerization also affected Dectin-1 uptake. Treatment of RAW Dectin-1 cells with an inhibitor of actin polymerization, Cytochalasin B, partially abolished the uptake of Dectin-1 stimulated by P-curdlan (**Figure 35**) and WGPsol (data not shown), as measured by the cell surface biotinylation assay. To validate the proper function of Cytochalasin B, treatment of RAW Dectin-1 cells with the compound completely disrupted the cortical actin cytoskeleton of the cells, as visualized by confocal microscopy using the actin probe, Alexa Fluor 488-phalloidin (data not shown). These experiments suggest that actin remodelling events play a role in the uptake of Dectin-1. Further investigations are needed to clarify if actin remodelling plays a direct role in clathrin-mediated endocytosis, or acts in a separate uptake mechanism such as macropinocytosis.

To this point, we have demonstrated that the soluble β-glucan-stimulated uptake of Dectin-1 is a dynamin-, most likely clathrin-, and potentially actin-dependent process.

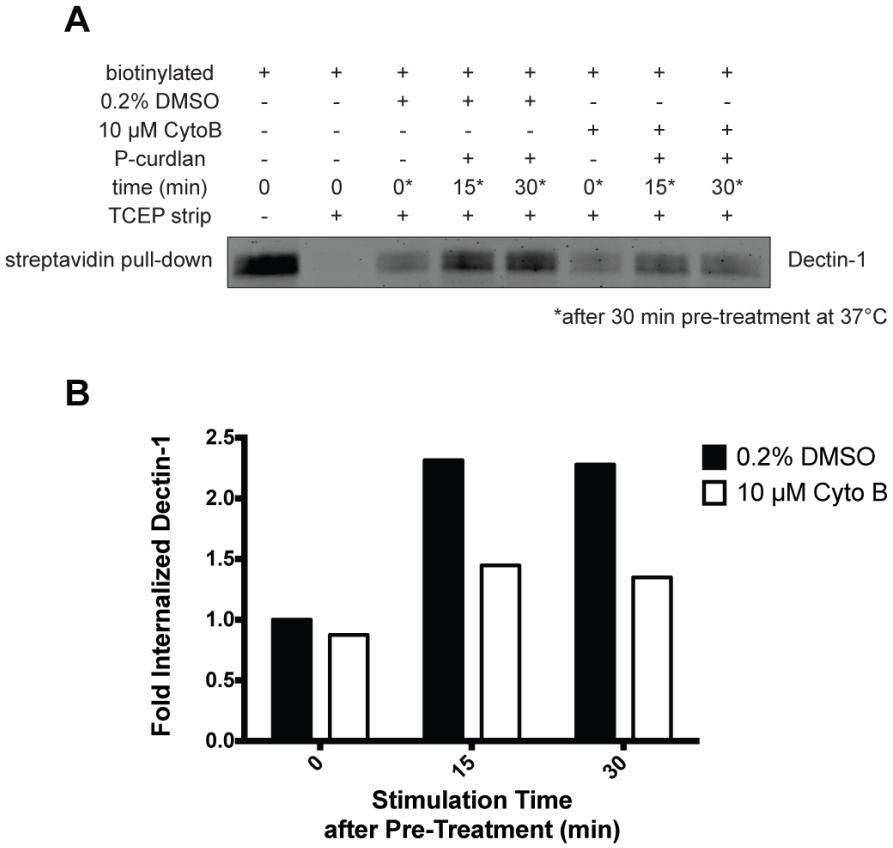

Figure 35: Cytochalasin B Treatment Partially Inhibits P-Curdlan-Stimulated Dectin-1 Uptake

RAW Dectin-1 cells were surface-biotinylated and pre-treated for 30 minutes at 37°C in media containing 0.2% DMSO (vehicle control) or 10 µM cytochalasin B (Cyto B). The cells were then incubated in the same solutions containing P-curdlan at 100 µg/mL or not for 15 or 30 minutes. Subsequent preparation of cell lysates, streptavidin pull-down, SDS-PAGE, immunoblotting for Dectin-1, and quantification were performed according to the cell surface biotinylation protocol. An immunoblot of Dectin-1 in the streptavidin pull-down fraction is presented in (**A**), whereas the quantification of relative uptake is presented in (**B**). The data presented here are from one experiment.

3.5. Dectin-1 Trafficking

3.5.1. WGPsol-Stimulated Dectin-1 is Trafficked to Early Endosomes and the Recycling Compartment

Having dissected the mechanisms involved in Dectin-1 uptake, we were now interested in determining which trafficking routes Dectin-1 would follow upon its entry into the cell. It is well established that after receptor-mediated endocytosis, internalized receptors traverse the endosomal-lysosomal pathway (Sorkin and Zastrow, 2009). This pathway encompasses several membrane-

bound compartments in the cell, including the early endosomes, recycling endosomes, and late endosomes/lysosomes. Endocytic vesicles fuse with the early endosome (the biogenesis of which is likely due to the homotypic fusion of multiple endocytic vesicles), which serves as a sorting station that dictates the fate of the receptors (Huotari and Helenius, 2011; Jovic et al., 2010). Receptors of similar fates are selected and concentrated in microdomains or tubular extensions of the early endosome. The tubular extensions can bud off, either recycling directly to the plasma membrane, or fusing with the perinuclear vesiculotubular recycling compartment, of which recycling endosomes develop from and eventually also fuse with the plasma membrane (Grant and Donaldson, 2009; Jovic et al., 2010). On the other hand, receptors diverted to microdomains in the early endosome are encapsulated in intraluminal vesicles while the early endosome undergoes maturation to a late endosome (Huotari and Helenius, 2011; Saftig and Klumperman, 2009). The late endosome eventually fuses with the lysosome, encountering hydrolytic enzymes that result in the degradation of its luminal contents (Saftig and Klumperman, 2009). Therefore, the canonical fates of endocytosed cargoes are their trafficking to either late endosomes and lysosomes for degradation of the receptors and their ligands, or to recycling compartments for their return to the plasma membrane (Grant and Donaldson, 2009; McMahon and Boucrot, 2011; Sorkin and Zastrow, 2009).

The compartments of the endo-lysosomal pathway are each distinguished by specific markers that are required for their function (Grant and Donaldson, 2009; Jovic et al., 2010; Saftig and Klumperman, 2009; Stenmark, 2009). To identify which compartments Dectin-1 traversed upon WGPsol stimulation, we performed experiments comparing the localization of ligand-bound Dectin-1 to these markers. We chose the following markers: Rab5, a marker of endocytic vesicles and the early endosome (Jovic et al., 2010; Stenmark, 2009); Rab7, a marker of the late endosome (Huotari and Helenius, 2011; Stenmark, 2009); LAMP2 (lysosome-associated membrane protein 2), a marker of lysosomes (Saftig and Klumperman, 2009); Rab4, Rab11, and VAMP3 (vesicle-associated membrane protein 3), markers of the recycling compartment (Bajno et al., 2000;

Daro et al., 1996; Galli et al., 1994; Grant and Donaldson, 2009; McMahon et al., 1993); and Giantin, a marker of the Golgi apparatus (Linstedt and Hauri, 1993). Each marker was either transfected into RAW Dectin-1 cells as a GFP-tagged construct or labeled by immunofluorescence after cell fixation. RAW Dectin-1 cells were stimulated with WGPsol for 15 minutes at 37°C, after which several washes were performed to remove unbound ligand. The cells were then kept at 37°C for various times to chase the "pulse" of WGPsol-bound Dectin-1. After fixation, the cells were then immunostained for Dectin-1 or β-glucan (and if needed, the compartmental marker).

We observed that Dectin-1 strongly colocalized with $Rab5^+$, $Rab4^+$, $Rab11^+$, and $VAMP3^+$ vesicular structures at 15 and 30 minutes of internalization (**Figure 36**), suggesting that upon WGPsol binding, Dectin-1 immediately enters early endosomes and then the recycling compartment. Remarkably, there was little colocalization of Dectin-1 and $Rab7^+$ or $LAMP2^+$ structures at 15 minutes or even 120 minutes after the pulse of WGPsol, suggesting that most of Dectin-1 is not targeted for degradation upon stimulation with WGPsol (**Figure 37**). Co-staining for the presence of WGPsol with the anti-β-glucan antibody revealed that the ligand remained bound to the receptor and similarly avoided lysosomes at both 15 and 120 minutes, although the 120-minute timepoint also featured some puncta that were only $WGPsol^+$, suggesting some separation between ligand and receptor had occurred (**Figure 37B**).

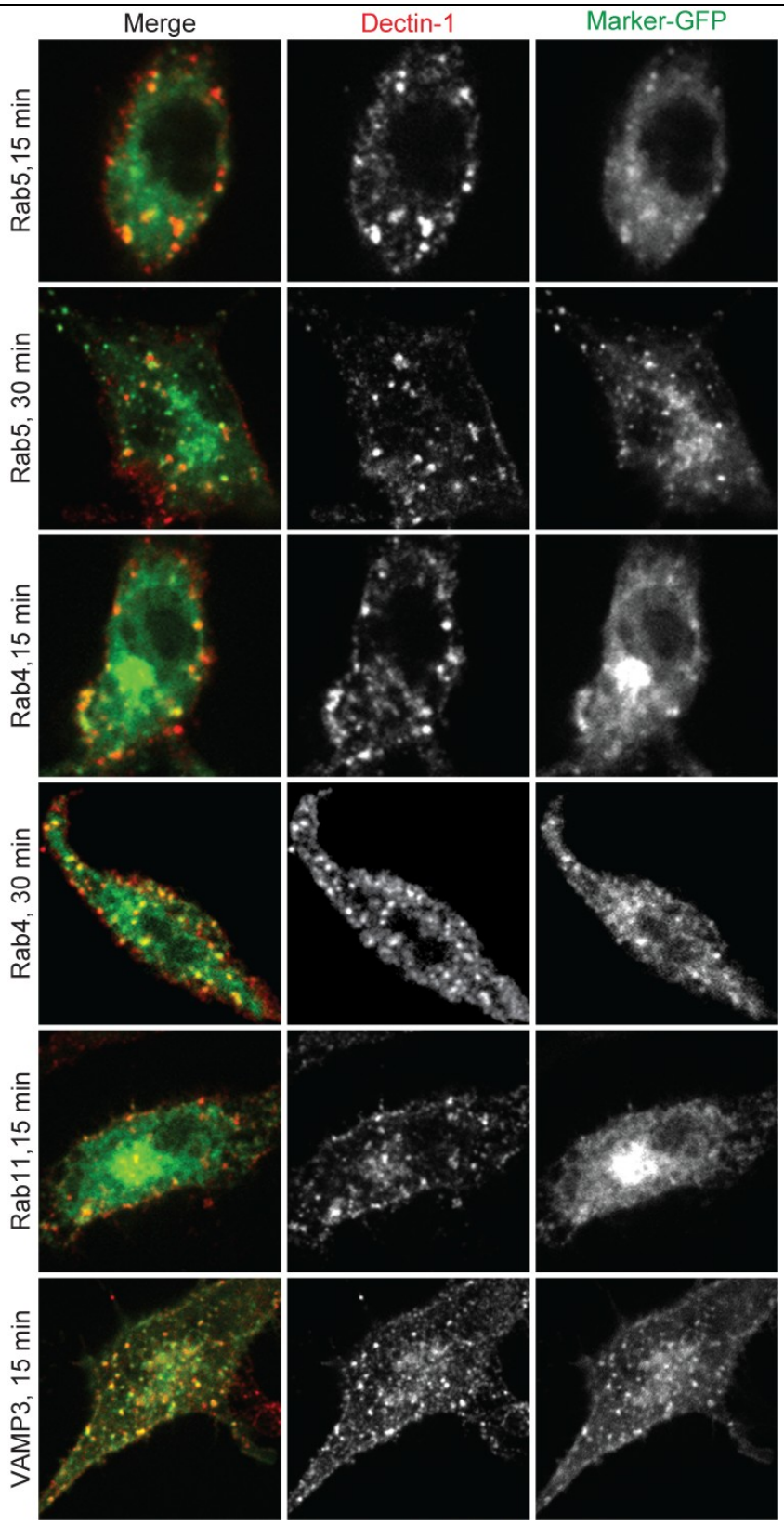

Figure 36: WGPsol-Stimulated Dectin-1 Colocalizes with Markers of Early Endosomes and the Recycling Compartment

Figure 36: WGPsol-Stimulated Dectin-1 Colocalizes with Markers of Early Endosomes and the Recycling Compartment (Cont'd)

RAW Dectin-1 cells were transfected with the appropriate GFP-tagged constructs (Rab5-, Rab4-, Rab11-, VAMP3-GFP; indicated on the left). The cells were then stimulated for 15 minutes with 100 μg/mL WGPsol at 37°C. For the 30-minute timepoint, the ligand was washed away with several PBS rinses, and the cells were chased in plain media at 37°C for the remaining 15 minutes. At the end of the indicated timepoints (left label), the cells were fixed, permeabilized in 0.1% saponin, and immunostained for Dectin-1 before visualization on the confocal microscope. The images presented are representative of WGPsol-induced trafficking at both 15 and 30 minutes after the initiation of WGPsol stimulation. They are representative of the results from two (Rab11, VAMP3) or three (Rab4, Rab5) independent experiments.

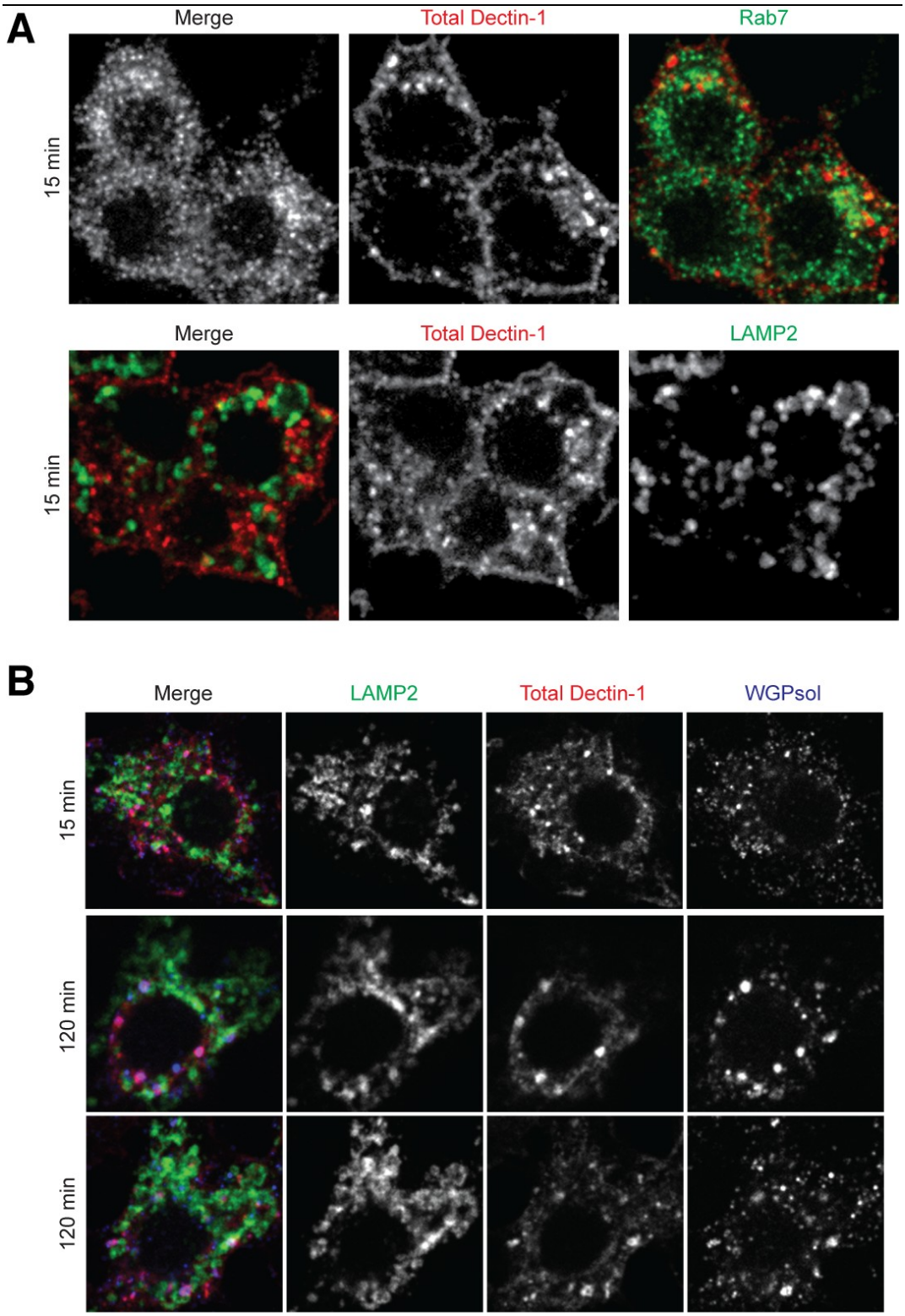

Figure 37: WGPsol-Stimulated Dectin-1 Does Not Colocalize with Markers of Late Endosomes and Lysosomes

RAW Dectin-1 cells were all stimulated for 15 minutes with 100 µg/mL WGPsol at 37°C. For the 120-minute timepoint, however, the ligand was washed away with several PBS

rinses, and the cells were chased in plain media at 37°C for the remaining 105 minutes. At the end of the indicated timepoints, the cells were fixed, permeabilized with 0.1% saponin, and immunostained for the organellar marker of interest (Rab7 or LAMP2) and either Dectin-1 (**A**) or Dectin-1 and soluble β-glucan (**B**) before visualization on the confocal microscope. The images shown are representative of the results from one experiment (Rab7) or three independent experiments (LAMP2).

