University of Alberta

Examination of the Regulation of Phosphorylation Events in Macrophage Adhesion and Response to Zymosan

by

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ABSTRACT

Macrophages play a central role in innate immunity, most notably in tissue repair, phagocytosis of dead or infected cells, secretion of chemokine and cytokines at sites of infection, as well as the activation of other immune cells. These roles are highly dependent on their capacity to migrate throughout the body by mediating intercellular adhesion and adhesion to the ECM. A comprehensive understanding of the molecular mechanisms of adhesion and motility of macrophages is thus critical to better manipulate the innate immune response. Pyk2 and paxillin are key components of the macrophage cytoskeleton. I demonstrated that Pyk2 is found as two biochemically and spatially distinct populations in macrophages, based on their binding partners, serine/threonine phosphorylation status, and post-translational modifications. I further investigated the role of the protein tyrosine phosphatase CD45 on the regulation of the macrophage cytoskeleton. I found that the absence of CD45 in macrophages leads to defects in adhesion and motility; these defects are possibly due a decrease in paxillin expression. Moreover, inhibition of calpain and Pyk2 activity partially restored paxillin expression in these cells. Finally, I examined whether the absence of CD45 led to defects in macrophage responses to the yeast-derived particle zymosan. No significant differences in cytokine secretion, pathogen recognition receptor expression and rate of phagocytosis were observed in CD45-deficient macrophages. Changes in tyrosine phosphorylation and increased particle binding, however, were observed upon treatment with zymosan. In all, these results underline the contribution of Pyk2 and CD45 in the regulation

of macrophage adhesion as well as the role of CD45 in the regulation of macrophage responses to fungal stimulus.

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LIST OF ABBREVIATIONS

aa	amino acid
Ab	antibody
ALLN	N-Acetyl-L-leucyl-L-leucyl-L-norleucinal
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N′ ,N′ -tetraacetic acid tetrakis(acetoxymethyl ester)
BMDM	bone marrow-derived macrophages
BSA	bovine serum albumin
CHO	chinese hamster ovary
CI-II	caspase inhibitor II
CIAP	calf intestinal alkaline phosphatase
Col I	collagen type I
Col IV	collagen type IV
CR3	complement receptor 3
CRD	carbohydrate recognition domain
DMEM	Dubelcco's modified Eagle's medium
DMSO	dimethyl sulphoxide
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
Erk	extracellular-regulated kinase
FAK	focal adhesion kinase
FcR	Fc receptor
FcγR	Fc gamma receptor
FcRγ	Fc receptor common gamma chain
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FN	fibronectin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
IFN	interferon
IL	interleukin
IP	immunoprecipitation
ITAM	immune tyrosine activation motif

JNK	c-Jun N-terminal kinase
KO	knock-out
Lam	laminarin
LD	leucine-aspartic acid domains
LIM	Lin-11 Isl-1 Mec-3
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MEF	mouse embryonic fibroblasts
MHC	major histocompatibility
MR	mannose receptor
MTOC	microtubule organizing centre
NEM	N-ethylmaleimide
NK	natural killer
NP-40	Nonidet P-40
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PE	phycoerythrin
PI3K	phosphoinositide 3-kinase
PLC-γ	phospholipase C-γ
PRR	pathogen recognition receptor
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
Pyk2	proline-rich tyrosine kinase 2
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RSB	reducing sample buffer
RT	reverse transcriptase
RT-PCR	reverse-transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFK	Src family kinase
SH1	Src homology 1
SH2	Src homology 2
SH3	Src homology 3
TC	tissue culture
Th1	T helper 1
Th2	T helper 2

TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
WGP	whole glucan particles
WT	wild-type

CHAPTER ONE: INTRODUCTION

1.1 Overview of the Immune System

The immune system of vertebrates has evolved as a means to protect against stresses such as disease and injury and to maintain homeostasis. It is divided into two components: the innate immune system, which is qualified as non-specific as it can only recognize pathogens with a limited number of germline-encoded receptors, and the adaptive immune system, which is considered highly specialized as it can adapt to recognize virtually any non-self molecule (termed antigen) (reviewed in Medzhitov, 2007). The innate immune system is comprised of cells and anatomical barriers that prevent infection. The first barrier encountered is the epithelium, which provides a physical barrier between the environment and the body. If infectious agents make it through the epithelium and enter the tissue, they then encounter cells of the innate immune system such as macrophages, which function to recognize and destroy them. Molecules conserved within groups of pathogens, which are referred to as pathogen-associated molecular patterns (PAMPs), allow for recognition of microbes by the innate immune cells (and Akira et al., 2006; reviewed in Janeway and Medzhitov, 2002). PAMPs are recognized either directly by pathogen recognition receptors (PRRs) expressed by immune cells, or by soluble factors that are subsequently bound by receptors on the cell surface of immune cells (Janeway and Medzhitov, 2002). Once innate immune cells recognize pathogens or infected cells, they can act in several different ways to eliminate them. Natural Killer (NK) cells destroy infected cells by releasing toxic molecules that induce cell death. Phagocytes,

which include macrophages and neutrophils, can engulf microbes or dead cells at sites of infection. Basophils stimulate vasodilatation through the release of histamine and play a role in controlling parasitic infections. Eosinophils release free radicals and toxic proteins that can kill pathogens. Moreover, all of these cells are capable of secreting cytokines and chemokines, which can recruit or activate other immune cells.

Another key function of the innate immune system is to activate the adaptive immune system. This process starts by the presentation of antigen by innate immune cells, notably dendritic cells (DCs) and macrophages, to cells of the adaptive immune system. After the antigen is loaded onto the major histocompatibility (MHC) molecules on these antigen presenting cells (APCs) it can bind the appropriate receptor on the adaptive immune cell, notably the T-cell receptor (TCR) on the surface of T-cells and the B-cell receptor (BCR) on B-cells. Activation of a specific subset of T-cells termed cytotoxic T-cells (CTL) allows for destruction of infected cells, a process known as cell-mediated immunity while the activation of B-cells leads to the secretion of antibodies, which is known as humoral immunity. The activation of a second subset of T-cells called helper T-cells leads to cytokine secretion that assists these processes. Type 1 helper T cells (Th1) produce interferon-γ (IFN-γ) and tumor necrosis factor- β (TNF-β), which assist killing of microbes by macrophages and stimulate the proliferation of CTL. Type 2 helper T cells (Th2) produce interleukins (IL)-4, -5, -6, and -13 and aid in the humoral response by stimulating B-cell proliferation and neutralizing antibody production. The highly specific nature of the adaptive immune system

stems from the capacity of these cells to undergo somatic hypermutation and V(D)J recombination. This leads to the generation of BCRs and TCRs capable of recognizing a large number of antigens. Another key aspect of the adaptive immune system is its capacity to generate immunological memory, which insures rapid protection in case of future attacks from the same pathogen.