A recent report found that the soluble β-glucan glucan phosphate was trafficked to the Golgi apparatus beginning 15 minutes after stimulation and up to at least 24 hours (Ozment et al., 2012). In our system, we were unable to detect significant colocalization of WGPsol with the Golgi apparatus at 15, 60 or 120 minutes post-stimulation (**Figure 38**), although ligand and cell-type differences may contribute to the discrepancy.

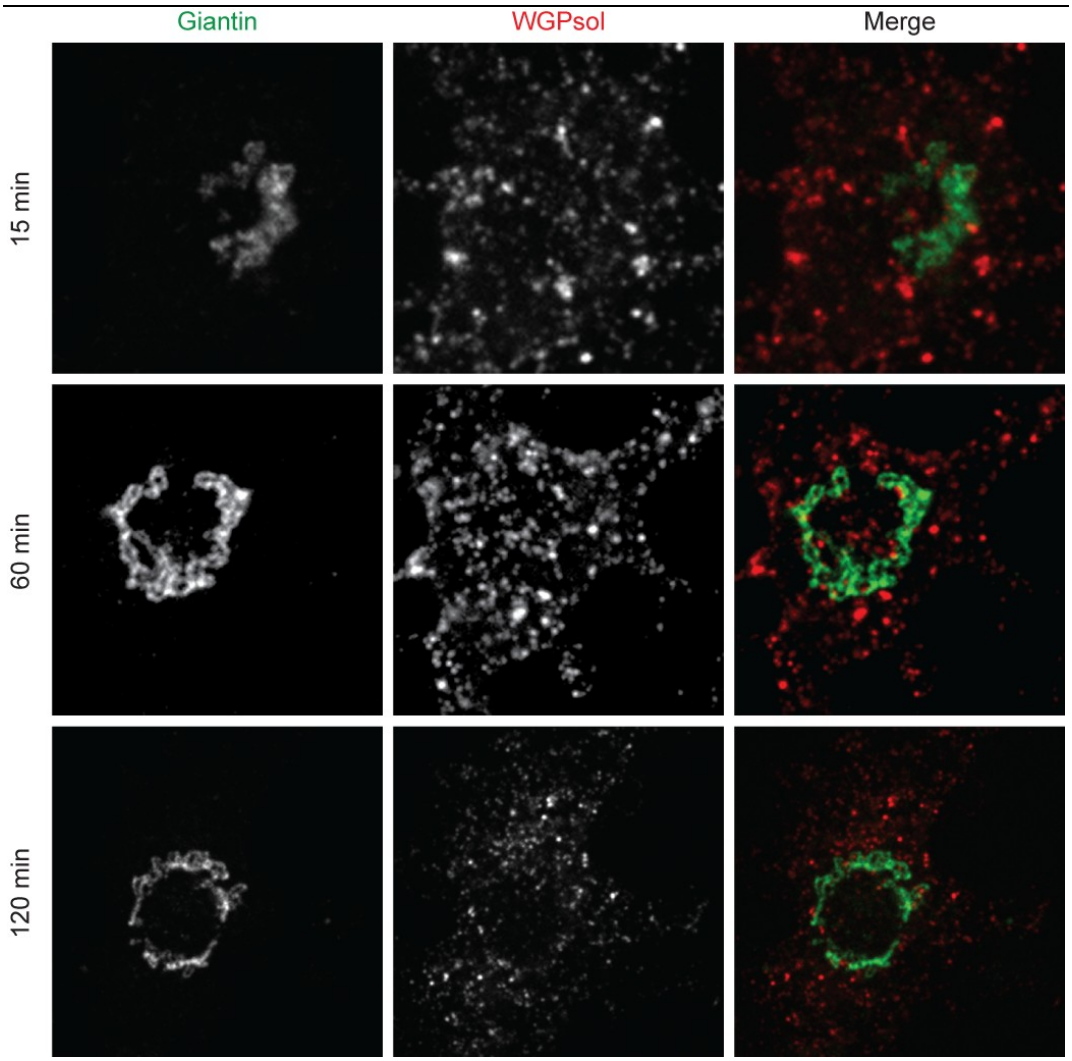

Figure 38: Internalized WGPsol Does Not Colocalize with the Golgi Apparatus Marker Giantin

RAW Dectin-1 cells were all stimulated for 15 minutes with 100 μg/mL WGPsol at 37°C. For the 60- and 120-minute timepoints, however, the ligand was washed away with several PBS rinses, and the cells were chased in plain media at 37°C for the remaining 45 or 105 minutes. At the end of the indicated timepoints, the cells were fixed, permeabilized with 0.1% saponin, and immunostained for the Golgi marker giantin and soluble β-glucan prior to visualization on the confocal microscope. The images shown are representative of the results from two independent experiments.

Thus, our confocal images suggest that upon binding WGPsol, Dectin-1 is targeted to the early endosome and then to the recycling compartment at least up to 30 minutes post-stimulation. This is consistent with Dectin-1 not appearing to traffic to late endosomes or lysosomes. We also found that WGPsol is not trafficked to the Golgi apparatus. However, it would be interesting to determine if the majority of WGPsol eventually separates from Dectin-1 or remains bound to

the receptor during its time in the cell.

3.5.2. Dectin-1 is Not Targeted to Lysosomes Upon Laminarin- and WGPsol-Stimulated Uptake

Given the lack of colocalization between Dectin-1 and lysosomes, it is likely that Dectin-1 does not traffic to lysosomes upon ligand stimulation. As added confirmation, we conducted the cell surface biotinylation assay to measure Dectin-1 uptake in the presence or absence of two lysosomotropic agents: chloroquine, which interferes with the low pH of the lysosome, which is required for the hydrolytic activities of the enzymes that reside there, and leupeptin, which inhibits a class of proteases with members that residue in the lysosome. The use of either inhibitor should prevent lysosome-associated degradation. We reasoned that if Dectin-1 was trafficked to lysosomes and degraded within them, the addition of either inhibitor would increase the levels of internalized Dectin-1 detected by the cell surface biotinylation assay. Indeed, treatment with leupeptin was unable to alter the levels of internalized Dectin-1 upon WGPsol stimulation (**Figure 39A**), lending support to the receptor's observed trafficking through the recycling compartment. Chloroquine did slightly increase levels of internalized Dectin-1, although we believe this could be due to it having general alkalinizing effects on the endosomal system, the compartments of which all exhibit acidic luminal pHs (Casey et al., 2010). Perhaps the alkalinization altered the function of the recycling compartment, resulting in more Dectin-1 being retained intracellularly. A similar surface biotinylation experiment was performed with laminarin as the stimulating β-glucan (**Figure 39B**). Similar to WGPsol, leupeptin treatment did not drastically alter the levels of internalized Dectin-1 stimulated by laminarin, although chloroquine treatment slightly increased it. Importantly, both these experiments are preliminary results and require more replicates for true conclusions to be drawn. However, the results from leupeptin treatment support the imaging data showing little colocalization of Dectin-1 to $LAMP2^+$ compartments (**Figure 37**), suggesting that Dectin-1 does not traffic to late endosomes and lysosomes.

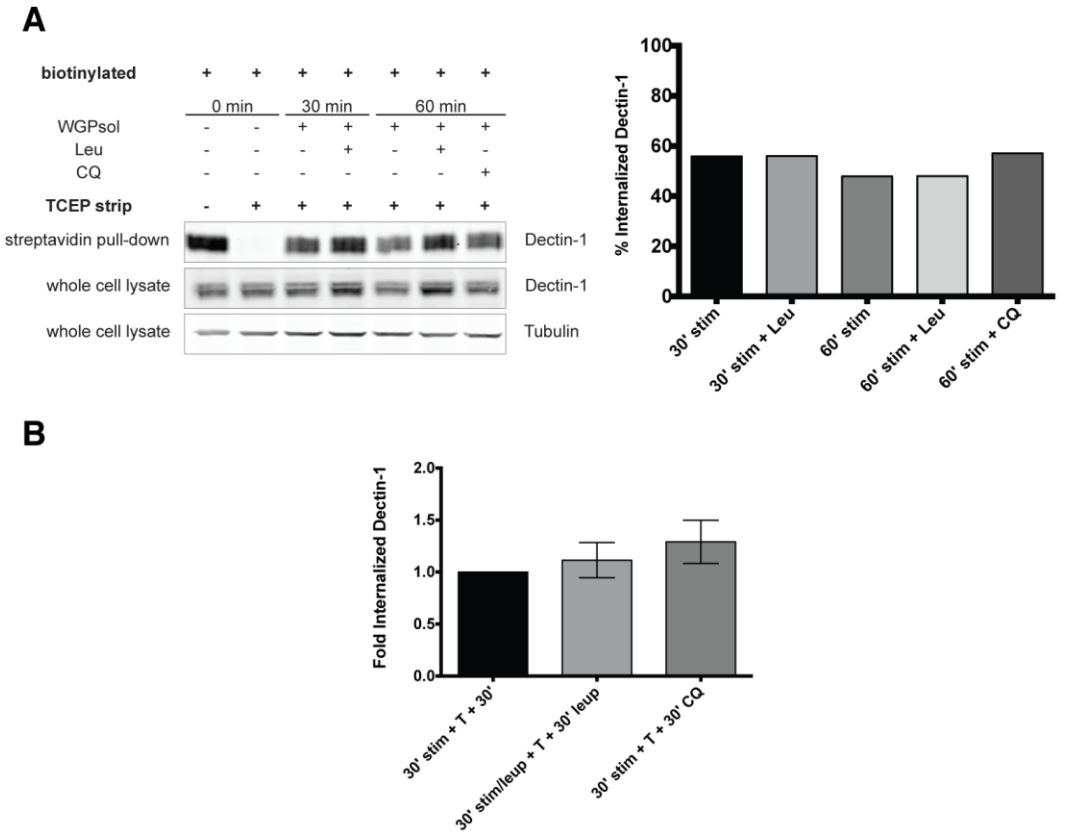

Figure 39: Disruption of Lysosomal Function on the Levels of Internalized Dectin-1

(**A**) RAW Dectin-1 cells were pre-treated for 2 hours with 250 μM leupeptin if needed, and then surface-biotinylated. Dectin-1 uptake was stimulated with 100 μg/mL WGPsol for 30 or 60 minutes at 37°C. For the leupeptin-treated conditions (Leu), 250 μM leupeptin was present during WGPsol uptake. For the chloroquine-treated condition (CQ), chloroquine was added to the media to a final concentration of 250 μM during the last 30 minutes of WGPsol internalization to avoid inhibiting the initial internalization process. After TCEP stripping, the cells were lysed and processed according to the cell surface biotinylation protocol. The detection of increased Dectin-1 levels in the whole cell lysate suggested that leupeptin pretreatment increased the overall amount of Dectin-1 prior to surface biotinylation. This corresponded to an increase in biotinylated Dectin-1 in the streptavidin pull-down. Normalizing the levels of biotinylated Dectin-1 to that of Dectin-1 in the whole cell lysate revealed that leupeptin treatment had no effect on the levels of internalized Dectin-1 (right, graph). The results presented are from one experiment.

(**B**) RAW Dectin-1 cells were pre-treated for 2 hours with 250 μM leupeptin, if needed, and then surface-biotinylated. Dectin-1 uptake was stimulated with 100 μg/mL laminarin for 30 minutes at 37°C. The cells were stripped with TCEP (T) on ice to restrict the biotin label to a pool of internalized Dectin-1, then were warmed to 37°C in plain media for another 30 minutes. For the leupeptin-treated condition (Leu), 250 μM leupeptin was present during both incubations, whereas in the chloroquine-treated condition (CQ), 250 μM chloroquine was only present during the second 30 minute incubation. The cells were lysed and processed according to the cell surface biotinylation protocol. Shown is the quantification averaging the results from two independent experiments.

3.5.3. Dectin-1 is Recycled to the Plasma Membrane Upon Laminarin-, WGPsol-, and P-Curdlan-Stimulated Uptake

The colocalization of Dectin-1 with markers of recycling endosomes suggested it was being recycled to the plasma membrane. To demonstrate this trafficking event, we performed a modified cell surface biotinylation assay that utilized two TCEP stripping steps: the first to restrict the biotin label to only a pool of internalized Dectin-1 in the cell, and the second to assess the return of Dectin-1 to the plasma membrane. RAW Dectin-1 cells were biotinylated on ice and Dectin-1 uptake was triggered for 30 min by incubating the cells with laminarin, WGPsol, or P-curdlan at 37°C. To restrict the biotin label to membrane proteins that had been internalized, the cells were chilled on ice and stripped with TCEP. The cells were then incubated in plain medium for another 30 minutes at 37°C, allowing intracellular trafficking to occur for the biotinylated pool of endocytosed proteins. To determine if recycling to the plasma membrane had occurred, the cells were either TCEP-stripped again or not stripped prior to cell lysis. If any biotinylated proteins had recycled to the plasma membrane, they would become sensitive to the TCEP reduction and be stripped of their biotin label.

The levels of biotinylated Dectin-1 were quantified from these experiments. We observed that 30 minutes of treatment with laminarin, WGPsol, or P-curdlan stimulated Dectin-1 uptake (**Figure 40**). The first TCEP strip was then performed, and followed by a 30-minute incubation in plain media. We are not able to explain why a slight increase in biotinylated Dectin-1 was detected consistently after this incubation. Nevertheless, when the second TCEP strip was applied, the amount of biotinylated Dectin-1 was dramatically reduced (**Figure 40**). This result would only occur if part of the pool of internalized Dectin-1 had returned to the plasma membrane, rendering the biotinylated receptor accessible to TCEP. This recycling event was observed regardless of which ligand was used to stimulate the receptor, with 65.5 ± 11.2% (laminarin), 61.9 ± 5.5% (WGPsol), and 74.8 ± 16.1% (P-curdlan) of internalized receptor arriving back at the plasma membrane. In support of receptor recycling, inhibiting intracellular trafficking by

keeping the cells on ice for the 30 minutes after the first TCEP strip prevented the internalized pool of Dectin-1 from acquiring TCEP resistance (**Figure 40B**).

Combining the results from surface biotinylation assays (**Figure 39**, **Figure 40**) with the colocalization studies (**Figure 36**, **Figure 37**, **Figure 38**), we conclude that Dectin-1 does not traffic to lysosomes upon internalization. Instead, a portion of the receptor traffics to lysosomes and is recycled to the plasma membrane. Interestingly, the initial biotinylation assays measuring Dectin-1 internalization kinetics also detected a decrease in internalized receptor after 30 minutes of ligand stimulation (**Figure 19**, **Figure 20**). The results from this section suggest the decrease was due to Dectin-1 recycling to the cell surface, and not due to lysosomal degradation.

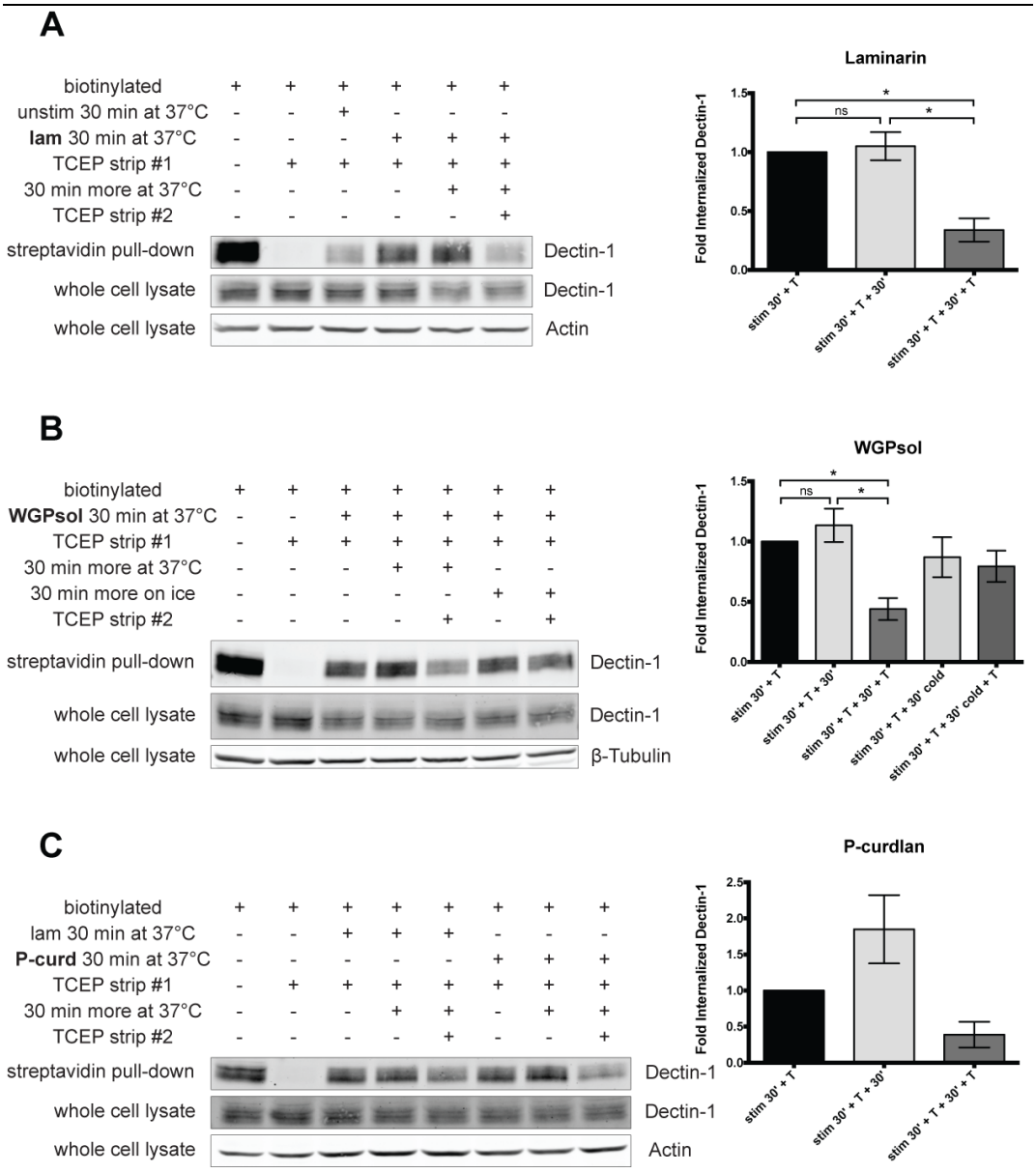

Figure 40: After Internalization, Dectin-1 Recycles to the Plasma Membrane, as Measured by the "Double-Strip" Method of Reversible Cell Surface Biotinylation

RAW Dectin-1 cells were biotinylated on ice. Dectin-1 uptake was triggered for 30 min by incubating the cells with 100 μg/mL laminarin (lam) (**A**), WGPsol (**B**), or P-curdlan (P-curd) (**C**) at 37°C. The cells were next chilled on ice and stripped with 100 mM TCEP (T), then incubated in plain medium for another 30 minutes at 37°C. Following this, they were chilled on ice and TCEP-stripped again (T) or not prior to homogenization. Cell viability was monitored during both stripping events to ensure removal of the biotin tag was not due to loss of plasma membrane integrity. The lysates were processed according to the cell surface biotinylation protocol for detection and quantification of biotinylated Dectin-1. Immunoblots shown are representative results from multiple replicates. The quantifications of the recycling events are shown to the right, and are averages of six (**A**), three (**B**), and two (**C**) independent experiments.

3.6. The Connection Between Dectin-1 Endocytosis and Signaling

3.6.1. Upon Ligand Stimulation, Syk Accumulates on Dectin-1$^+$ Vesicles and is Active as a Kinase

We had previously determined that laminarin, WGPsol, and P-curdlan were all capable of activating Dectin-1 signaling, albeit to different extents (Section 3.2). Having explored the internalization kinetics, required endocytic machinery, and intracellular trafficking pathways of Dectin-1, we were now interested to determine if a link existed between the endocytic and signaling events; in other words, if signaling regulated internalization and if internalization regulated signaling.

Upon ligand binding, Dectin-1 is phosphorylated at the hemITAM tyrosine of its intracellular tail, and this is sufficient to recruit Syk (Gantner et al., 2003; Rogers et al., 2005). Given that Dectin-1 is internalized into endocytic vesicles and endosomes upon ligand binding, we wondered if Syk would be localized to these Dectin-1$^+$ organelles. To test this, RAW Dectin-1 cells were transfected with GFP-tagged Syk, and the localization of Syk-GFP over time during WGPsol stimulation was evaluated by confocal imaging (**Figure 41**). Syk-GFP was cytoplasmic when the cells were left unstimulated, but addition of WGPsol altered its distribution, with some Syk accumulating at distinct puncta within the cytoplasm. These puncta were present 15 and 60 minutes after the start of ligand stimulation. Interestingly, the Syk-GFP puncta colocalized with puncta of internalized Dectin-1 and WGPsol (**Figure 41**), suggesting that Syk localized only to endocytic vesicles or endosomes that were ferrying WGPsol-bound Dectin-1, presumably binding to the cytoplasmic, tyrosine-phosphorylated hemITAM of the receptor. Similar results were obtained with the ligands laminarin and P-curdlan, although we did not stain for the presence of β-glucan in those experiments (**Figure 42**).

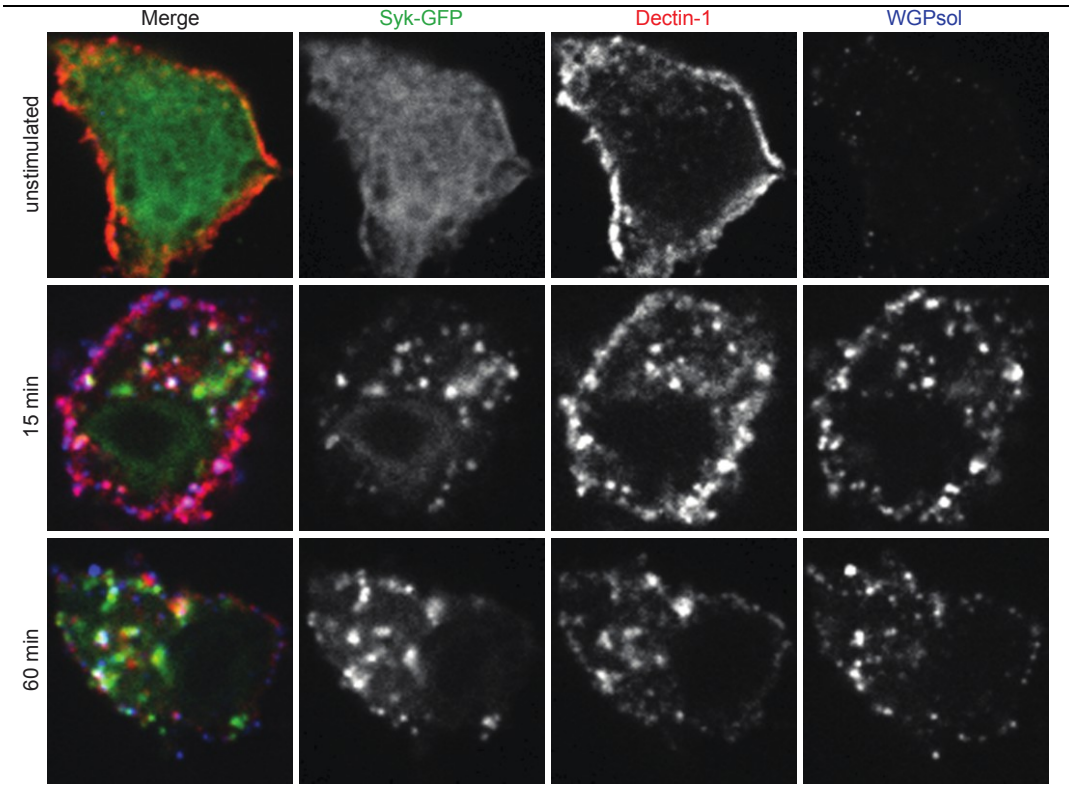

Figure 41: Syk Accumulates on Dectin-1$^+$ Endocytic Vesicles upon WGPsol Stimulation

RAW Dectin-1 cells were transfected with Syk-GFP and incubated at 37°C with media alone (unstimulated) or containing 100 μg/mL WGPsol. For the 15-minute stimulation timepoint, WGPsol stimulation was maintained for the entire duration. For the 60-minute timepoint, cells were pulse-stimulated with media containing WGPsol for 15 minutes, then rinsed with PBS and chased for 45 minutes in plain media. Prior to confocal imaging, the cells were fixed, permeabilized, and immunostained for Dectin-1 and β-glucan. Images shown are representative of the results from one experiment.

In B Cell receptor signaling, the interaction of Syk with the phosphorylated ITAM tyrosine residues of the receptor is sufficient to induce Syk's kinase activity (Geahlen, 2009). To confirm if the Syk localized to Dectin-1 puncta was actively signaling as a kinase, we performed another imaging experiment (**Figure 42**). Here, mApple-tagged Dectin-1 and Syk-GFP were co-transfected into RAW WT cells, and P-curdlan and laminarin were used to stimulate Dectin-1 endocytosis for various times. After fixation, the cells were immunostained for Syk phosphorylated on residue Y352, an autophosphorylation site that plays a stimulatory role in signaling events downstream of Syk by positively regulating Syk activity. Phosphorylation also converts Y323 into a docking site for Syk-

interacting proteins (Geahlen, 2009). Indeed, Syk that had accumulated on Dectin-1^+ vesicles and endosomes colocalized strongly with the phosphorylated Syk signal, suggesting that the majority of Syk was only active as a kinase when bound to internalizing Dectin-1. This occurred with both P-curdlan and laminarin stimulation and at all timepoints observed up to 60 minutes. On unstimulated cells, immunostaining for phosphorylated Syk revealed a less intense signal that was localized uniformly in the cytoplasm.

Taken together, stimulation with laminarin, WGPsol, or P-curdlan induces Dectin-1 internalization. Syk accumulates on Dectin-1^+ endocytic vesicles and endosomes containing the ligands and is active as a kinase at these locations, probably a result of binding the phosphorylated tails of Dectin-1. Active Syk is localized to Dectin-1^+ endosomes/vesicles for at least 60 minutes after the start of ligand stimulation.

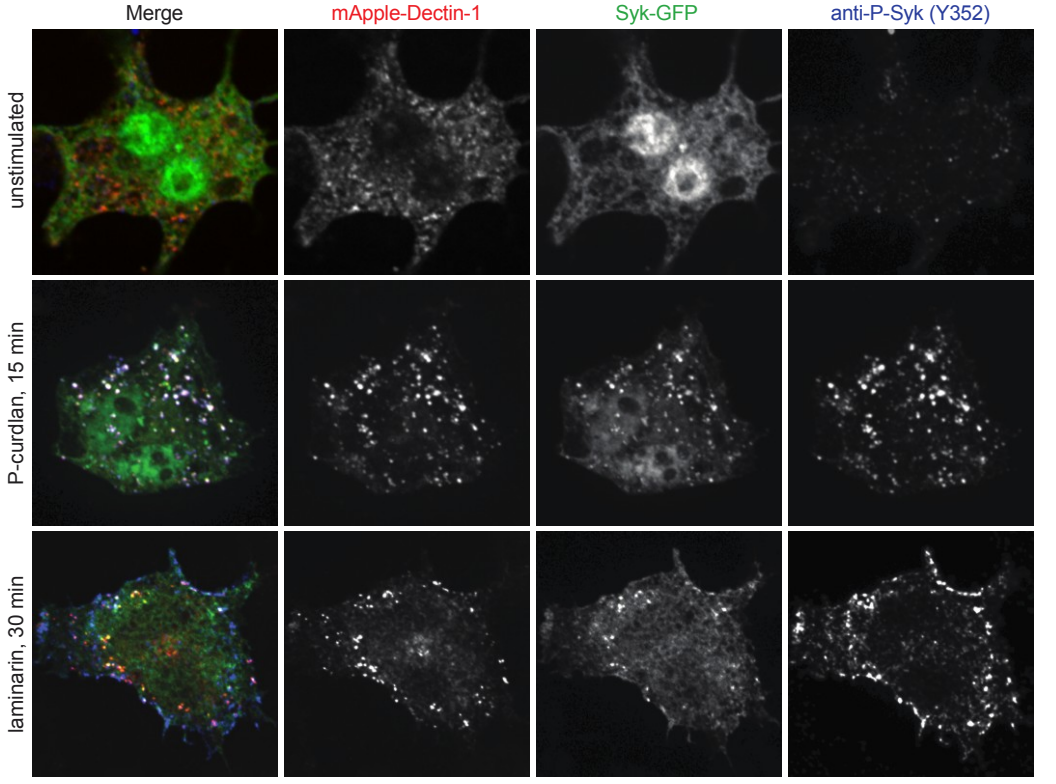

Figure 42: The Syk Accumulated on Dectin-1^+ Endocytic Vesicles is Active as a Kinase

RAW WT cells were transfected with Syk-GFP and mApple-Dectin-1 (mApple is a red fluorescent protein with peak fluorescence emission at 592 nm). The mApple-Dectin-1

fusion protein was targeted to the plasma membrane, but in some cases a large pool was retained intracellularly, unlike for mEmerald-Dectin-1 fusions (**Figure 8A**). After serum starvation, the cells were stimulated continuously with 100 μg/mL P-curdlan, laminarin, or neither for the indicated times at 37°C. Next, the cells were fixed, permeabilized, and immunostained for phosphorylated Syk (at residue Y352) before visualization on the confocal microscope. Images shown are representative of cells observed at the 15-, 30-, 45-, and 60-minute timepoints over two independent experiments.

3.6.2. Dectin-1 Signaling is Not Required for Receptor Internalization

The accumulation of active Syk on Dectin-1$^+$ endocytic vesicles led us to question if this localization served a purpose or if the molecule was simply recruited to the activated receptor at the plasma membrane and remained bound during endocytosis. A potential function of Syk's association to endocytic vesicles could be the regulation of Dectin-1 endocytosis, as Syk has been shown to involved in the internalization of antigen-antibody immune complexes, Shiga toxin and human rhinovirus and directly binds to clathrin heavy chain (Lau et al., 2008; Lauvrak et al., 2006; Sedlik et al., 2003; Utskarpen et al., 2010; Wälchli et al., 2009). To determine if Syk's kinase activity was necessary for Dectin-1 uptake, we used reversible cell surface biotinylation to measure the amount of endocytosed Dectin-1 in cells treated with WGPsol (**Figure 43**) or P-curdlan (**Figure 44**) in the presence of the Syk kinase inhibitors piceatannol or Syk inhibitor IV. Src family kinases likely phosphorylate the tail of Dectin-1 (Brown and Williams, 2009; Geahlen, 2009; Underhill and Goodridge, 2007), allowing initiation of Dectin-1-dependent signaling cascades and Syk activation (Elsori et al., 2011; Olsson and Sundler, 2007; Underhill et al., 2005) (**Figure 5**, **Figure 11**). Thus, we also treated the cells with the Src family kinase inhibitor PP2 to determine if the initiation of Dectin-1 signaling in general was required for uptake. While the inhibitors were determined to be active and functional (**Figure 11**), none were able to reduce the amount of Dectin-1 internalized after 30 minutes of WGPsol or P-curdlan stimulation (**Figure 43** and **Figure 44**). In fact, a slight increase of receptor uptake was observed upon signaling inhibition. This suggests that neither the kinase activity of Syk, nor Dectin-1 signaling in general, play a role in Dectin-1 endocytosis, at least when WGPsol or P-curdlan are used

as ligands.