Although the adaptive immune system is highly specific and can generate immunological memory in comparison to the innate immune system, the lag time between the pathogen encounter and immune reaction takes days, whereas the innate immune system is capable of immediate action. Therefore, in the case of a pathogenic attack, it is essential for the innate immune system to contain infection until the adaptive immune system is fully activated to eliminate the pathogen. Macrophages are key innate immune cells with both the capacity to eliminate pathogens and infected cells as well as the ability to activate the adaptive immune response, making them an essential player in the fight against infection.

1.2 Macrophage Biology

1.2.1 Development and activation

As is the case of all immune cells, macrophages originate from hematopoietic stem cells (HSCs) in the bone marrow (and reviewed in Gordon and Taylor, 2005; and Lawrence and Natoli, 2011; Volkman and Gowans, 1965). A portion of these HSCs has the potential to differentiate successively into common myeloid progenitor cells, granulocyte-macrophage precursors and macrophage/DC progenitors. From there, macrophage/DC progenitors can

differentiate either into monocytes, which will eventually mature to become macrophages, or into the common DC progenitor, which will give rise to classical DCs. Monocytes then exit the bone marrow into the blood stream where they will circulate until they are recruited into tissues under inflammatory conditions. In these conditions, monocytes can give rise to inflammatory DCs or macrophages (Van Furth et al., 1973). It is also thought that local proliferation of macrophages also contributes to population density in tissues (Murray and Wynn, 2011).

Depending on the cytokines and the pathogenic stimuli present in the environment, macrophages can be activated through two general pathways: the classical (M1) or alternative (M2) macrophage activation pathways (reviewed in Gordon, 2003; and Lawrence and Natoli, 2011). M1 macrophages are generally considered highly microbicidal, phagocytic, pro-inflammatory and promote extracellular matrix (ECM) destruction whereas M2 macrophages promote tissue repair by stimulating ECM construction, angiogenesis, and cell proliferation, as well as aiding in combatting extracellular and parasitc pathogens. M1 macrophages arise in a T helper 1 (Th1) environment where IFN-γ "primes" macrophages and are subsequently activated by stimuli such as TNF-α or LPS (Gordon, 2003; Lawrence and Natoli, 2011; Mosser, 2003). This mode of activation induces transcription factors involved in the transcription of IFN-α/-β, which promote an antiviral response, as well as genes involved in the production of nitric oxide, which promote an antibacterial response. Macrophages are alternatively activated in the presence of Th2 cytokines IL-4 and IL-13 (Gordon and Martinez, 2010; Lawrence and Natoli, 2011). These cells activate signal

transducer and transcriptional activator 6 (STAT6), a transcription factor associated with Th2-related processes, and activate the enzyme arginase type I, which promotes collagen production (Gordon and Martinez, 2010; Lawrence and Natoli, 2011; Rutschman et al., 2001). It is worth noting that although designations such as M1 and M2 exist, it is becoming increasingly evident that macrophages display heterogeneity and plasticity among subpopulations (and Geissmann et al., 2010; reviewed in Taylor et al., 2005). The M1 and M2 designations can instead be viewed as describing opposite ends of a spectrum in which macrophages are polarized more or less strongly to one of these (Mosser and Edwards, 2008). Therefore, it is increasingly difficult to clearly assign functions and markers to specific macrophage populations.

1.2.2 Functions

1.2.2.1 Pathogen Recognition

The innate immune system is able to recognize a wide variety of infectious agents due to the expression of PRRs (reviewed in Akira et al., 2006). These receptors are germline-encoded, non-clonal and independent of immunological memory. They have evolved to recognize PAMPs that are often essential for pathogen survival, therefore are not subjected to high mutation rates. These include LPS, peptidoglycan, flagellin, double-stranded RNA, beta-glucans and mannans. PRRs can be divided in functionally diverse classes: endocytic, signalling, and secreted PRRs (Janeway and Medzhitov, 2002; Jeannin et al., 2008). This classification system merely distinguishes between the main

functions and capacities of PRRs, as endocytic PRRs and secreted PRRs are also capable of triggering signalling events. Representative examples of these receptors are illustrated in Figure 1.1. Macrophages express both endocytic and signalling PRRs on their surface, in the cytoplasm and in endocytic compartments, whereas secreted PRRs are used as an intermediary between pathogens and receptors expressed on the cell surface. Recognition of microbes by these receptors culminates in a variety of inflammatory and anti-microbial responses that include phagocytosis and the secretion of inflammatory factors (Janeway and Medzhitov, 2002).

1.2.2.1.1 Endocytic Pathogen Recognition Receptors

Endocytic PRRs lead to engulfment of microbes following their recognition. They include Dectin-1, Dectin-2, the mannose receptor (MR) and the complement receptor 3 (CR3; also known as CD11b/CD18, macrophage-1 antigen, integrin $\alpha_M\beta_2$) (Figure 1.1a). Dectin-1 and Dectin-2 are type II transmembrane proteins of the C-type lectin family that recognize fungal beta-glucan and mannans, respectively, through their single carbohydrate recognition domain (CRD) in their extracellular region (Adachi et al., 2004; McGreal et al., 2006). Signalling by Dectin-1 occurs through its immunorecptor tyrosine- based activation motif (ITAM) located on its cytoplasmic tail whereas Dectin-2 associates with an adaptor, the ITAM-containing Fc receptor common γ