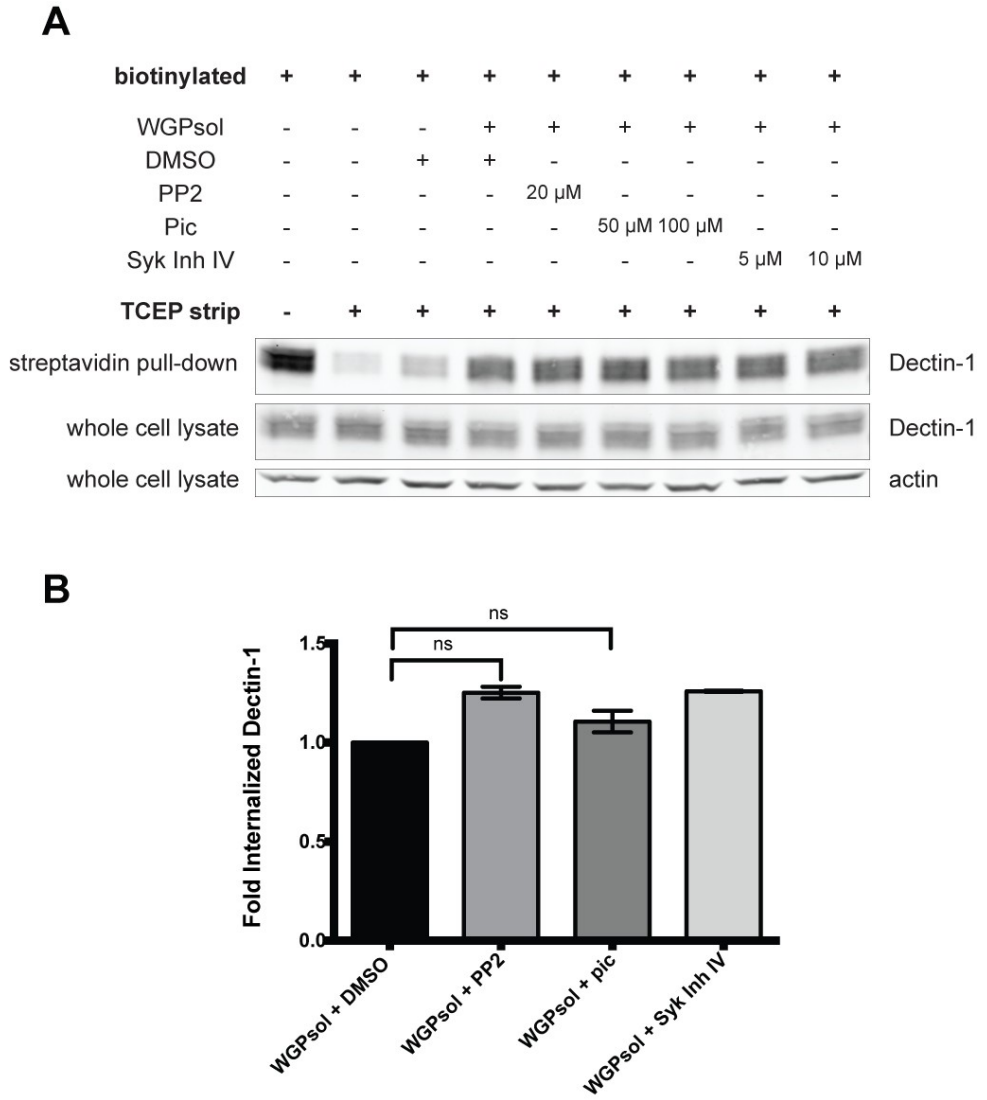

Figure 43: Inhibition of Src Family Kinase or Syk Signaling Does Not Inhibit WGPsol-Stimulated Dectin-1 Internalization

RAW Dectin-1 cells were surface-biotinylated, pre-treated with various concentrations of PP2, piceatannol (pic), and Syk Inhibitor IV (Syk Inh IV), or 0.1% DMSO (as the vehicle control) for 30 minutes at 37°C, and incubated with 100 µg/mL WGPsol in the same solutions for 30 more minutes. Afterwards, the cells were TCEP stripped, lysed, and processed according to the cell surface biotinylation protocol. Immunoblots of actin in the whole cell lysate, along with Dectin-1 in the streptavidin pull-down fraction and whole cell lysate from one experiment are presented in (**A**). The quantification of relative uptake is presented in (**B**) as averaged from three independent experiments (except for the Syk Inhibitor IV-treated condition, which was from two independent experiments), with inhibitor concentrations as follows: 20 µM for PP2, 50 µM for piceatannol, and 10 µM for Syk Inhibitor IV.

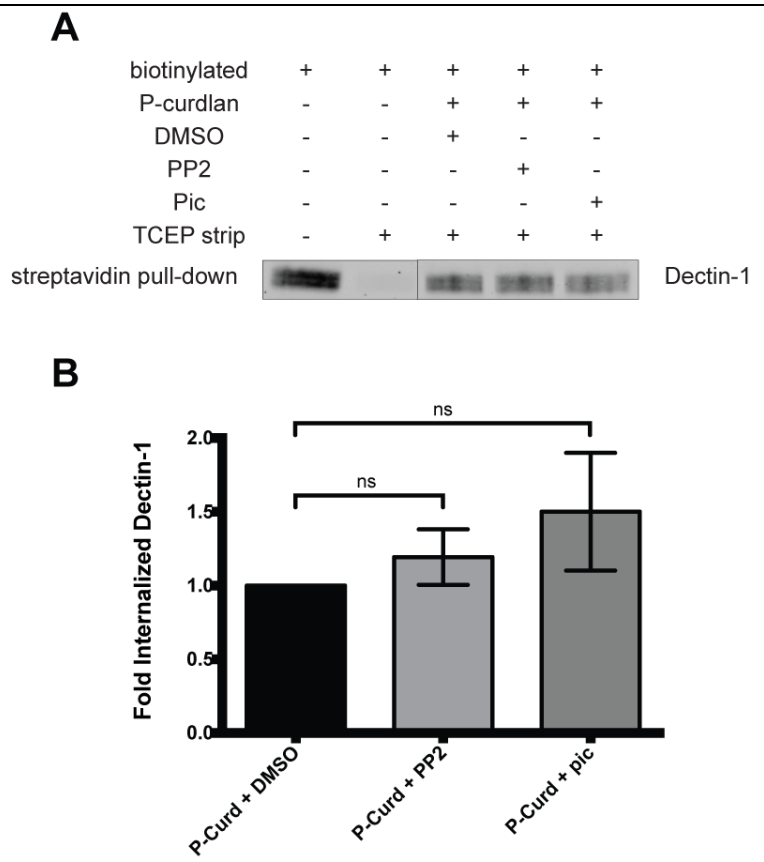

Figure 44: Inhibition of Src Family Kinase or Syk Signaling Does Not Inhibit P-Curdlan-Stimulated Dectin-1 Internalization

RAW Dectin-1 cells were surface-biotinylated, pre-treated with 20 μM PP2, 50 μM piceatannol (pic), or 0.1% DMSO (as the vehicle control) for 30 minutes at 37°C, and incubated with 100 μg/mL P-curdlan (P-curd) in the same solutions for 30 more minutes. Afterwards, the cells were TCEP stripped, lysed, and processed according to the cell surface biotinylation protocol. A representative immunoblot of the Dectin-1 in the streptavidin pull-down fraction are presented in (**A**), whereas the quantification of relative uptake is presented in (**B**) as averaged from four independent experiments.

To further support the notion that Dectin-1 signaling is not required for its endocytosis, we generated a signaling-deficient mutant of Dectin-1 where the hemITAM tyrosine residue in the cytoplasmic tail of the receptor was substituted with alanine (Y15A), similar to previous studies (Brown et al., 2003; Gantner et al., 2003; Herre et al., 2004; Rogers et al., 2005; Underhill et al., 2005). Unlike RAW Dectin-1 cells, WGPsol stimulation of RAW 264.7 cells expressing the mutated Dectin-1 did not activate Src family kinases or Syk (**Figure 45**), confirming the inability of the mutant receptor to initiate signaling upon ligand binding.

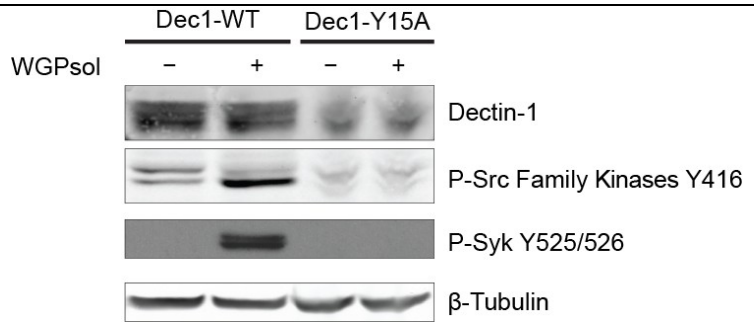

Figure 45: The Y15A Substitution Mutant of Dectin-1 is Unable to Induce Src Family Kinase and Syk Activation Upon Stimulation with WGPsol

RAW Dectin-1 (Dec1-WT) or RAW Dectin-1-Y15A (Dec1-Y15A) cells were stimulated for 15 minutes with or without 100 μg/mL WGPsol at 37°C. The cells were promptly lysed, processed by SDS-PAGE, and immunoblotted for the presence of phosphorylated Src family kinases and Syk, or β-tubulin as a loading control. The immunoblots presented are representative of two independent experiments.

The signaling-deficient mutant was then expressed in RAW 264.7 cells and stimulated with laminarin and WGPsol in parallel to RAW Dectin-1 cells, which expressed the wild-type receptor. By reversible cell surface biotinylation, signaling-deficient Dectin-1 exhibited similar and not drastically decreased amounts of β-glucan-induced endocytosis compared to wild-type Dectin-1, although the experiments will need to be replicated to confirm the findings (**Figure 46**). Still, combined with the data that showed inhibition of Dectin-1 signaling did not inhibit its endocytosis, it is clear Dectin-1 signaling is not required for its endocytosis to occur. Perhaps the act of ligand binding the receptor is sufficient to stimulate internalization.

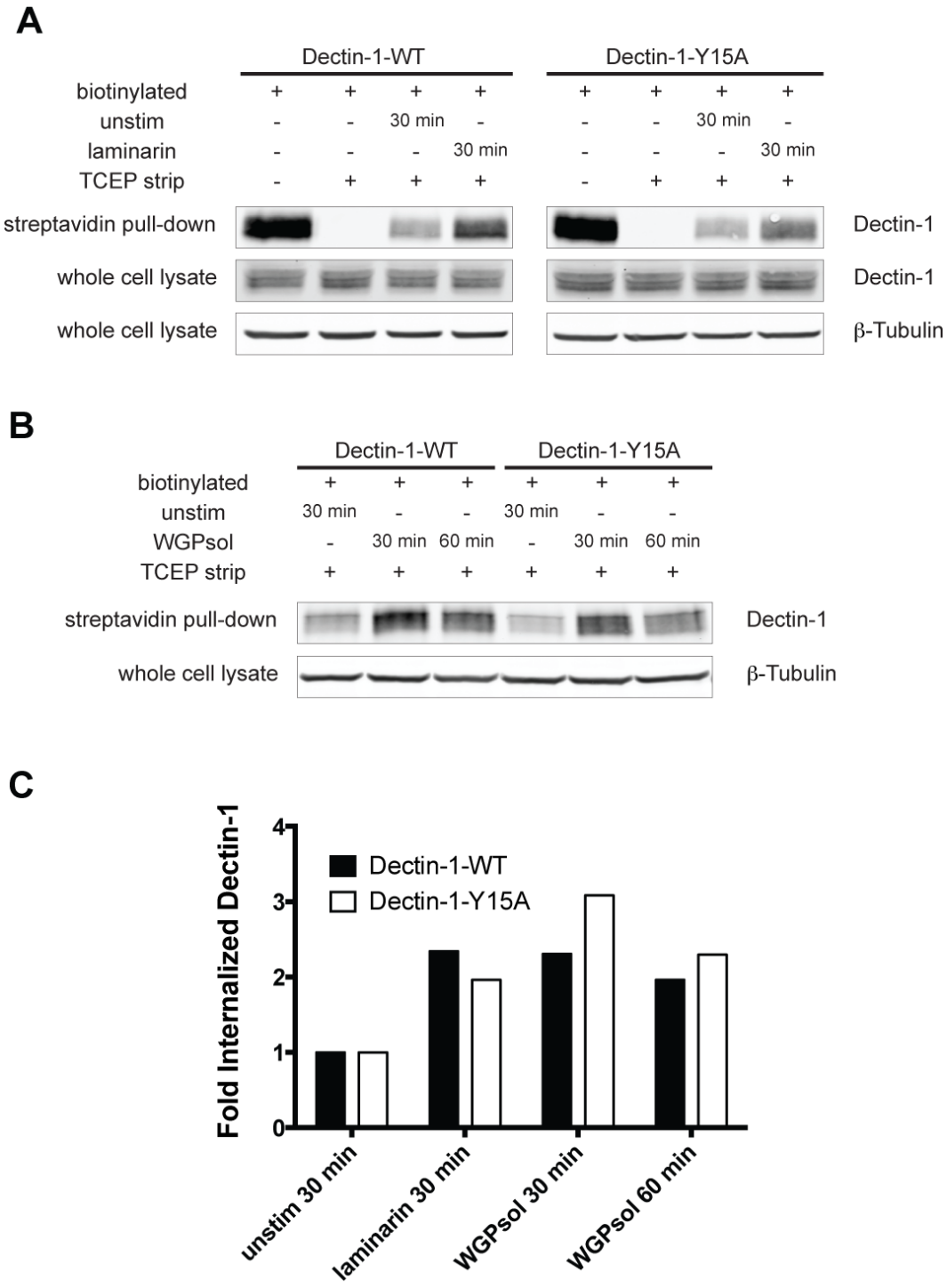

Figure 46: Signaling-Deficient Dectin-1 is Endocytosed as Efficiently as Wild-Type Dectin-1

RAW Dectin-1 (expressing wild-type (WT) Dectin-1) and RAW Dectin-1-Y15A (expressing signaling-deficient Y15A mutant of Dectin-1) cells were surface-biotinylated and incubated with plain media or media containing 100 μg/mL laminarin for 30 minutes (**A**) or 100 μg/mL WGPsol for 30 or 60 minutes (**B**) at 37°C. Afterwards, the cells were

TCEP stripped, lysed, and processed according to the cell surface biotinylation protocol. Immunoblots of Dectin-1 in the streptavidin pull-down fraction and whole cell lysate, and the β-Tubulin loading control in the whole cell lysate, are presented in (**A**) and (**B**), whereas the quantification of relative uptake in both experiments is presented in (**C**). (**A**) and (**B**) are the results from separate experiments each performed once.

To summarize the findings so far, soluble β-glucan binding induces Dectin-1 internalization and the Dectin-1-dependent activation of Src family kinases and Syk. While Syk accumulates and is active as a kinase on Dectin-1^+ endocytic vesicles and endosomes, we have clearly demonstrated that this Dectin-1-activated signaling is not required for the internalization of the receptor. The observed accumulation of Syk on Dectin-1^+ vesicles could then simply be a result of its recruitment to Dectin-1 at the plasma membrane early on, and it remaining bound to the receptor during Dectin-1 internalization. However, the fact that Syk is active suggests that it might be phosphorylating target proteins that are recruited to it. Perhaps, then, Dectin-1^+ vesicles serve as scaffolds for the formation of multiprotein complexes that function in Dectin-1 signaling. We also have not ruled out if Syk activity could be involved in regulating Dectin-1 trafficking to the recycling compartment. In fact, Syk has been found to regulate the trafficking of the Fc and B cell receptors to the lysosome and for antigen presentation (Bonnerot et al., 1998; Le Roux et al., 2007; Sedlik et al., 2003).

3.6.3. Endocytosis May Regulate Dectin-1 Signaling

Having learned that the endocytic uptake of Dectin-1 does not depend on the initialization of its signaling, we asked if, instead, Dectin-1 internalization could regulate its ability to signal. Perhaps endocytosis could be necessary for the initiation or transmission of the signal through the multiple molecules involved in its signaling cascades (**Figure 5**), and the recruitment of Syk to Dectin-1^+ vesicles would be required for the formation of endosomal multiprotein complexes that function in signaling. To answer this question, we chose to use P-curdlan as the soluble β-glucan since it induced full activation of Dectin-1 signaling, down to the level of the transcription factor NF-κB (**Figure 14**). We then stimulated RAW Dectin-1 cells with P-curdlan for various times in media depleted or supplemented with potassium. (Recall that potassium depletion was an effective treatment to

inhibit Dectin-1 uptake in Section 3.4.) We then lysed the cells and performed SDS-PAGE and immunoblotting to detect activation of an upstream and a downstream event in the Dectin-1 signaling pathway (**Figure 47**). These events were the activation of Syk, as observed by phosphorylation of Syk residues Y323 and Y525/Y526, and that of NF-κB, detected by the phosphorylation and degradation of IκBα and the phosphorylation of p65. Indeed, stimulation with P-curdlan in the potassium-supplemented condition resulted in Syk phosphorylation that increased up to 20 or 30 minutes, then decreased towards 60 minutes, similar to the kinetics exhibited by WGPsol stimulation (**Figure 10B**). NF-κB activation was also detected, as IκBα became phosphorylated, then degraded, with P-curdan stimulation, events concurrent to the phosphorylation of p65. Therefore, P-curdlan activated both upstream and downstream Dectin-1 signaling as expected. However, in the potassium-depleted condition, P-curdlan-induced Syk phosphorylation was stronger and sustained, not exhibiting the phosphorylation kinetics observed in the potassium-supplemented condition. Furthermore, the activation of NF-κB was dramatically diminished and exhibited delayed kinetics compared to the potassium-supplemented condition. This was observed for all the events of IκBα phosphorylation, IκBα degradation and p65 phosphorylation. Importantly, potassium depletion alone did not affect the signaling components examined (please see the unstimulated lanes), indicating that the effects observed were not due to non-specific effects produced by the treatment. Thus, when endocytosis was inhibited, P-curdlan was able to initiate strong Dectin-1 signaling upstream (as evidenced by the heightened Syk activation), but was unable to have its signals transduced downstream to NF-κB. The finding suggests endocytosis regulates Dectin-1 signaling, both by promoting the propagation of signals to the downstream activation of transcription, and downregulating upstream Syk activation to avoid hyperstimulation of the signaling cascades overall.

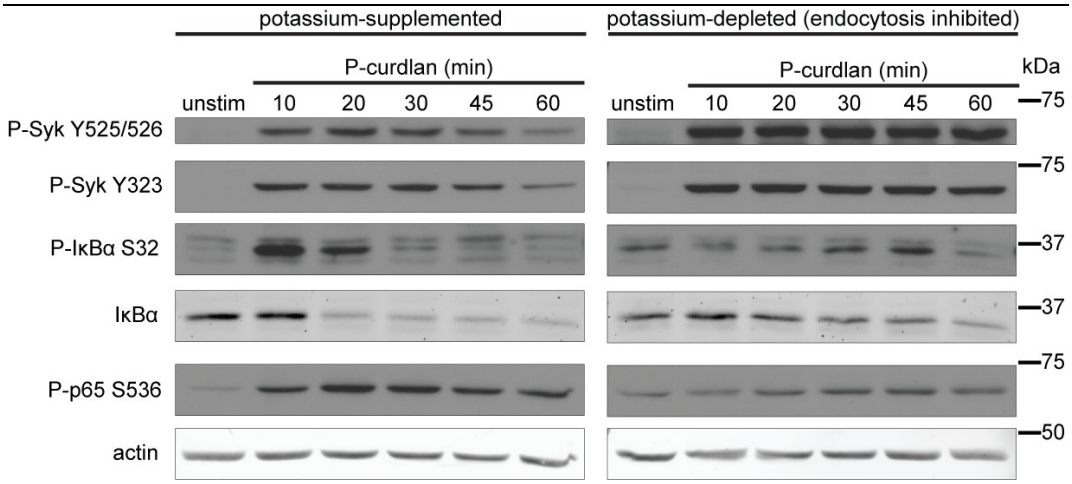

Figure 47: Endocytic Inhibition by Potassium Depletion Affects Upstream and Downstream Dectin-1 Signaling Events During P-Curdlan Stimulation

RAW Dectin-1 cells were treated with media supplemented or depleted with potassium for 30 minutes at 37°C. They were then stimulated continuously in the same solutions at 37°C in the presence of 100 μg/mL P-curdlan for the indicated times, or left unstimulated. The cells were promptly lysed, processed by SDS-PAGE, and immunoblotted for the presence of phosphorylated Syk (on residues Y525/Y526 and Y323), IκBα, and the NF-κB subunit p65. The presence of total IκBα was also detected by immunoblot to assess IκBα degradation. Immunoblots of actin served as the loading control. Blots from both potassium-depleted and potassium-supplemented conditions were developed at the same time at identical exposure times. The immunoblots presented are from one experiment.

To see if endocytic inhibition might affect NF-κB activation from a different perspective, we examined the ability for P-curdlan to induce NF-κB translocation (Lipinski et al., 2013) in the absence or presence of dynasore, another inhibitor of Dectin-1 endocytosis, by confocal imaging (**Figure 48**). While P-curdlan was able to induce NF-κB translocation, this event was inhibited in the presence of dynasore. Importantly, dynasore treatment alone did not alter the cellular localization of NF-κB. Therefore, endocytosis is required for P-curdlan to induce NF-κB translocation.

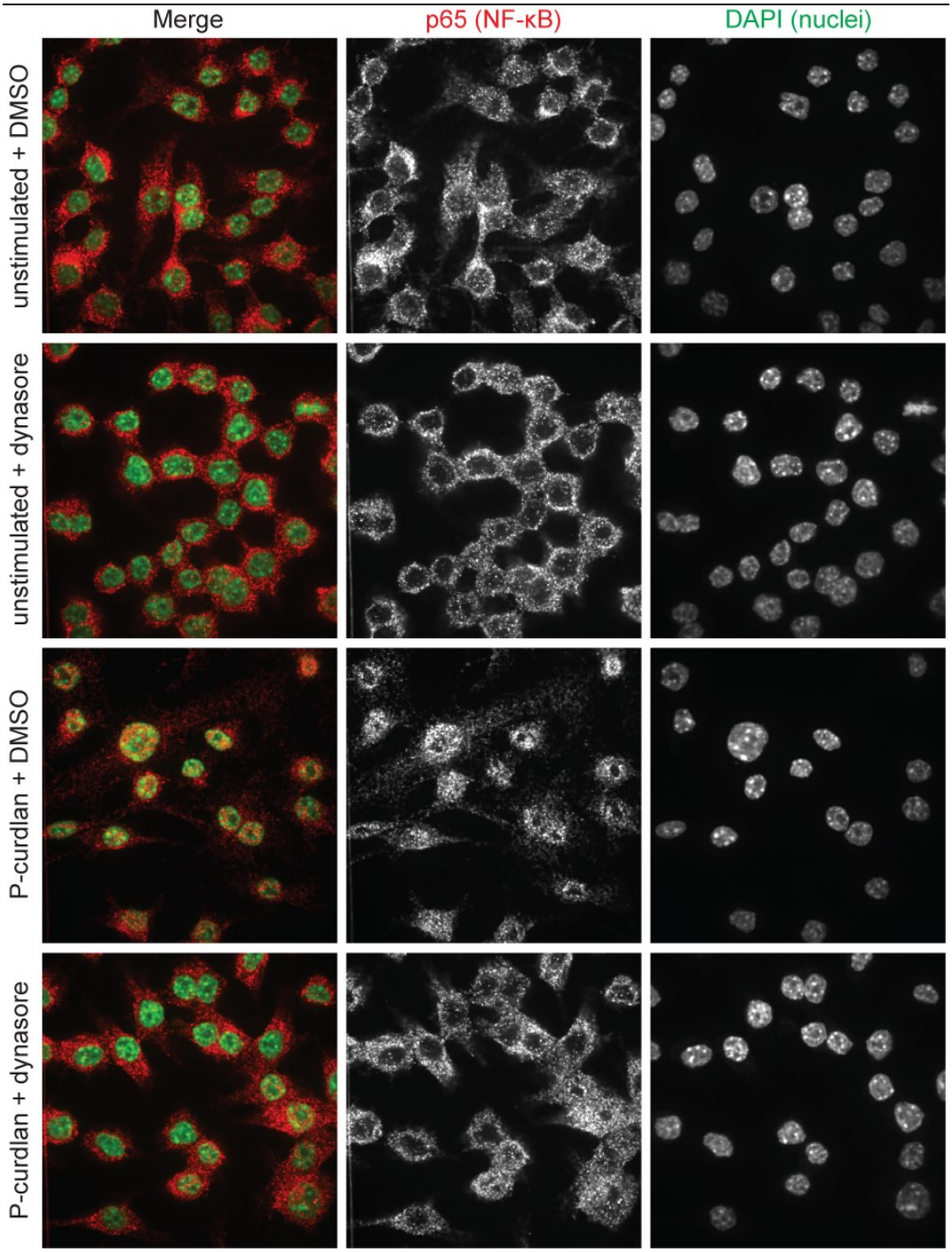

Figure 48: Dynasore Treatment Inhibits P-Curdlan Stimulated NF-κB Translocation

RAW Dectin-1 cells were pre-treated with either 80 μM dynasore or 0.13% DMSO (vehicle control) for 30 minutes at 37°C. The cells were then pulse-stimulated with the same solutions supplemented with 100 μg/mL P-curdlan or not for 10 minutes, after which they were rinsed with PBS and incubated for 20 more minutes in plain media containing DMSO or dynasore. The cells were fixed, permeabilized, immunostained for p65 NF-κB (red), and labeled with DAPI (green) to visualize cellular nuclei. Images

were acquired on the confocal microscope, and are representative of the results from three independent experiments.

With both approaches, we have preliminary observations that the endocytosis of Dectin-1 regulates its ability to signal as a receptor. Future studies will seek further support for this idea, as well as provide precise mechanistic details as to how endocytosis regulates the molecules involved in Dectin-1 signaling. While we have observed that active Syk is localized to endocytic vesicles and endosomes containing ligand-bound Dectin-1, the function of Syk recruitment does not appear to be for regulation of receptor internalization, as inhibition of signaling did not affect Dectin-1's ability to be taken into the cell. Taking the latest findings into consideration, the endosomal localization of Syk may be a sign that endocytosis is critical for the transmission of upstream Dectin-1 signals downstream. Perhaps multiprotein complexes that mediate Dectin-1 signaling are assembled on endosomes, and inhibiting receptor internalization prevents these complexes from forming and relaying the signals of Dectin-1 activation downstream.

Chapter 4. DISCUSSION

4.1. Introduction

For decades, soluble and particulate β-glucans have been well documented for their immunostimulatory effects (Brown and Gordon, 2003; Brown and Williams, 2009; Chan et al., 2009; Chen and Seviour, 2007; DI CARLO and FIORE, 1958; Vannucci et al., 2013). The identification of Dectin-1 as a *bona fide* β-(1,3)-glucan receptor (Brown and Gordon, 2001b), with widespread expression on a variety of leukocytes (Willment et al., 2005), suggested that soluble β-glucans might elicit their biological effects through the binding and activation of Dectin-1 (Brown and Gordon, 2003; Brown and Williams, 2009). We were particularly interested in this idea from the perspective of endocytosis, the process by which receptors, their ligands, and extracellular fluid are taken into the cell by the generation of membrane-bound bodies from the plasma membrane (Doherty and McMahon, 2009). Most research into Dectin-1-dependent internalization has focused on particulate, water-insoluble β-glucans and their phagocytosis (Goodridge et al., 2011; Heinsbroek et al., 2009; Hernanz-Falcón et al., 2009; Herre et al., 2004; Hino et al., 2012; Ma et al., 2012; Mansour et al., 2013; McCann et al., 2005; Nakamura and Watanabe, 2010; Rosas et al., 2008; Shah et al., 2009; Strijbis et al., 2013; Underhill et al., 2005; Xu et al., 2009a), the actin-dependent process where particles ≥0.5 μm in size are engulfed after binding to cell surface receptors (Flannagan et al., 2012). Therefore, we wished to shed light on how Dectin-1 would be internalized in the presence of soluble β-glucans. Given their small size (certainly <0.2 μm) and water-soluble nature, we predicted that soluble β-glucans would be endocytosed via small vesicles derived from plasma membrane invaginations (Swanson, 2008). The presence of soluble β-glucans in the blood during invasive fungal infection (Mennink-Kersten and Verweij, 2006), as well as the efficacy of experimental anti-fungal vaccines composed of soluble β-glucans (Lipinski et al., 2013; Pietrella et al., 2010),

highlight the importance of understanding what cellular events occur when such compounds interact with Dectin-1.

To study Dectin-1 endocytosis, we successfully expressed full-length human Dectin-1 in RAW 264.7 monocyte/macrophage cells, which alone expressed no detectable Dectin-1 (**Figure 6**). Since expression of Dectin-1 conferred the ability of the cells to bind soluble β-glucans (**Figure 8**), it is unlikely that other β-glucan receptors (Albeituni and Yan, 2013; Brown and Williams, 2009; Goodridge et al., 2009b) were expressed to significant levels to contribute to β-glucan recognition. Dectin-1 was appropriately glycosylated (**Figure 7**) and targeted to the plasma membrane (**Figure 8**, **Figure 16**, and **Figure 18**). It was also functional as a receptor; upon binding soluble β-glucan ligands (**Figure 8**), it induced intracellular signaling events consistent with the known pathways of Dectin-1 signaling (**Figure 10**, **Figure 12**, **Figure 5**). We obtained three soluble β-glucans to use as ligands to the receptor: laminarin, soluble WGP (WGPsol) and phosphorylated curdlan (P-curdlan), each of which exhibited different capacities to activate components of the Dectin-1 signaling pathway (**Figure 13**, **Figure 14**). Since it is considered that the molecular weight of β-glucans affects the extent of their biological activity (Batbayar et al., 2012; Brown and Gordon, 2003; Brown and Williams, 2009), we were curious if ligands of different size induced different endocytic rates and trafficking fates for Dectin-1. Perhaps these differences could offer explanations to the size-dependent discrepancies in β-glucan activity. Laminarin was chosen to represent small β-glucans, in comparison to the larger ligands, WGPsol and P-curdlan.

Next, we sought to develop tools to visualize the intracellular trafficking of Dectin-1. A reversible cell surface biotinylation procedure was established to monitor how much of a pool of surface Dectin-1 was internalized within a given time (**Figure 18**). Immunofluorescence staining protocols were also developed to distinguish between surface and intracellular receptor pools (**Figure 16**, **Figure 17**), and track the location of internalized receptor relative to various subcellular compartments (**Figure 36**, **Figure 37**, **Figure 38**). To our benefit, we were also able to visualize the soluble β-glucans themselves by fluorescent imaging (**Figure**

8). This was accomplished by fluorescently labeling the soluble ligands or by immunofluorescent detection with an anti-β-glucan antibody.

With these tools at hand, we proceeded to explore answers to the following questions: 1) Is Dectin-1 internalized upon ligand stimulation? If so, what are the kinetics of Dectin-1 uptake? 2) What cellular machinery contributes to Dectin-1 internalization? 3) Where is Dectin-1 trafficked in the cell, and what is the fate of the receptor? 4) Is the endocytosis of Dectin-1 related to its ability to signal as a receptor, and *vice versa*? The following sections discuss answers to these and other questions based on our experimental findings. We also speculate to the biological and therapeutic significance of the results, and offer ideas for future studies.

4.2. Soluble β-Glucans Can Be Biologically Active Dectin-1 Ligands

An area of debate in β-glucan research is whether soluble β-glucans are biologically active ligands of Dectin-1 (Batbayar et al., 2012; Brown and Gordon, 2003; Brown and Williams, 2009), or functionally inactive (Brown, 2006; Brown et al., 2003; Goodridge et al., 2011; Kennedy et al., 2007; Michalek et al., 1998; Underhill et al., 2005). We interpret biological activity as the ability to induce intracellular signaling events that produce cellular responses, such as transcriptional activation for the production of cytokines. On one hand, there are numerous instances where soluble β-glucans have been shown to have biological activity (Batbayar et al., 2012; Brown and Williams, 2009; Chan et al., 2009; Chen and Seviour, 2007), although it is not known if they elicited their effects through Dectin-1. For example, macrophages that ingested particulate curdlan discharged biologically active soluble β-glucan fragments into the culture media. These fragments were then able to induce cytokine production in macrophages that had not previously encountered β-glucan (Hino et al., 2012). In addition, glucan phosphate, a soluble β-glucan preparation from *Saccharomyces cerevisiae* (Williams et al., 1991a), increased survival in mice challenged with *Staphylococcus aureus* or *Candida albicans* infection (Rice et al., 2005).

Numerous other examples of soluble β-glucans activating an immune response have been documented (Engstad et al., 2002; Hetland et al., 2000; Muramatsu et al., 2012; Patchen et al., 1998; Pretus et al., 1991; Qi et al., 2011; Stashenko et al., 1995; Williams et al., 1991b). More recently, some reports have found a role for Dectin-1 in mediating the cellular responses to soluble β-glucans isolated from spores and fruiting bodies of mushrooms. These soluble β-glucans were certainly biologically active, since they were able to induce MAP kinase or NF-κB activation along with the secretion of TNF-α and IL-6 (Fang et al., 2012; Guo et al., 2009; Wang et al., 2013).

On the contrary, Goodridge and coworkers purport that unlike particulate β-glucans, soluble β-glucans are unable to activate Dectin-1 unless they are immobilized on a solid surface or spherical beads greater than 0.5 μm in size (Goodridge et al., 2011). They generated soluble β-glucan by hydrolyzing particulate WGP to fractions ranging from 16 – 400 kDa in molecular weight, and observed that none of the products were able to induce ROS production, TNF-α secretion, or Syk or p38 MAPK phosphorylation in Dectin-1-expressing RAW 264.7 cells, bone marrow-derived macrophages, and bone marrow-derived dendritic cells. Importantly, the soluble β-glucans produced were able to bind to Dectin-1 and inhibit responses elicited by the particulate β-glucans (Goodridge et al., 2011). While the inactivity of the soluble β-glucans used was demonstrated very clearly, it is uncertain if different results would have been obtained had they generated soluble β-glucan from other organisms, used established immunoactive preparations such as glucan phosphate (Williams et al., 1991a), or generated soluble β-glucans of higher molecular weight. It is well known that the chemical properties of soluble β-glucans can affect their activity (Bohn and BeMiller, 1995). For example, increases in molecular weight and the presence of branches can strengthen the binding affinity of soluble β-glucans to Dectin-1 (Adams et al., 2008), perhaps enhancing the ability to activate the receptor.