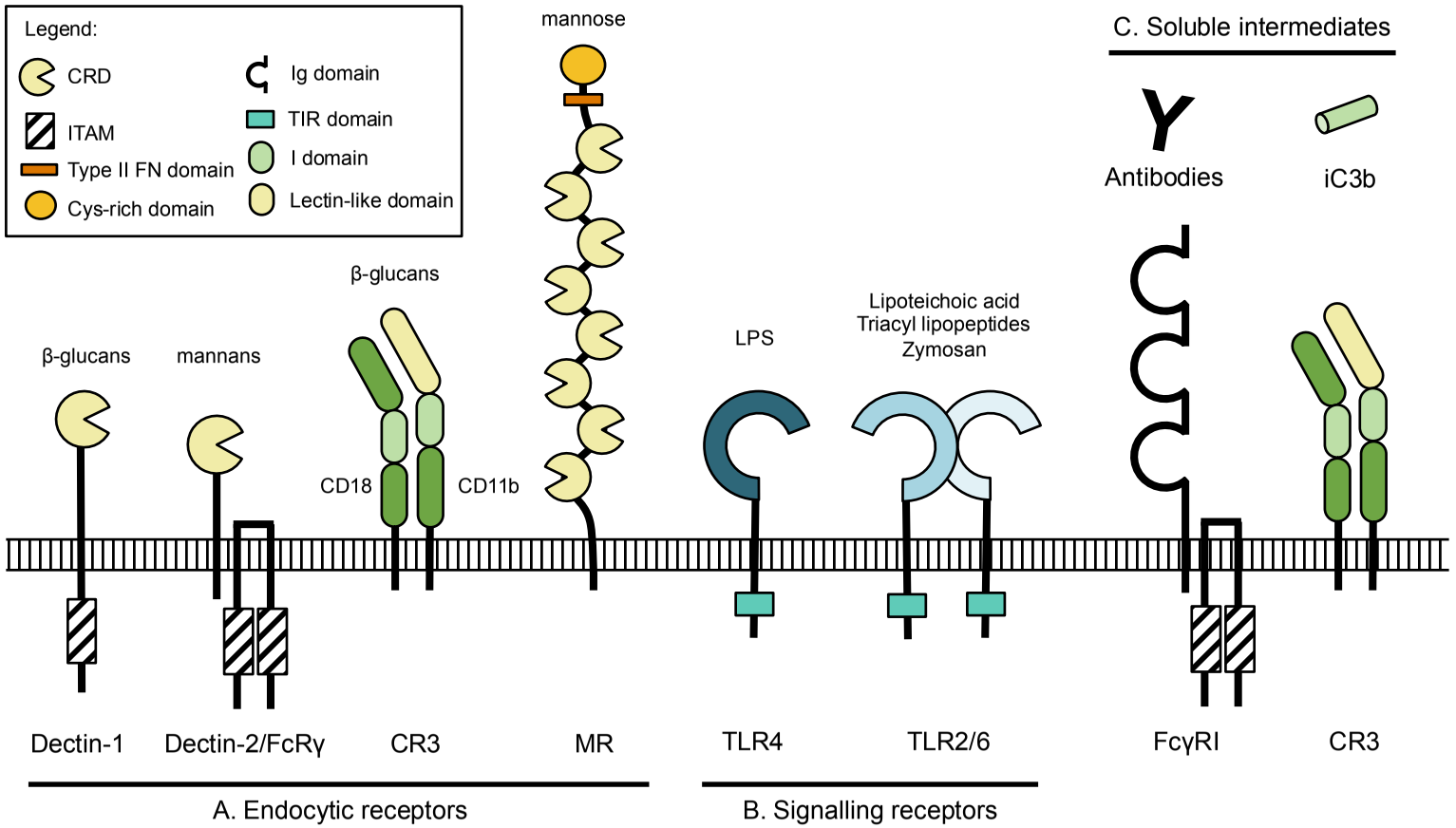

Figure 1.1. Types of pattern recognition receptors expressed by macrophages and their ligands. A. Endocytic PRRs trigger the phagocytosis of microbial ligands by macrophages. Examples of endocytic PRRs include the C-type lectins Dectin-1, Dectin-2 and the mannose receptor. B. Signalling PRRs trigger the release of inflammatory molecules upon ligand binding. The TLR family is an example of signalling receptors. C. Antibodies and iC3b are examples of soluble PRRs found in plasma that link microbes to transmembrane receptors. CR3 can act as an endocytic receptor, as it can directly bind to β-glucan, or as a receptor for the soluble intermediate iC3b.

chain (FcRγ) (Ariizumi et al., 2000; Gantner et al., 2003; and reviewed in Saijo and Iwakura, 2011; Sato et al., 2006). The many signalling pathways downstream of these receptors lead to phagocytosis, cytokine production and ROS production and involve spleen tyrosine kinase (Syk) and Src Family Kinases (SFK) (Brown, 2006; Robinson et al., 2009; Rogers et al., 2005; Saijo and Iwakura, 2011; Sato et al., 2006).

The MR is also a C-type lectin and possesses eight CRDs of which CRDs 4 to 8 are necessary for binding and internalizing mannosy/fucosyl or N-acetylglucosamine conjugates on the surface of pathogens (Taylor et al., 1992; Taylor and Drickamer, 1993). Downstream events following pathogen recognition by this receptor include the release of reactive oxygen intermediates (ROI) and cytokines such as IL-1, IL-6, TNF-α and GM-CSF (Berton and Gordon, 1983; Ezekowitz et al., 1991; Garner et al., 1994; Stein and Gordon, 1991; Yamamoto et al., 1997). The extracellular portion of MR also includes a cysteine-rich region and a type II fibronectin (FN) domain in its extracellular portion (reviewed in Fraser et al., 1998). The cysteine-rich domain has been shown to bind to sulphated carbohydrates found on glycoprotein hormones such as thyrotropin and lutropin and is thought to be involved in hormone homeostasis (Fiete and Baenziger, 1997; Fiete et al., 1998). It also mediates MR binding to sulphated forms of sialoadhesins, a macrophage-specific membrane molecule and the leukocyte common antigen, CD45 (Pomares et al., 1999). It has been proposed that the soluble form of the MR participates in antigen transport as it binds pathogens through its CRDs and delivers them to sialoadhesin- or CD45-

positive cells in lymphoid organs through interaction with the cysteine-rich domain (Martinez-Pomares and Gordon, 1999).