Our findings are supportive of soluble β-glucan having biological activity. P-curdlan was able to induce Dectin-1 signaling down to the level of NF-κB activation (**Figure 14**). Beyond P-curdlan, laminarin and WGPsol exhibited

partial biological activity. Both ligands were able to induce strong, immediate activation of upstream Dectin-1 signaling events, despite not being able to activate NF-κB (**Figure 13**). WGPsol even exhibited a slight ability to induce ERK activation (**Figure 12**). In fact, the ability for laminarin to induce upstream Dectin-1 signaling is one that we are first to report, and is quite surprising as laminarin has frequently been found to be an inactive ligand to Dectin-1 (Fang et al., 2012; Gantner et al., 2003; 2005; Goodridge et al., 2011; Lowe et al., 2001). A remaining question, then, is how WGPsol, which had a much higher molecular weight than laminarin, was unable to activate NF-κB, unlike P-curdlan, which was also a large soluble β-glucan. Clearly, ligand size may not be the only determinant of the ability to stimulate Dectin-1. Further physicochemical characterization of the ligands should provide molecular explanations to their different capacities to induce complete Dectin-1 signaling.

While WGPsol and laminarin were found to be only partially active biologically, covalently linking multiple laminarin molecules onto a protein anchor proved otherwise. In fact, the BSA-17-laminarin conjugate was able to induce high levels of ERK and NF-κB activation like P-curdlan (**Figure 13**), despite consisting of what was originally a partially active soluble β-glucan. This suggests the spatial orientation and density of β-glucan molecules may play a role in the initiation and propagation of the Dectin-1 signal. Perhaps the close proximity of the multiple anchored laminarin molecules nucleated denser Dectin-1 clusters than free laminarin, resulting in stronger receptor activation. Despite their soluble nature, our findings comparing laminarin and the laminarin-protein conjugates are consistent with the phagocytic synapse model of Dectin-1 activation proposed by Goodridge *et al*. In this model, the binding of soluble β-glucan, immobilized at high density, allows large clusters of Dectin-1 to form on the plasma membrane (Goodridge et al., 2011). These clusters recruit the activating Src family kinases and exclude the inhibitory membrane-embedded protein tyrosine phosphatases, CD45 and CD148, promoting tyrosine phosphorylation and preventing dephosphorylation. As a result of this segregation, the hemITAM motif of Dectin-1 is continuously phosphorylated,

facilitating the recruitment of Syk and the initiation of Dectin-1 signaling (Goodridge et al., 2011). We predict similar segregation events might occur upon P-curdlan and BSA-17-laminarin binding, and not with laminarin and WGPsol binding. It will be interesting to see if such events can be detected by conventional fluorescence microscopy or even super-resolution microscopy for our soluble β-glucan ligands.

Thus, we have found that soluble β-glucans can be biologically active ligands to Dectin-1, as demonstrated by the ability for P-curdlan and the laminarin-protein conjugates to activate full Dectin-1 signaling down to the transcriptional level. We also identified other soluble β-glucans, laminarin and WGPsol, that activate strong Dectin-1 signaling upstream but somehow are not able to have that signal transduced downstream. Perhaps Goodridge *et al.*'s phagocytic synapse model of Dectin-1 activation also applies to soluble β-glucans, providing a molecular explanation to the differential abilities for our ligands to induce Dectin-1 signaling.

4.3. Dectin-1 is Internalized Upon Stimulation by Soluble β-Glucan Ligands

By reversible cell surface biotinylation, we observed that Dectin-1 exhibited a basal level of internalization in the absence of ligand stimulation (**Figure 19**, **Figure 20**). However, in the presence of soluble β-glucan ligands, the amount of internalized receptor increased approximately 2.5-fold to three-fold at the time of maximal uptake (30 minutes, **Figure 19C**). Critically, this increase did not occur when the polysaccharide dextran, a glucan not recognized by Dectin-1 (Brown and Gordon, 2001b; Palma et al., 2006; Willment, 2001), was used to stimulate the cells (**Figure 20**), demonstrating the specificity of the increase in uptake to stimulation by β-glucan. Immunofluorescent imaging revealed similar results, where the amount of intracellular Dectin-1 increased noticeably when the cells were incubated with laminarin, WGPsol, and P-curdlan (**Figure 16**, **Figure 17**). Puncta of the ligands themselves were also observed inside the cells, with colocalization to Dectin-1 puncta (**Figure 16**), demonstrating that the ligands

were internalized upon binding to Dectin-1. Thus, Dectin-1 and soluble β-glucan ligands (by binding Dectin-1), are internalized into RAW 264.7 cells, and increased Dectin-1 uptake is induced upon ligand binding.

What aspect of ligand binding could stimulate endocytosis? While this was not investigated in depth, some of our experiments may provide insight. It was clear that the initialization of Dectin-1 signaling was not required for receptor uptake (**Figure 43**, **Figure 44**, and **Figure 46**). However, cell surface chemical cross-linking revealed that in the presence of soluble β-glucan ligands, the proximity of the receptor to neighbouring Dectin-1 molecules increased (**Figure 9**), suggesting that ligand binding induces clustering of the receptor. This event is similar to observations of the receptor CLEC-2, which also signals via a hemITAM (Fuller et al., 2007; Hughes et al., 2010; Suzuki-Inoue, 2006). At the steady state, CLEC-2 exists in monomeric and dimeric forms, but is converted to dimers and oligomers in the presence of the ligand rhodocytin, as detected by chemical cross-linking (Hughes et al., 2010). Perhaps receptor clustering then is a stimulus for Dectin-1 endocytosis, similar to how clustering of the transferrin receptor induces the formation of clathrin-coated pits (Liu et al., 2010). In support of this, the binding of a polyclonal mixture of anti-Dectin-1 antibodies, in the absence of β-glucan ligand, was able to stimulate uptake of the receptor (**Figure 21**), although we could not distinguish if the uptake could have also been mediated by a conformational change produced by antibody binding. Taken together, our findings lead us to propose that the trigger for endocytosis is not receptor-activated signaling, but could be ligand-induced clustering of Dectin-1, a conformational change, or a combination of both.

With the knowledge that Dectin-1 was endocytosed, we sought to examine the kinetics of its uptake. Two sets of surface biotinylation experiments were performed to measure the internalization kinetics of Dectin-1, the first comparing treatment with no ligand or laminarin to WGPsol (**Figure 19**), and the second to P-curdlan (**Figure 20**). When the amount of internalized receptor was plotted as a function of time, the shapes of the β-glucan ligand-stimulated curves were slightly different between sets. The first set exhibited a sharp increase in internalized

Dectin-1 early on in uptake at 15 minutes, followed by a slower increase to 30 minutes (**Figure 19B**), in contrast to the second set, which exhibited a relatively linear increase in internalized receptor from 0 to 30 minutes (**Figure 20B**). While this difference may have resulted from the use of different batches of RAW Dectin-1 cells between the sets, a consistent pattern emerged between both sets: under continuous stimulation with soluble β-glucan, the net amount of internalized Dectin-1 increased until 30 minutes, peaking at between 40 – 50% internalized receptor, after which it decreased over the remaining 30 or 60 minutes (**Figure 19B** and **Figure 20B**). Thus, we successfully used reversible cell surface biotinylation to measure the endocytic kinetics of Dectin-1 upon soluble β-glucan stimulation, determining that a maximum of internalized receptor could be observed after 30 minutes of stimulation.

We originally predicted that there would be prominent ligand-dependent differences in Dectin-1 internalization kinetics. We reasoned that WGPsol and P-curdlan, being much higher molecular weight β-glucans than laminarin, would bind more Dectin-1 molecules at once and trigger a larger quantity of receptors to enter the cell relative to laminarin. Although laminarin did induce less Dectin-1 uptake than WGPsol (**Figure 19B**), neither WGPsol nor P-curdlan (**Figure 20B**) stimulated substantially higher amounts of Dectin-1 to enter the cells. It is possible that the concentration of ligands chosen (100 μg/mL) was saturating for Dectin-1 expressed on the cell surface, causing all receptors to be occupied by ligand. Perhaps stimulation with lower concentrations will produce more obvious ligand-dependent kinetic differences. Alternatively, to ensure the cells are stimulated with the same number of β-glucan molecules, regardless of the ligand, experiments could be performed where the molar concentration of the ligands was controlled instead of the mass concentration.

While we have observed rapid, soluble β-glucan-stimulated Dectin-1 uptake, these findings are consistent with previous observations. Other groups have reported the endocytosis of soluble β-glucans upon binding to an β-glucan receptor on macrophages (Konopski et al., 1995; 1994; Müller et al., 1996), the internalization of Dectin-1 itself (Brown et al., 2002; Herre et al., 2004; Ozment-

Skelton et al., 2006), as well as endocytosis of a complex of both Dectin-1 and soluble β-glucan (Ozment et al., 2012). For example, Muller and colleagues observed that radioactively labeled glucan phosphate, bound to an unidentified β-glucan receptor on cultured monocyte/macrophages at 37°C for a period of time, could not be competed off with a 50-fold excess of unlabeled glucan phosphate, suggesting the receptor had internalized and become inaccessible to the cell surface (Müller et al., 1996). Similarly, Brown and coworkers found that briefly warming macrophages to 37°C after glucan phosphate or laminarin binding reduced the ability of an anti-Dectin-1 antibody to bind to the cell surface, compared to when the cells were kept at 4°C, again suggestive of Dectin-1 endocytosis (Brown et al., 2002). Our study adds support to the overwhelming body of evidence that soluble β-glucans do promote internalization of Dectin-1 after binding to the receptor.

4.4. Dissection of Molecules Involved in Dectin-1 Endocytosis

To determine which cellular components played a role in Dectin-1 uptake, we treated RAW Dectin-1 cells with acute, reversible pharmacological treatments and observed if Dectin-1 endocytosis was affected via confocal microscopy and cell surface biotinylation. Complete endocytosis of the receptor was noticeably blocked when dynamin was inhibited by the compounds dynasore (**Figure 22B**, **Figure 24B**) and dyngo-4A (**Figure 23**), and clathrin, by the compound pitstop 2 (**Figure 32**) and the depletion of potassium from the cell media (**Figure 30**, **Figure 31**). However, these inhibitors may have had non-specific effects on the cells. To our knowledge, off-target effects of dynasore and dyngo-4A have not yet been reported, but this is not the case for the modes of clathrin inhibition. We originally chose potassium depletion as an inhibitor of clathrin-mediated endocytosis due to its specificity to the pathway relative to other classical pharmacological treatments, which were considered more non-specific (Ivanov, 2008). Yet, potassium depletion was recently found to inhibit the uptake of lactosylceramide, a cargo for clathrin-independent uptake mechanisms

(Vercauteren et al., 2010). To avoid drawing conclusions from potassium depletion alone, we also treated cells with pitstop 2, a recently characterized potent inhibitor of the interaction between the terminal domain of clathrin and amphiphysin, an adaptor protein involved in clathrin-mediated endocytosis (Kleist et al., 2011). Although it was reported as a specific inhibitor of clathrin-mediated uptake (Kleist et al., 2011), the proposed mechanism of action for pitstop 2 was recently questioned (Lemmon and Traub, 2012). Moreover, pitstop 2 was found to inhibit the endocytosis of various clathrin-independent cargoes as effectively as that of transferrin, even when clathrin heavy chain, the intended target of the inhibitor, was depleted in the cells (Dutta et al., 2012). The suggestion that the inhibitor is non-specific is supported by the observation that pitstop 2 treatment produces a general immobilization of the plasma membrane (Dutta et al., 2012; Kleist et al., 2011). Based on our inhibitor studies alone, the notion of Dectin-1 uptake being clathrin-dependent must be taken with caution.

To complement results obtained by pharmacologic treatment, one can use genetic manipulations targeting the molecular players of interest, such as the expression of dominant-negative constructs and short interfering RNAs, to block endocytosis (Vercauteren et al., 2010). Indeed, expression of a dominant-negative dynamin mutant (Damke et al., 1994; van der Bliek et al., 1993) and RNA interference of clathrin heavy chain completely inhibited Dectin-1 uptake in immunofluorescence experiments (**Figure 22A**, **Figure 24A** and **Figure 34**), reiterating that the event is dynamin- and clathrin-dependent. A caveat of these manipulations is that they were prolonged and sustained (24 – 48 hours), potentially causing up- or downregulation of other compensatory molecules in the cells and confounding interpretation of the observed effects (Kleist and Haucke, 2012; McMahon and Boucrot, 2011; Thompson and McNiven, 2006). Still, the dramatic inhibition of Dectin-1 uptake observed upon dynasore and dyngo-4A treatment and expression of the dynamin mutant leave us confident of the role of dynamin in Dectin-1 uptake, a finding that we are first to report. Additionally, we assert that Dectin-1 uptake is likely primarily clathrin-mediated, based on its dependence on dynamin, which is required for the formation and scission of

clathrin-coated pits (Damke et al., 1994; Hill et al., 2001; Kosaka and Ikeda, 1983; Macia et al., 2006; van der Bliek et al., 1993); the co-internalization of the receptor with transferrin (**Figure 25**), the receptor of which is a cargo for clathrin-mediated uptake; and colocalization of Dectin-1 with clathrin puncta upon stimulation of endocytosis (**Figure 26**, **Figure 27**, **Figure 28**). More replicates of clathrin depletion experiments (**Figure 34**) will ultimately confirm the dependence of Dectin-1 uptake on clathrin. Other methods to strengthen this notion could include expression of dominant-negative constructs of the clathrin hub fragment (Liu et al., 1998), or of accessory molecules for clathrin-mediated endocytosis, such as adaptor protein 180 (Ford et al., 2001), epidermal growth factor receptor pathway substrate 15 (Eps15) (Benmerah et al., 1999), amphiphysin (Wigge et al., 1997), or the adaptor protein 2 complex (AP-2) (Nesterov et al., 1999), and observing if endocytosis was inhibited. Corroborating these findings could involve depletion of AP-2 (Motley et al., 2003), a molecule considered crucial to clathrin-mediated uptake (McMahon and Boucrot, 2011), or mis-targeting AP-2 to mitochondria by rapamycin-inducible protein hetero-dimerization (Robinson et al., 2010).

A recent report also dissected cellular components required for Dectin-1 and soluble β-glucan endocytosis (Ozment et al., 2012). In confirmation of our findings of the involvement of clathrin, Ozment and colleagues found that Dectin-1 and soluble β-glucan uptake could be blocked by treatment with hypertonic sucrose (Ozment et al., 2012), another classical, although likely non-specific, method of inhibiting clathrin-mediated endocytosis (Hansen et al., 1993a; Ivanov, 2008). Since cells can utilize clathrin-independent pathways for endocytosis (Mayor and Pagano, 2007; Sandvig et al., 2011), some of which are cholesterol-dependent (Doherty and McMahon, 2009; Kumari et al., 2010; Mayor and Pagano, 2007), Ozment and colleagues also treated macrophages with methyl-β-cyclodextrin (MβCD), which extracts cholesterol from cells, and evaluated the effects on endocytosis. They found that β-glucan and Dectin-1 endocytosis were blocked in the presence of MβCD, although this was an indirect effect of the treatment preventing proper surface targeting of the receptor (Ozment et al.,

2012). One of the clathrin-independent endocytic pathways is mediated by caveolae, which are flask-shaped invaginations of 60 – 80 nm in the plasma membrane (Parton and Simons, 2007). Caveolin proteins are markers of caveolae, and as their coat proteins, make up the majority of the protein component in caveolae (Parton and Simons, 2007; Stan, 2005). With the exception of muscle tissue, the expression of caveolin-1 is essential for the formation of caveolae in cells (Stan, 2005). Ozment and colleagues obtained macrophages from wild-type and caveolin-1 knockout mice, and found no effect of the absence of caveolin-1 on Dectin-1 or soluble β-glucan internalization (Ozment et al., 2012). By immunoblot, we detected marginal expression of caveolin-1 in RAW 264.7 macrophages relative to HeLa cells (data not shown), consistent with another report (Patlolla et al., 2004). Given that caveolin-1 is required for caveolar biogenesis (Stan, 2005), it is unlikely that caveolin-1 and caveolae are involved in Dectin-1 uptake in RAW 264.7 cells. Consolidating our data with Ozment *et al.*'s findings (Ozment et al., 2012), Dectin-1 and soluble β-glucans are likely internalized by a dynamin- and clathrin-dependent, caveolin-1-independent mechanism. On the other hand, further investigation is needed to assess the involvement of cholesterol and the clathrin-independent endocytic pathways in Dectin-1 uptake.