CR3 is a heterodimeric protein consisting of the integrin subunits β_2 (CD18) and α_M (CD11b) (reviewed in Ehlers, 2000). In addition to its role as an endocytic receptor, it functions as an adhesion receptor and a receptor for secreted PRR, which will be reviewed in a later section. Its role as an endocytic receptor is possible due to the direct recognition of PAMPs such as beta-glucans and LPS on the surface of fungi or bacteria, respectively; the C-terminal lectin-like domain of the α_M subunit is responsible for beta-glucan binding whereas the binding site for LPS is thought to be within the "inserted" (I) domain (Matsuno et al., 1998; Ross et al., 1985; Ross et al., 1987; Thornton et al., 1996; Wright and Jong, 1986; Xia and Ross, 1999). Receptor recognition of these PAMPs by CR3 has been shown to trigger phagocytosis, nitric oxide production and superoxide production in phagocytes (Le Cabec et al., 2000; Matsuno et al., 1998; Steadman et al., 1990). In addition, the recognition of beta-glucans by CR3 has the unique capacity of "priming" this receptor, i.e. inducing a higher affinity state (Vetvicka et al., 1996). This results in increased phagocytosis of pathogens coated with iC3b, a secreted PRR that is also a ligand for CR3. Ligation of CR3 through the lectin site as well as via iC3b thus mediates the most efficient response to pathogens. It is important to note, however, that this beta-glucan-induced priming is distinct from the usual high affinity state induced by "inside-out" signalling during integrin-mediated adhesion, which will be discussed in a latter section.

1.2.2.1.2 Signalling Pathogen Recognition Receptors

Upon recognition of their ligand, signalling PRRs trigger cell activation responses, such as gene transcription and cytokine secretion. The Toll-like receptors (TLRs) represent the prototypical family of signalling PRRs (Janeway and Medzhitov, 2002; Kawai and Akira, 2010). Thirteen TLRs have been identified in mice and humans thus far. Although they are similar in structure in that they are type I transmembrane receptors with N-terminal leucine-rich repeats and a C-terminal Toll/interleukin-1 receptor (TIR) domain (Figure 1.1b), they differ in ligand specificity, cellular expression and cellular localization. To allow for recognition of PAMPs and to induce signalling, they can either homodimerize, heterodimerize, or associate with a variety of other receptor or adaptor molecules.

The first mammalian TLR characterized as a PRR, and probably the most extensively studied, is TLR4 whose homodimerization allows for signalling downstream of LPS (Medzhitov et al., 1997; Ozinsky et al., 2000a; Shimazu et al., 1999). An example of a TLR heterodimer is TLRs 2 and 6, which recognize a variety of PAMPs including diacylated lipopeptides of gram-positive bacteria and the fungal-derived particle zymosan (Ozinsky et al., 2000a; Ozinsky et al., 2000b). Upon recognition of their ligands, TLRs trigger the production of inflammatory cytokines and chemokines as well as the expression of costimulatory, MHC and antimicrobial molecules on the ligated APC (Akira et al., 2006; Janeway and Medzhitov, 2002). In macrophages, this is accomplished through one of two signalling pathways: the MyD88-dependent and MyD88-independent (or TRIF-dependent) pathways (Akira and Takeda, 2004) (Figure 1.2). The MyD88

pathway is used by all TLRs (except TLR3) whereas the MyD88-independent pathway is used by TLRs 3 and 4 (Akira and Takeda, 2004; Kawai and Akira, 2010). In the MyD88-dependent pathway, the MyD88 adaptor molecule is recruited via its TIR domain to the cytoplasmic tail of TLRs where it mediates a homophilic interaction with the TLR TIR domain. For TLR2 and TLR4, an additional TIR-containing adaptor molecule is recruited MyD88 then recruits a variety of kinases that lead to the activation of the mitogen-activated protein kinase (MAPK) signalling cascade and the activation of the transcription factor NF-κB, which lead to the transcription of genes encoding for pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6. The MyD88-independent pathway signals through the TIR-containing TRIF and TRAM adaptor molecules. It also leads to the activation of MAPK signalling cascade and NF-kB, although the kinetics of this activation are delayed in comparison to the MyD88-dependent pathways. In addition, the MyD88-independent pathway leads to the activation of the IRF3 transcription factor, which is involved in the transcription of type I interferons (IFN) and IFN-inducible genes involved in the anti-viral response.

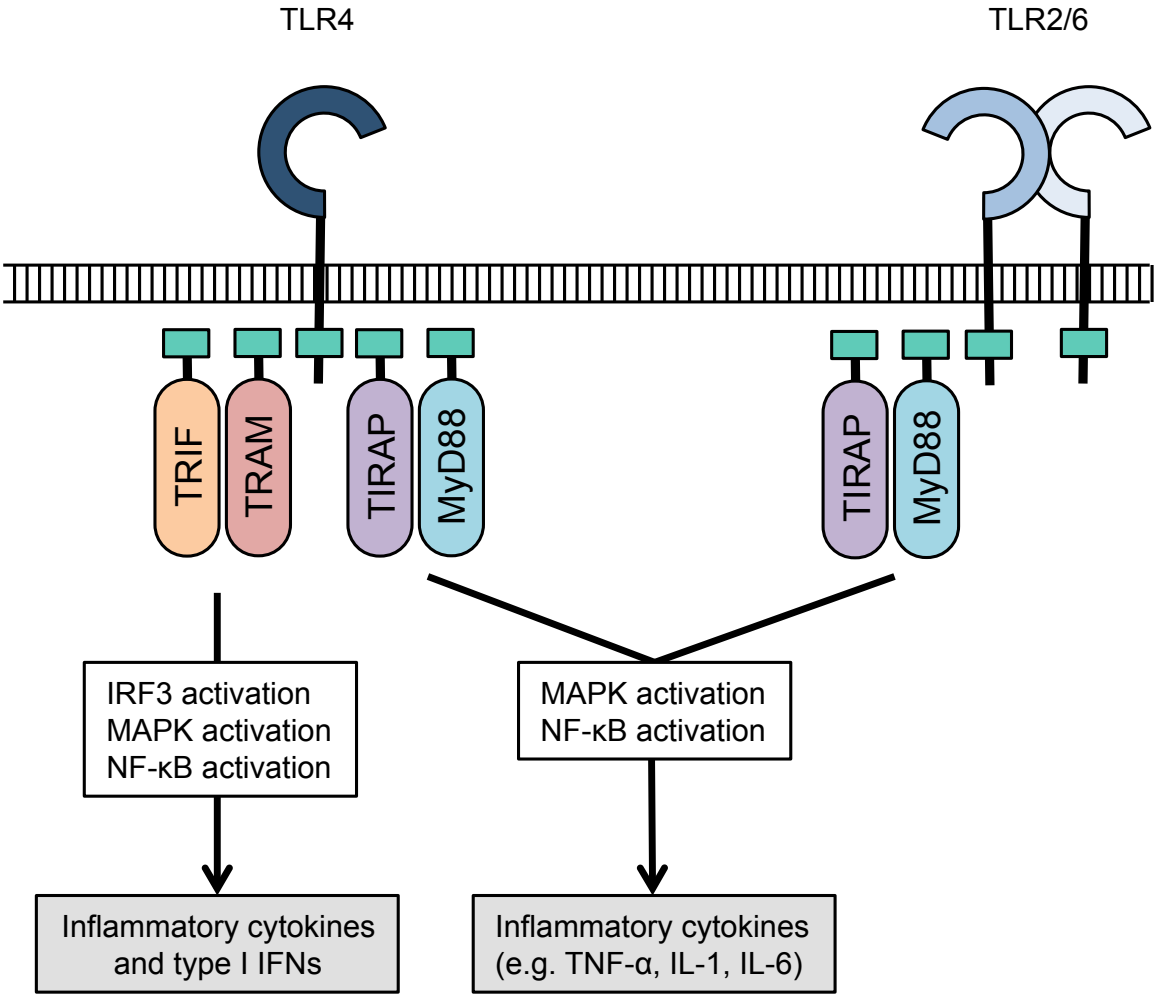

Figure 1.2. Diagram of TLR signalling. TLRs can signal through two signalling pathways: The MyD88-dependent pathway and the TRIF-dependent pathway. All TLRs except TLR3 signal through the MyD88-dependent pathway whereas TLRs 3 and 4 can signal through the TRIF-dependent pathway. In the MyD88-dependent pathway, the MyD88 adaptor, as well as in some cases the TIRAP adaptor, are recruited to the TLR TIR domain. In the TRIF-dependent pathway, adaptors TRIF and TRAM are recruited to the TLR TIR domain. Both these pathways result in activation of the MAPK signalling cascade and the transcription factor NF-κB, and culminate in inflammatory cytokine production. The TRIF-dependent pathway additionally activates the transcription factor IRF3 and results in the secretion of type I IFNs.

1.2.2.1.3 Secreted Pathogen Recognition Receptors

Several humoral components act as PRRs and assist in the recognition and elimination of microbes by phagocytes. They include antibodies, which are recognized by Fc family of receptors (FcRs), and complement components, which are bound by complement receptors of phagocytes (Figure 1.1c). The FcRs responsible for the phagocytosis of IgG-coated pathogens and involved in phagocytosis are the FcγRI, FcγRIIA and FcγRIIIA receptors in human macrophages and FcγRI and FcγRIII in murine macrophages (reviewed in Nimmerjahn and Ravetch, 2007). Apart from FcγRIIA, which possesses an ITAM in its cytoplasmic tail, FcγRs must associate with adaptor molecules such as FcRγ to induce signalling (Ernst et al., 1993; Kurosaki and Ravetch, 1989). Signalling is similar for all of these receptors: 1) ITAMs are tyrosine phosphorylated by various SFKs, followed by 2) recruitment of Syk to phosphorylated ITAMs and 3) activation of various signalling pathways and molecules downstream of Syk, including pathways culminating in gene transcription, cytokine secretion, cytoskeletal rearrangement and phagocytosis (Ghazizadeh et al., 1994; Kiener et al., 1993; Wang et al., 1994).

The complement system consists of over 30 secreted and transmembrane proteins and is involved in the innate immune response to a plethora of pathogens (reviewed in Gasque, 2004). Most of the secreted complement proteins are produced by hepatocytes and circulate in serum until they recognize and bind to microbial particles. Upon recognition, these proteins are activated, cleaved, and mediate phagocytosis of the coated particle by phagocytes bearing the appropriate

complement receptor. One of the key complement receptors on macrophages is CR3; it binds to iC3b, a stable fragment of the complement component 3 (C3) (Ehlers, 2000). Unlike other PRRs, however, the CR3 receptor requires prior "priming" (i.e. conformation change) in order to induce phagocytosis of iC3b-coated particles. This can be prompted by stimulating cells with cytokines (ex. TNF-α, GM-CSF), attachment to extracellular matrix substrates (ex. Laminin, fibronectin) or binding of the CR3 lectin domain by beta-glucans (Ehlers, 2000; Gasque, 2004; Pommier et al., 1983; Vetvicka et al., 1996; Wright et al., 1983; Wright and Griffin, 1985). Moreover, in contrast to other phagocytic receptors, iC3b-mediated responses by macrophages are limited to phagocytosis and do not elicit the release of inflammatory mediators (Aderem et al., 1985; Wright et al., 1983).

1.2.2.2 Phagocytosis

Phagocytosis is the process of internalization of particles of more than 0.5 μm in diameter (Jaumouille and Grinstein, 2011). It is essential for the uptake of microbes or dead cells by macrophages but also participates in development, tissue remodelling and inflammation (Aderem and Underhill, 1999). From an adaptive immunity perspective, this process is essential for the uptake and presentation of antigens to cells of the adaptive immune system (Soehnlein and Lindbom, 2010). A large number of receptors trigger phagocytosis in macrophages, resulting in diverse signalling pathway leading to particle uptake (Jaumouille and Grinstein, 2011; Underhill and Ozinsky, 2002). The basic steps,

however, remain similar: 1) binding of the cell receptors to their ligand on the surface of the particle, 2) receptor clustering and subsequent signalling, 3) engulfment of the particle by the phagocyte and is contained in the phagosome followed by complete containment in the phagosome, 4) maturation of the phagosome through fusion events with late endosomes and lysosomes (forming the phagolysosome) and 5) break down of the particle through acidification and activation of hydrolytic enzymes and other bactericidal compounds (Jaumouille and Grinstein, 2011; Vieira et al., 2002).