We also briefly investigated the role of actin remodelling events in Dectin-1 uptake. We found that treatment with the actin polymerization inhibitor cytochalasin B partially inhibited Dectin-1 endocytosis (**Figure 35**). This effect is not due to inhibition of phagocytosis, as the process does not occur for the internalization of soluble ligands due to their small size (Swanson, 2008). However, the role of actin remodelling in clathrin-mediated uptake is well established (Mooren et al., 2012), and we believe the effects of actin inhibition on Dectin-1 uptake are at least in part due to perturbation of clathrin-mediated endocytosis. Actin remodelling is also required for macropinocytosis, an endocytic mechanism for fluid-phase uptake. Here, rearrangements of the actin cytoskeleton extend plasma membrane ruffles or protrusions known as lamellipodia into the extracellular fluid. When the ruffles fuse with the plasma

membrane, large volumes of fluid as well as membrane become non-selectively engulfed by the cell (Lim and Gleeson, 2011). As professional antigen presenting cells, macrophages and dendritic cells utilize constitutive macropinocytosis to constantly sample their environments, engulfing pathogens and digesting them into antigens to be presented to the adaptive immune system (Kerr and Teasdale, 2009; Lim and Gleeson, 2011). Activation of receptors, such as the epidermal growth factor receptor, can upregulate the frequency and rate of macropinocytic events (Bryant et al., 2007; Haigler et al., 1979; Racoosin and Swanson, 1989; Schmees et al., 2012; West et al., 1989). Perhaps Dectin-1 activation could increase the frequency of macropinocytosis, leading to more efficient internalization of soluble β-glucans loosely bound to the receptor or in the fluid phase, or fungal pathogens in the vicinity of the cell. Increased macropinocytosis would also cause more Dectin-1 to be taken up into the cell due to the increased influx of plasma membrane. Therefore, it is possible that Dectin-1 is internalized by macropinocytosis, offering an alternative explanation to how cytochalasin B treatment could have inhibited entry of the receptor. While it is clear actin remodelling is involved in Dectin-1 uptake, we will need to assess if these events are acting on clathrin-mediated uptake, macropinocytosis, or other internalization mechanisms.

In summary, our data reveal conclusively that Dectin-1 uptake is dynamin-dependent and most likely clathrin-dependent. This was obtained by a combination of pharmacologic and genetic approaches to inhibit both processes, all of which substantially inhibited Dectin-1 uptake. Perhaps future studies will clarify the contributions of actin, cholesterol, and clathrin-independent endocytosis to the uptake of Dectin-1.

4.5. Intracellular Trafficking of Dectin-1 and Soluble β-Glucans

It is known that during Dectin-1-mediated phagocytosis, zymosan and yeast particles traffic to LAMP1 (lysosome-associated membrane protein 1)-enriched lysosomal compartments (Herre et al., 2004; McCann et al., 2005). However,

what happens to the receptor during the endocytosis of soluble β-glucans has not been established. After determining the molecular machinery Dectin-1 uses for uptake of soluble β-glucans, we wanted to investigate which intracellular compartments the receptor traffics to once inside the cell.

Upon WGPsol stimulation, Dectin-1 puncta colocalized with GFP-tagged markers of early endosomes and the recycling compartment, and not to $LAMP2^{+}$ lysosomal compartments (**Figure 36**, **Figure 37**). Furthermore, reversible cell surface biotinylation experiments demonstrated that a pool of internalized Dectin-1 was recycled to the plasma membrane, regardless of the soluble β-glucan used for stimulation. High or low molecular weight, all three ligands stimulated this recycling behaviour, with similar amounts of the internalized receptor returning to the plasma membrane (**Figure 40**). In addition, Dectin-1 did not appear to be degraded in lysosomal compartments; inhibition of lysosomal proteolytic activity did not increase the levels of internalized Dectin-1 detected by the surface biotinylation assay, at least when laminarin and WGPsol were used to stimulate the receptor (**Figure 39**). Thus, it seems stimulation with soluble β-glucans promotes Dectin-1 internalization and then its recycling to the cell surface.

Herre and colleagues also examined Dectin-1 recycling in murine macrophages. They stimulated the cells with soluble β-glucans for various times and monitored the levels of surface-localized Dectin-1 (Herre et al., 2004). They observed a bifurcation in intracellular trafficking events depending on the size of the β-glucan ligand used. Laminarin (7.7 kDa (Mueller et al., 2000)) stimulation caused substantial recovery of Dectin-1 at the cell surface within 160 minutes. This occurred even when the protein synthesis inhibitor cycloheximide was present, implying that the recovery was due to internalized receptors returning to the plasma membrane and not due to biosynthesis of new Dectin-1 molecules (Herre et al., 2004). Interestingly, little Dectin-1 surface recovery occurred when the cells were stimulated with the high molecular weight β-glucan glucan phosphate (156 kDa (Mueller et al., 2000)). The recovery that was observed was abrogated upon inhibition by cycloheximide, suggesting that it was due to newly synthesized Dectin-1 and not recycled receptor (Herre et al., 2004). This

suggested that glucan phosphate stimulation resulted in retention of the receptor in intracellular compartments, unlike laminarin. Ultimately, they concluded that the fate of Dectin-1 trafficking could depend on properties of the soluble β-glucan ligand, such as polymer size (Herre et al., 2004). Unlike Herre *et al*., we did not observe any ligand size-dependent bifurcations in receptor trafficking, with all of our ligands inducing a similar recycling behaviour (**Figure 40**) to their findings with laminarin stimulation (Herre et al., 2004). The discrepancy could be a result of structural or size differences between our high molecular weight ligands (WGPsol and P-curdlan) and glucan phosphate, or differences in the cell types used for experimentation.

Ozment and coworkers also examined the localization of soluble β-glucan to Dectin-1 and various intracellular compartments in murine macrophages by confocal microscopy (Ozment et al., 2012). Similar to our results with Dectin-1 (**Figure 36**), they observed a colocalization of soluble β-glucan with a marker of early endosomes (Ozment et al., 2012), although this occurred only up to 15 minutes of stimulation, unlike our colocalization between Dectin-1 and $Rab5^{+}$ compartments, which was observed up to at least 30 minutes after ligand stimulation. They also observed little colocalization of soluble β-glucan with the lysosomal marker LAMP1 at every timepoint up to 30 minutes and even at 24 hours (Ozment et al., 2012), a finding consistent with the lack of colocalization we observed with LAMP2 up to two hours post-internalization (**Figure 37**). Unexpectedly, Ozment *et al.* detected dissociation of soluble β-glucan from Dectin-1 after five minutes of internalization, although colocalization between the two was restored by 15 minutes and was sustained to 24 hours (Ozment et al., 2012). They also observed a strong colocalization of soluble β-glucan with Golph4 (Golgi phosphoprotein 4), a marker of the Golgi apparatus, at 15 and 30 minutes and 24 hours after internalization (Ozment et al., 2012). Although we used a different protein as a marker of the Golgi apparatus, we did not find substantial colocalization between β-glucan and the Golgi apparatus at any of the timepoints observed (**Figure 38**). It is noteworthy that Ozment's group used a 20 kDa branched, water-soluble yeast β-glucan from Biotec Pharmacon (Tromsø,

Norway) (Aarsaether et al., 2006; Lehne et al., 2006) as their principal ligand, a possible cause for the discrepancies with our findings. They also bound the ligand to their cells at 4°C and at high concentration (100 mg/mL) for 3 hours prior to initiating endocytosis at 37°C (Ozment et al., 2012). Perhaps these conditions may explain why their ligand appeared to have an aggregated, particulate appearance in their images and localized to the Golgi apparatus during trafficking. In addition, the differences in the trafficking observed could be a result of cell type; Ozment's group used thioglycollate-elicited macrophages for their experiments, while we used the leukemic cell line RAW 264.7.

The observation that Dectin-1 recycles upon internalization is fascinating but puzzling. It is tempting to speculate that soluble β-glucan ligands dissociate from the receptor in the acidic environment of endosomal compartments (Casey et al., 2010), such that the unligated receptor is free to internalize more soluble β-glucans from the extracellular milieu upon returning to the surface. However, in our immunofluorescence images, internalized Dectin-1 and WGPsol were still quite associated 120 minutes post-internalization (**Figure 37B**). It could be that the prolonged association of ligand and receptor, induced by continual cycling between the plasma membrane, early endosome, and recycling compartment, serves to sustain Dectin-1 signaling (Rajagopalan, 2010). Indeed, localization in endosomes and endocytic vesicles would confine the soluble β-glucan in a small volume, serving to maintain the ligand-receptor interaction required for Syk activation (Geahlen, 2009; Rajagopalan, 2010; Sorkin and Zastrow, 2009). Relatedly, Dectin-1's avoidance of the lysosome would assist in signal potentiation by preventing degradation of the receptor and thus termination of the signal. In support of this hypothesis, Syk-GFP was recruited to endosomes containing Dectin-1 and WGPsol even 60 minutes after ligand stimulation, and was active (**Figure 41**), suggesting Dectin-1 signaling was still proceeding at that time. More work is certainly needed to verify this hypothesis. Observing live instead of fixed cells would help, allowing the ability to track the behaviour of individual Dectin-1^+ endosomes with high temporal resolution, perhaps enabling visualization of the recycling event. Additionally, experiments with cells

expressing dominant-negative constructs that inhibit recycling events could help delineate the importance of recycling to the propagation of Dectin-1 signaling.

While Dectin-1 puncta colocalized with WGPsol puncta 120 minutes post-stimulation, we did find some WGPsol puncta that were not colocalized with the receptor (**Figure 37B**). It might be that soluble β-glucans do eventually dissociate from Dectin-1, and that timepoints longer than 120 minutes would reveal a more distinct separation between receptor and ligand. A cause for this dissociation might be the acidic luminal pH of the endo-lysosomal system (Casey et al., 2010), leading to disruption of the non-covalent interactions between Dectin-1 and β-glucan. To determine if slight acidification could induce dissociation of soluble β-glucans from the receptor, we could investigate if, *in vitro*, the binding affinity between Dectin-1 and soluble β-glucans is reduced during changes in pH.

Despite working with only three ligands, we believe that our findings apply to other soluble β-glucans, whether they are biologically active or not. We used three structurally diverse ligands, each produced by a different organism and with different abilities to induce Dectin-1 signaling. Clearly, the endocytic routes the ligands and Dectin-1 followed were not correlated to the ability for the ligands to signal. While we expected each to produce different internalization and trafficking behaviours as a result of their structure, based on the findings of Herre *et al*. (Herre et al., 2004), each triggered receptor endocytosis with similar kinetic profiles and the formation of endocytic vesicles that accumulated Syk. All three ligands caused Dectin-1 to recycle to the plasma membrane after internalization. Both WGPsol and P-curdlan were internalized by a dynamin- and clathrin-dependent mechanism, and both WGPsol and laminarin did not appear to stimulate Dectin-1 trafficking to lysosomes. The similarities in Dectin-1 internalization and trafficking induced by each ligand suggest other soluble β-glucans should induce similar behaviour with the receptor in our system. It will be interesting to see if this prediction holds true in future investigations.

To summarize, we have observed that up to two hours after ligand binding, internalized Dectin-1 avoids trafficking to lysosomes and instead recycles to the

plasma membrane, regardless of whether it is stimulated by laminarin, WGPsol, or P-curdlan. We speculate that this diversion from the lysosome could be a mechanism to sustain Dectin-1 signaling. Further work should examine the localization of soluble β-glucans relative to Dectin-1 over longer durations of time, and explore the trafficking of Dectin-1 upon stimulation by β-glucans other than laminarin, P-curdlan, and WGPsol.

4.6. The Connection Between Dectin-1 Endocytosis and Signaling

We are the first to report that kinase-active Syk accumulates on Dectin-1^+ endosomes upon soluble β-glucan stimulation (**Figure 41**, **Figure 42**). This hinted at an intimate connection between Dectin-1 endocytosis and signaling, with endocytosis requiring signaling to occur and/or signaling requiring endocytosis to occur. Endocytosis was originally proposed as a mechanism to terminate signaling by removing activated receptors from the cell surface, preventing further association with their ligands and targeting the receptors for lysosomal degradation (Gould and Lippincott-Schwartz, 2009; Rajagopalan, 2010). However, a recent paradigm shift is the notion that endosomes can be platforms for receptor signal potentiation, with signals emanating from the endosomes themselves and not from the plasma membrane. Several receptors that signal from endosomes include the hepatocyte growth factor receptor Met, the epidermal growth factor receptor, the transforming growth factor β receptor, TLR4, and the neurotrophin receptor TrkA, and they are the subject of numerous reviews (Pálfy et al., 2012; Sigismund et al., 2012) (Disanza et al., 2009; Gould and Lippincott-Schwartz, 2009; Rajagopalan, 2010; Scita and Di Fiore, 2010; Sorkin and Zastrow, 2009). The novelty of endosomes to promote signaling could be their small volume, which entraps ligands to their receptors, their enrichment in specific lipids or scaffold proteins, or their acidic internal pH (Rajagopalan, 2010; Sorkin and Zastrow, 2009). On the other hand, Syk signaling has been known to mediate endocytosis and intracellular trafficking. Syk directly associates with clathrin upon the binding of Shiga toxin or human rhinovirus to their receptors

and mediates their uptake into the cell (Lau et al., 2008; Lauvrak et al., 2006; Wälchli et al., 2009). Shiga toxin also induces the Syk-dependent formation of clathrin-coated pits (Utskarpen et al., 2010). Other ITAM-containing receptors, such as the B cell receptor (Le Roux et al., 2007) and the Fc receptor γ chain (Bonnerot et al., 1998), require Syk for the trafficking of ligands for antigen presentation and to lysosomes, respectively. With these ideas in mind, we began exploring if Dectin-1 signaling regulated endocytosis, and if endocytosis of the receptor regulated its ability to signal.

We observed that Dectin-1 signaling was not required for receptor endocytosis. This was confirmed by two complementary approaches. The first involved substituting the hemITAM tyrosine residue of Dectin-1, Y15 (Brown et al., 2003; Gantner et al., 2003; Herre et al., 2004; Rogers et al., 2005; Underhill et al., 2005), with an alanine residue, a mutation that prevented activation of Syk and Src family kinases upon stimulation of Dectin-1 (**Figure 45**). The second approach involved inhibiting Dectin-1 signaling by treatment with inhibitors of the Syk and Src family kinases. When Dectin-1 signaling was inhibited by either method, Dectin-1 was still internalized as efficiently as the uninhibited condition (**Figure 46**, **Figure 43**, **Figure 44**). This is in contrast to the findings of Dectin-1 phagocytosis in macrophages, where residue Y15 was required for the phagocytosis of zymosan, even if Syk itself was not involved (Herre et al., 2004; Underhill et al., 2005). However, our results are consistent with studies of the B cell receptor and Fc receptor γ chain, which showed no requirement for Syk in receptor internalization, even if Syk regulated subsequent trafficking events (Bonnerot et al., 1998; Le Roux et al., 2007). Another study found the Fc receptor γ chain required Syk and Src family kinases for phagocytosis, but not endocytosis (Huang et al., 2006). Thus, it seems that in general, ITAM/hemITAM signaling is not required for receptor-mediated endocytosis but is required for phagocytosis. While we have shown that Dectin-1 signaling is not required for its uptake during stimulation with soluble β-glucans, we would like to determine if signaling (in particular Syk activation) plays a role in receptor trafficking. It would be interesting to see if RNA interference of Syk, or

overexpression of a kinase-dead mutant of Syk (Yokozeki et al., 2003), prevented Dectin-1 from recycling to the plasma membrane and/or resulted in its mis-targeting to lysosomes.

Another potential connection between signaling and endocytosis was apparent in the primary amino acid sequence of Dectin-1. Intriguingly, the hemITAM signaling sequence (YXXL) resembled the tyrosine-based internalization and trafficking motif YXXϕ, where ϕ is an amino acid with a bulky hydrophobic side chain (Bonifacino and Traub, 2003; Pandey, 2009). Internalization motifs facilitate the interaction of transmembrane receptors with endocytic adaptor proteins, allowing the receptors to be internalized. For YXXϕ, the motif interacts with the μ subunit of the AP-2 complex, a critical molecule in clathrin-mediated endocytosis (McMahon and Boucrot, 2011). Dectin-1 does not exhibit any other canonical internalization motifs (Bonifacino and Traub, 2003; Pandey, 2009) in its cytoplasmic tail, so a question that we had early in the study was if Dectin-1 uses its hemITAM as an internalization sequence. However, the fact that the Y15A mutant showed no defect in endocytosis (**Figure 46**) suggests the hemITAM does not function as a canonical YXXϕ internalization motif. Tyrosine phosphorylation on YXXϕ would also block interactions with the μ subunit of AP-2 and inhibit clathrin-dependent internalization (Bonifacino and Traub, 2003). That Dectin-1 is constantly phosphorylated on YXXL, inferred by the accumulation of Syk on Dectin-1$^+$ endosomes (**Figure 43**, **Figure 44**) (Underhill and Goodridge, 2007), and still is internalized efficiently adds support to the notion that YXXL is not a canonical YXXϕ motif. While studies have yet to be performed to identify true internalization motifs in the Dectin-1 tail, mutation of the triacidic cluster of residues (DED) preceding the hemITAM (Ariizumi, 2000) was found to completely inhibit phagocytic uptake by Dectin-1 (Underhill et al., 2005). Acidic triads have also been implicated in the internalization and/or trafficking of other receptors (Azad et al., 2008; Mahnke et al., 2000). Perhaps future efforts will clarify if the triacidic cluster in Dectin-1 functions as an internalization motif.