Given the major changes in cell morphology and shape that occur during phagocytosis, it is not surprising that the remodelling of the actin cytoskeleton plays a major part in the execution of phagocytosis. An abundance of signalling molecules are involved in actin dynamics during this process including scaffolding proteins, kinases and membrane traffic regulators (Underhill and Ozinsky, 2002). Some of the notable participants in this process include Syk, SFKs Hck, Fgr and Lyn, Focal Adhesion Kinase (FAK), Protein tyrosine kinase 2 (Pyk2) and paxillin (Table 1). It is noteworthy that the molecules listed above are also involved in cytoskeletal functions. Indeed, phagocytosis is intricately tied to cell adhesion as both use some of the same cell machinery, signalling proteins and receptors. Therefore, a better understanding of the mechanisms that govern the components involved in cell adhesion may provide valuable information on their role in phagocytosis, or vice versa.

Table 1. Role of signaling and adaptor molecules in macrophage phagocytosis and adhesion

Protein	Role in macrophage phagocytosis	References	Role in macrophage adhesion	References
FAK	Tyrosine phosphorylated during FcγR-mediated phagocytosis; Required for phagocytosis of *Yersinia pseudotuberculosis*;	Pan, Darby et al, 1999; Antonieta Cote-Vélez, Ortega and Ortega, 2001; Bruce-Staskal, Weldow et al, 2002; Hudson, Bliska, Bouton, 2005; Owen, Thomas, Bouton, 2007;	Localized and tyrosine phosphorylated in focal complexes; Required for the regulation of macrophage cell spreading, adhesion turn-over and chemotaxis; Required for *in vivo* migration of monocytes to sites of inflammation; Promotes chemotaxis downstream of M-CSF and haptotaxis to FN downstream of integrin α5β1;	Rovida, Lugli et al, 2005; Owen, Pixley et al, 2007; Abshire, Thomas et al, 2011;
Paxillin	Tyrosine phosphorylated during FcγR-mediated phagocytosis;	Greenberg, Chang, Silverstein, 1994; Kedzierska et al, 2001;	Localized in podosomes and focal complexes; Promotes chemotaxis downstream of integrin α4β1;	De Nichilo, Yamada, 1996; Duong and Rodan, 2000; Abshire, Thomas et al, 2011;
Pyk2	Tyrosine phosphorylated during FcγR-mediated phagocytosis; Required for phagocytosis of *Yersinia pseudotuberculosis*;	Kedzierska et al, 2001; Hudson, Bliska, Bouton, 2005; Owen, Thomas, Bouton, 2007;	Tyrosine phosphorylated downstream of β2 integrins; Localized to and tyrosine phosphorylated in podosomes; Required for macrophage spreading, adhesion turn-over and migration;	Duong and Rodan, 2000; Owen, Pixley et al, 2007;
SFKs	Participate in FcγR-mediated phagocytosis and phagocytosis of opsonized zymosan; Negatively regulates phagocytosis of opsonized particles;	Crowly, Costello et al, 1997; Pan, Darby et al, 1999; Gresham, Dale et al, 2000; Fitzer-Attas et al, 2000; Kedzierska et al, 2001; Suzuki, Kono et al, 2000; Strzelecka-Kiliszek, Kwiatkowska, Sobota, 2002; Adachi and Suzuki, 2007;	Hck/Lyn/Fgr triple KO macrophages are defective in spreading and motility *in vitro* and show impaired migration *in vivo*; Fgr and Lyn are negative regulators of β2-integrin-dependent signalling and cell spreading; Hck positively regulates podosome formation and size *in vitro* and migration to inflammatory sites *in vivo*;	Meng and Lowell, 1998; Suen, Ilic and al, 1999; Vines, Potter et al, 2001; Scholz, Cartledge and Dunn, 2000; Pereira, Lowell, 2003;Tang, Law and Tan, 2006; Cougoule, LeCabec et al, 2010;
Syk	Tyrosine phosphorylated during FcγR-mediated phagocytosis; Required for FcγR-, CR3-dependent phagocytosis;	Crowly, Costello et al, 1997; Kiefer, Brumell et al, 1998; Pan, Darby et al, 1999; Bruce-Staskal, Weldow et al, 2002; Strzelecka-Kiliszek, Kwiatkowska, Sobota, 2002; Shi, Tohyama et al, 2006;	Required for 'outside-in' signaling of β2-integrins	Reviewed in Tohyama and Yamamura, 2009

1.2.2.3 Other functions of macrophages

Microbe recognition and phagocytosis of microbes triggers a variety of macrophage responses that function to communicate with other cells, destroy microbes and activate the adaptive immune response. Cytokines are important soluble messengers secreted by all immune cell types. They can modulate the response of other cell types as well as act in an autocrine fashion. Macrophages secrete a number of cytokines under specific conditions, including the pro-inflammatory cytokines TNF-α, type I IFNs (IFN-α and -β), IL-6 and -12 as well as the anti-inflammatory cytokine IL-10. TNF-α and IL-6 are secreted in response to pathogenic infections and promote B- and T-cell proliferation (and Aggarwal, 2003; reviewed in Heinrich et al., 1998). Type I IFNs are typically secreted in response to viral infections and many PRRs that recognize viral ligands trigger IFN-α/β secretion by macrophages (Gonzalez-Navajas et al., 2012). They induce the expression of genes involved in resistance to viral replication in the responding cell types, activate NK cells, and increase antigen presentation by class I MHC (reviewed in Hervas-Stubbs et al., 2011). IL-12 secretion by macrophages is important for the polarization of Th1 cells and Th1-dependent immune responses to pathogens (Hsieh et al., 1993; Trinchieri, 1997). Finally, IL-10 is an immunomodulatory cytokine that serves as a negative feedback mechanism to suppress both the Th1 and Th2 responses; its secretion kinetics are generally slower compared to other cytokines (reviewed in Moore et al., 1993; and O'Garra et al., 2008).

Macrophages produce toxic molecules that contribute to microbial killing, which include nitric oxide (NO) and reactive oxygen species (ROS). One of the primary functions of ROS and NO is the destruction of engulfed bacteria in the phagolysosome (and Lambeth, 2004; reviewed in Nathan, 1992). ROS and NO production by macrophages and release in the phagolysosome is triggered by ligation of a variety of PRRs, such as TLR4, as well as in the presence of cytokines such as IFN-γ (Garotta et al., 1986; Ohashi et al., 2000; Ryan et al., 2004; Zughaier et al., 2004).

Although DCs have been coined as the principal APC, the significance of macrophages in antigen presentation has been demonstrated in several studies. Macrophages have been shown to have great importance in stimulating T-cell proliferation and activation, as well as generation of T-cell memory, in an antigen-specific manner (Desmedt et al., 1998; McCormack et al., 1993; McCormack et al., 1992; McCormack et al., 1991; Moser, 2001; Pozzi et al., 2005). For example, peptide-pulsed macrophages were able to stimulate naïve T-cells in the spleen to induce proliferation, effector functions and memory cell formation *in vivo* with the same potency as DCs (Pozzi et al., 2005). Moreover, antigen presentation by macrophages has been shown to generate both Th1 and Th2 T-cell responses *in vivo* (Desmedt et al., 1998; Moser, 2001).

1.3 Macrophage adhesion and motility

Monocytes and macrophages are highly motile cells that can migrate within the body to sites of infection and tissue damage. This migratory capacity is possible due to their dynamic regulation of cell adhesion to the extracellular matrix (ECM) and to other cells. Macrophages also utilize some of the same molecules involved in adhesion and motility to phagocytose microbes and dead cells (May and Machesky, 2001). These mechanisms are thus targets for therapies that limit or enhance the inflammatory response of macrophages. For example, monocytes/macrophages are known to participate in the initiation and progression of artherosclerosis; monocytes are recruited to the vascular endothelium where they differentiate into macrophages and contribute to the progression of the disease through their activation and release of inflammatory factors (Chi and Melendez, 2007). As such, multiple targets of monocyte/macrophage migration and adhesion are currently being studied as possible therapies for this disease (reviewed in Tiwari et al., 2008). On another note, the use of inhibitors aimed at containing the migration of other cell types can have important consequences on macrophages; knowledge of the possible influences of these drugs on macrophage function should be assessed in order to determine whether their effect is desirable in terms of immune modulation. For example, use of inhibitors of tumour cell invasion and metastasis in a pancreatic cancer model has been shown to also inhibit macrophage migration, which is desirable in this case as macrophages promote tumour proliferation and metastasis in this model (Stokes et al., 2011). In contrast, macrophages are also involved in

anti-tumour responses and inhibition of these macrophages could thus promote cancer progression in certain models (reviewed in Biswas and Mantovani, 2010). In all, the identification of the mechanisms that regulate macrophage adhesion and motility are crucial for the understanding of macrophage function and provide potential targets for the modulation of macrophage responses.

1.3.1 Cytoskeleton

The scaffolding of proteins responsible for maintaining cell shape is called the cytoskeleton. There are three main filaments that make up the cytoskeleton: microtubules, intermediate filaments and actin filaments (reviewed in Wang, 1991). Microtubules are generally involved in intracellular transport and contribute to the structure of the axoneme of cilia and flagella and the mitotic spindle. They emerge from the microtubule-organizing centre (MTOC), which is located near the nucleus and is the main site for microtubule nucleation. Intermediate filaments have many functions, which include tethering organelles in the cell, and maintaining cell shape, cell-cell and cell-matrix junctions (and Braga, 2002; reviewed in Schoenwaelder and Burridge, 1999). Actin filaments participate in maintaining cell shape, adhesion, migration and phagocytosis. Because these processes are intrinsically linked to the immune functions of macrophages, the adhesion receptors, protein components and signalling pathways involved in the regulation of the actin cytoskeleton will be described further. It is important to note that while many of the signalling pathways responsible for the regulation of the actin cytoskeleton have been described in

non-immune cell types, their function in macrophages has not been fully confirmed or elucidated. A review of some of noteworthy molecules involved in the regulation of macrophage cytoskeletal functions is found in Table I.

1.3.2 The integrin adhesome

Integrins are a family of cell-surface transmembrane adhesion receptors that function to link ligands of the cell microenvironment, such as ECM molecules, to the intracellular signalling pathways and elements of the actin cytoskeleton. This relay of information informs the cell on its environment and is termed "outside-in" signalling. In mammals, the combination of heterodimers composed two distinct subunits (α and β chains) give rise to 24 integrins (reviewed in Hynes, 2002). Macrophages express a variety of β_1 integrins (CD49c, CD49f, CD29), which are involved in binding to the ECM, and leukocyte-specific β_2 integrins (CD11a, CD11b, CD11c and CD18), which in addition to binding to ECM molecules, are able to bind to cellular and microbial ligands (Ammon et al., 2000). For example, integrin $\alpha_M\beta_2$ (CD11b/CD18) possesses a role in the recognition of microbial ligands as reviewed above, intercellular adhesion through its binding of intercellular adhesion molecules 1 and 2 (ICAM-1 and -2), as well as a role in cell adhesion through its binding of ECM molecules such as fibronectin (Diamond et al., 1990; Ehlers, 2000; Xie et al., 1995). In order to bind its ligand efficiently, integrins must be activated through a change in conformation that allows for high affinity binding of their ligands (and Abram and Lowell, 2009; reviewed in Hynes, 2002). This is triggered by intracellular activation pathways and is termed

"inside-out" signalling. This allows cells such as monocytes to regulate their adhesion state: when circulating through the body, these cells keep their integrins in a non-adhesive state and, once stimulated, can rapidly adhere to vessel walls and participate in the extravasation process. Examples of stimuli that induce inside-out signalling include cytokines, chemokines and microbial ligands. Inside-out signalling also allows cells to regulate cell-to-cell interaction, which is an important part of antigen presentation by macrophages. In the case of CD11b, a third activation state has been demonstrated in which β-glucan-treated macrophages bind iC3b-opsonized particles more efficiently without achieving the high affinity state required for endogenous ligand binding (Vetvicka et al., 1996).