After finding that signaling is not required for Dectin-1 endocytosis, we

wondered if endocytosis might be required for or regulate Dectin-1 signaling. Of interest to us, other groups have reported that phagocytosis serves to terminate Dectin-1 signaling. Most found that inhibiting the progression of phagocytosis pharmacologically or with large, non-phagocytosable particles resulted in sustained and/or dramatically heightened Dectin-1 signaling (Brown et al., 2003; Hernanz-Falcón et al., 2009; Rosas et al., 2008). Contrarily, other reports saw no effect of phagocytic inhibition on NF-κB translocation or TNF-α induction in RAW 264.7 macrophages (McCann et al., 2005) or IL-10 and IL-12 in murine dendritic cells (Rogers et al., 2005). When we inhibited endocytosis with potassium-depleted media during P-curdlan stimulation, a heightened Syk activation upstream and diminished NF-κB activation downstream was observed (**Figure 47**). This was suggestive of an enhanced signal at the cell surface (perhaps due to large quantities of P-curdlan interacting with the receptor, since endocytosis was blocked) that was somehow disconnected from activating NF-κB. We also observed an ability for dynasore treatment to inhibit the P-curdlan-stimulated nuclear translocation of NF-κB (**Figure 48**). While both these results are preliminary, they suggest that the endocytosis of Dectin-1 plays a major role in the regulation of Dectin-1 signaling events. It would be helpful to repeat the experiments using other methods of endocytic inhibition, pharmacological and genetic, to show the results can be reproduced regardless of how endocytosis is inhibited.

We speculate that early and recycling endosomes function as signaling organelles for Dectin-1. Blockage of internalization would prevent localization of the receptor to these areas, offering an explanation to how a higher level of Syk activation is not linked to an increase in NF-κB activation in **Figure 47**. Perhaps the small volume of these bodies and their recycling behaviour prolong ligand-receptor interactions and promote sustained signaling (discussed in Section 4.5). In addition, the localization of Dectin-1 to endosomes might facilitate activation of other signaling molecules. Maybe some of these molecules reside specifically on the endosome, or the endosome provides an effective platform to recruit and assemble active multiprotein complexes. It will be interesting to determine if

certain intermediary players in Dectin-1 signaling, such as PKCδ, the CARD9-Bcl-10-MALT1 complex, or MAP kinases, localize to endosomes during Dectin-1 activation in a manner similar to Syk. If they do, the observations would support the idea that Dectin-1^{+} endosomes are platforms for the assembly of signaling complexes.

4.7. Functions of Dectin-1 Endocytosis

Our results have demonstrated conclusively that Dectin-1 is rapidly taken up into the cell upon binding soluble β-glucans. What could be the purpose of this endocytosis, then, if the end result is to have the receptor back on the cell surface? We have already discussed potential roles of endosomes to function as small vessels to maintain receptor-ligand interactions (Sections 4.5), as well as platforms for the assembly of complexes for signal transduction (Section 4.6). We will now explore two other potential purposes of Dectin-1 endocytosis: digestion of soluble β-glucans, and antigen presentation.

One purpose of endocytosis could be digestion of the β-glucans into smaller fragments. Hino and colleagues found that RAW 264.7 macrophages that ingested particulate curdlan discharged soluble β-glucan fragments into the culture media (Hino et al., 2012). The conversion of curdlan to the smaller, soluble fragments was dependent on the production of ROS and the NADPH oxidase complex, a protein complex that produces ROS on the phagosome (Hino et al., 2012; Segal, 2008). In support of this, ROS have previously been implicated in the cell-mediated degradation of carbohydrates (Avci et al., 2011; Duan and Kasper, 2011). As well, mammals do not produce β-glucan hydrolyzing enzymes, preventing them from being able to digest β-glucans (Chan et al., 2009; Guan et al., 2013; Hong et al., 2004; Ishibashi et al., 2004; Stone and Clarke, 1992), so it is logical that β-glucans are degraded by other means. Furthermore, the activation of Dectin-1, as least by particulate ligands, is known to trigger the Syk-dependent production of ROS (Gantner et al., 2003; 2005; Underhill et al., 2005). Finally, beyond phagosomes, NADPH oxidase-dependent ROS production can also occur in endosomal compartments (Lamb et al., 2012).

Therefore, the function of Dectin-1 internalization and recycling could serve a digestive role, localizing the soluble β-glucans to endocytic compartments decorated with NADPH oxidase, where they are broken down by ROS to smaller fragments that are eventually released into the extracellular milieu, perhaps to active other immune cells (Hino et al., 2012).

The soluble β-glucans laminarin, WGPsol, and P-curdlan all seemed to induce recycling behaviour of Dectin-1, avoiding the lysosome (**Figure 40**). However, localization to lysosomal compartments is a crucial step toward antigen presentation, as this permits hydrolytic digestion of endocytosed components to form antigen fragments. The resulting antigen fragments are then loaded onto MHCII in the lysosome, which traffics back to the cell surface to present antigens to cognate T lymphocytes (Mantegazza et al., 2013). Without a lysosomal localization, it is improbable that our pure soluble β-glucans are loaded onto MHCII compartments for antigen presentation. However, mammals do have an ability to produce anti-β-glucan antibodies (Adachi et al., 1994; Ishibashi et al., 2005; 2010; 2013; 2011; LeibundGut-Landmann et al., 2007; Torosantucci et al., 2005), suggesting that antigen presentation of fungal β-glucans does occur. In support of this, it was observed recently that covalently coupling pure carbohydate to a carrier protein maximized the immune response to the carbohydrate antigen, much higher than carrier protein or carbohydrate alone (Avci et al., 2011). While T lymphocytes can recognize purely carbohydrate antigens, endosomally generated carbohydrates are not loaded onto MHCII unless they are conjugated to a carrier protein, presumably due to an inability to bind MHCII when unconjugated (Avci et al., 2011). Thus, antigen presentation of β-glucans (and other carbohydrates) can occur if the β-glucans are covalently linked to protein. Indeed, laminarin-protein conjugate vaccines have been found efficacious against fungal infection (Lipinski et al., 2013; Pietrella et al., 2010; Rachini et al., 2007; Torosantucci et al., 2005). Therefore, while improbable for pure soluble β-glucans, it is likely that protein-β-glucan conjugates are capable of being loaded onto MHCII complexes for antigen presentation. The hypothesized importance for Dectin-1-mediated endocytosis of protein-β-glucan conjugates,

then, would be to internalize them and target them to lysosomes, facilitating their loading onto MHCII complexes for antigen presentation. We have not yet tested if our laminarin-BSA conjugates are capable of being presented on MHCII complexes. However, experiments comparing the localization of our conjugates to lysosomes and MHCII will initiate these investigations.

In closing, we have discussed two other potential roles for Dectin-1 endocytosis in the biology of soluble β-glucans. 1) The endocytosis of soluble β-glucans could aid in their digestion prior to release into the extracellular environment. 2) For β-glucan-protein conjugate ligands, internalization by Dectin-1 could facilitate the necessary trafficking to lysosomes for antigen presentation.

4.8. Conclusions and Implications

A model of our experimental findings is presented in **Figure 49**. We have demonstrated that upon stimulation by three soluble β-glucan ligands, laminarin, WGPsol, and P-curdlan, Dectin-1 is rapidly internalized. The trigger for internalization is not the initiation of Dectin-1 signaling but could be ligand-induced clustering of the receptor. Dectin-1 internalization is mediated by dynamin and likely clathrin. Upon internalization, Dectin-1 and the β-glucans localize to $Rab5^+$ early endosomes and $Rab4^+$, $Rab11^+$, and $VAMP3^+$ recycling compartments and not to late endosomes and lysosomes. Syk is also recruited to these $Dectin\text{-}1^+$ compartments by binding the phosphorylated hemITAMs. While this appears to sustain Syk activation, the function of its recruitment is unknown at this time. Laminarin, WGPsol, and P-curdlan all stimulate upstream Dectin-1 signaling events, but only P-curdlan is able to signal downstream to the level of the transcription factor, NF-κB. While the role of endocytosis in Dectin-1 signaling needs further investigation, our preliminary data suggest endocytosis (resulting in Dectin-1's localization to early endosomes and the recycling compartment) regulates the transduction of upstream signaling at the Syk level downstream to NF-κB. Internalized Dectin-1 is then recycled to the plasma membrane. We speculate that the localization of Dectin-1 to the recycling

compartment serves to promote continued signaling from the receptor.

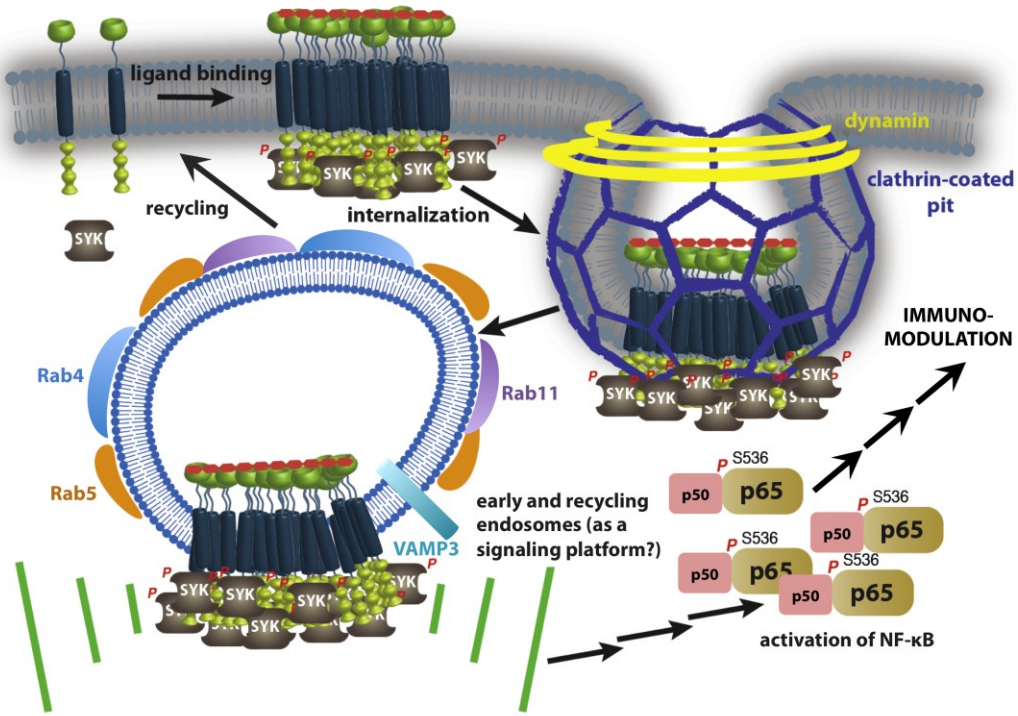

Figure 49: Model – Endocytosis, Trafficking, and Signaling of Dectin-1 Upon Binding Soluble β-Glucans

The schematic diagram above summarizes our major experimental findings into a model. Please see the text for a detailed description of the illustrated events.

Our findings give rise to three major implications for the field of β-glucan biology. Firstly, soluble β-glucan-stimulated Dectin-1 internalization provides an additional reason why soluble β-glucans block the activity of particulate β-glucans when bound to Dectin-1 prior to stimulation with the particulate ligands (Brown and Gordon, 2001a; Brown et al., 2002; 2003; Gantner et al., 2005; Kataoka et al., 2002; Rogers et al., 2005; Taylor et al., 2002; Underhill et al., 2005; Willment, 2001). While soluble β-glucans certainly occupy the receptor, preventing the particulate ligands from binding, the observed induction of receptor internalization cannot be ignored, as the process further removes and limits the number of receptors available to bind the particulate ligands.

Secondly, our work demonstrates that Dectin-1 can function as an endocytic receptor for soluble β-glucan, on top of its established ability as a phagocytic

receptor for particulate β-glucans (Goodridge et al., 2011; Heinsbroek et al., 2009; Hernanz-Falcón et al., 2009; Herre et al., 2004; Hino et al., 2012; Ma et al., 2012; Mansour et al., 2013; McCann et al., 2005; Nakamura and Watanabe, 2010; Rosas et al., 2008; Shah et al., 2009; Strijbis et al., 2013; Underhill et al., 2005; Xu et al., 2009a). Thus, the linkage of soluble β-glucan to vaccines could allow for their specific targeting to cells that express Dectin-1, such as dendritic cells, resulting in more of the vaccine molecules being delivered appropriately. Indeed, several groups have linked soluble β-glucan or anti-Dectin-1 antibodies to vaccines, and this has resulted in enhanced efficacy of the vaccines (Carter et al., 2006; Lipinski et al., 2013; Ni et al., 2010; Xie et al., 2010). This receptor-specific targeting technique has been shown to also improve the efficiency of antigen presentation when vaccines are targeted to other C-type lectin receptors, such as DEC-205, CLEC-9A, and the mannose receptor (Bonifaz et al., 2002; Jiang et al., 1995; Schreibelt et al., 2012; Tan et al., 1997). As we have demonstrated, Dectin-1 uptake occurs rapidly after ligand binding. Our findings suggest that soluble β-glucan-coupled vaccines would not only be targeted to bind Dectin-1-expressing cells, but that the endocytic capacity of the receptor would improve the efficiency by which they would be internalized and directed into the endo-lysosomal pathway, ensuring their entry into the target cells of choice. This provides a rationale for the design of soluble β-glucan-conjugated vaccines, as Dectin-1-mediated uptake of the conjugates should always occur, irrespective of how efficient the vaccine component itself is bound to and internalized by its cognate receptors, or even if those receptors are absent on dendritic cells. In summary, the covalent addition of soluble β-glucan to vaccines may increase the likelihood that they are delivered to and internalized by dendritic cells (and other cells expressing Dectin-1), improving vaccine efficacy and decreasing the required dosage for an immune response. One can envisage future applied research where soluble β-glucans are attached to vaccines and modified structurally to maximize their affinity to Dectin-1 and the ability to induce its endocytosis.

Thirdly, we have confirmed that some soluble β-glucans can have biological activity, as exemplified by P-curdlan being able to induce NF-κB activation. This

lends support to the notion that soluble β-glucans can induce immunostimulatory effects (Brown and Williams, 2009; Chan et al., 2009; Chen and Seviour, 2007) through Dectin-1, an ability that has been questioned in the field (Brown, 2006; Brown et al., 2003; Goodridge et al., 2011; Kennedy et al., 2007; Marakalala et al., 2013; Michalek et al., 1998; Underhill et al., 2005). Our data also suggest a key role for endocytosis in mediating these immunostimulatory effects, since inhibiting Dectin-1 endocytosis was able to inhibit P-curdlan-stimulated signaling events. Our findings imply that the soluble β-glucans released in the bloodstream during invasive fungal infection may actually activate the immune system. One could imagine them being internalized by Dectin-1 and activating Dectin-1 signaling, leading to further anti-fungal immune responses, just as immune cells react to large fungal particles.

Finally, our knowledge of soluble β-glucan signaling, endocytosis and trafficking could be applied to the design of anti-fungal vaccines. Since some soluble β-glucans are biologically active and induce signaling, they could be added to existing vaccines as adjuvants to prime the immune response. On the other hand, as β-glucans are components of the fungal cell wall, specific soluble β-glucan molecules from invasive fungal species could be incorporated into anti-fungal vaccines directly. If our predictions are correct, perhaps covalent linkage of a soluble β-glucan to a carrier protein anchor would be enough to promote lysosomal localization and hence, its antigen presentation (Avci et al., 2011), instead of the ligand-receptor recycling we observed with purified β-glucan. Dectin-1-mediated uptake would allow the vaccine to be internalized into dendritic cells and be targeted into the endo-lysosomal system. Upon presentation of the soluble β-glucan antigens to the adaptive immune system, the eventual production of anti-β-glucan antibodies could result, aiding rapid eradication of the fungal pathogen of interest upon a future encounter with the organism. It is our hope that this knowledge can be used to help combat the recent rise in invasive fungal infections (Armstrong-James et al., 2014; Brown et al., 2012a; 2012b).

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