Integrins have limited actin-binding capacities and do not possess any catalytic activity; they rely on a large network of intracellular proteins to relay signals to the cell and to link the ECM to the cytoskeleton (reivewed in Liu et al., 2000; and Zamir and Geiger, 2001). Some proteins are capable of direct interaction with the cytoplasmic portion of either the α or β chains, and more are recruited through additional protein-protein interactions. Recent *in silico* analysis have linked over 180 components through more than 680 interactions in this network, appropriately coined the "adhesome" (and Zaidel-Bar and Geiger, 2010; reviewed in Zaidel-Bar et al., 2007). The adhesome is involved in several macrophage functions such as maintenance of cell shape, motility and extravasation. Some of the notable components of the integrin adhesome include the adaptor protein paxillin and the signalling proteins of the SFK and FAK

family. The role of these proteins in macrophage adhesion and function will be reviewed below.

1.3.3 Focal complexes

Macrophages can form several types of adhesion structures including focal complexes and podosomes (Linder et al., 2000; Neumeister et al., 2003; Pixley, 2012; Pixley et al., 2001). Focal complexes are formed upon adhesion to substrate and function to link the ECM to the plasma membrane and intracellular components of the cytoskeleton (Linder and Kopp, 2005; Zamir and Geiger, 2001). They appear as punctate structures of approximately 0.5μm, although their size, appearance and distribution vary even within a single cell. These structures are integrin-based and contain cytoskeletal-associated proteins, such as paxillin, talin, vinculin and FAK (Linder and Kopp, 2005; Zaidel-Bar et al., 2003; Zamir and Geiger, 2001; Zimerman et al., 2004). In sessile cells, focal complexes can mature into the more stable and robust focal adhesions, which include some of the same cytoskeletal-associated proteins (reviewed in Zaidel-Bar et al., 2003). Macrophages, however, tend to form the smaller focal complexes rather than focal adhesions in order to promote motility (Neumeister et al., 2003). Macrophages can also bind to ECM proteins via podosomes, which are integrin-rich ring-like structures of 0.5 to 1μm diameter that contain actin and many cytoskeletal-associated proteins (Buccione et al., 2004; Linder and Aepfelbacher, 2003; Linder and Kopp, 2005; Spinardi and Marchisio, 2006). In addition to cell adhesion, these structures are involved in matrix degradation and transcellular diapedesis.

Podosomes bare resemblance to invapodia found in v-Src-transformed fibroblasts (Marchisio et al., 1987; Tarone et al., 1985). These dynamic adhesion complexes undergo constant remodeling and reorganization of protein complexes. The main mechanisms that ensure the assembly and turnover of these complexes include proteolytic cleavage and phosphorylation.

Proteolytic cleavage of cytoskeletal and cytoskeletal-associated proteins promotes rapid adhesion complex turnover and is mediated by cellular proteases. Caspases are cysteine-aspartic acid proteases, which are initiators and effectors of the apoptotic process. The maintenance of cellular adhesion is essential for cell survival and, inversely, triggering of apoptosis leads to a loss in adhesion. FAK and paxillin are involved in both processes as they have been shown to mediate signals of cell survival downstream of cellular adhesion, and are both caspase substrates (Chay et al., 2002; Gervais et al., 1998; Shim et al., 2001). During apoptosis, proteolysis of FAK and paxillin by caspases leads to focal adhesion disassembly, cell rounding and detachment (Chay et al., 2002; Levkau et al., 1998; Shim et al., 2001). Calpains are calcium-dependent cysteine proteases that regulate adhesion turnover through cleavage of cytoskeletal and cytoskeletal-associated proteins. For example, calpain cleavage of FAK, Pyk2 and paxillin has been demonstrated in multiple cell types and has been linked to focal adhesion and podosome turnover (Carragher et al., 2001; Carragher et al., 1999; Cooray et al., 1996; Raja et al., 1997).

Adhesion is critically dependent on phopshorylation events that regulate protein interactions and activity. A large number of studies have indeed shown

that both protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) regulate phosphorylation at sites of adhesion in a coordinated and tightly controlled fashion. These enzymes act at different steps of the adhesion process: 1) Upstream of adhesion complex assembly via regulation of signalling, 2) Proximally to integrin engagement assembly and 3) Downstream of adhesion complex signalling. The regulation of these pathways can influence many aspects of adhesion, such as adhesion assembly and disassembly, as well as the rate of adhesion complex turnover. Although the roles of many PTKs have been defined in macrophages, the role and contribution of PTPs in macrophage adhesion and motility, however, remains largely unknown.

1.4 Signalling components of macrophage function

1.4.1 Src Family Kinases

The Src-family kinases represents the largest group of cytoplasmic PTKs as it contains eight known members: Src, Lyn, Fyn, Hck, Lck, Fgr, Blk and Yes (Korade-Mirnics and Corey, 2000; Roskoski, 2005). They regulate many pathways involved in cell differentiation, survival and adhesion. Abnormal function of SFKs has been associated with cell transformation, tumorigenesis and metastasis (Summy and Gallick, 2003). Hck, Fgr and Lyn are highly expressed in macrophages and their roles in macrophage adhesion and phagocytosis are briefly outlined in Table 1 (Yamanashi et al., 1989; Yi and Willman, 1989; Ziegler et al., 1988). Studies with Hck/Fgr/Lyn triple KO macrophages have underlined the importance of these kinases in macrophage spreading and morphology; these

macrophages displayed defective adhesion and rounded morphology on fibronectin (FN)-coated surfaces *in vitro* and impaired migration upon intraperitoneal injection of thioglycollate *in vivo* (Meng and Lowell, 1998). Further dissection of the roles of these SFKs, however, has shown opposing effects of these kinases. For example, Hck/Fgr double KO macrophages fail in adhesion structure formation and proper polarization during migration whereas Lyn KO neutrophils are hyperadhesive (Pereira and Lowell, 2003; Suen et al., 1999). In addition, SFKs are involved in signalling pathways downstream of many PRRs. For example, Hck, Fgr and Lyn are involved in FcγR-mediated macrophage responses as triple KO macrophages showed reduced FcγR-mediated phagocytosis, respiratory burst, actin cup formation and tyrosine phosphorylation events (Fitzer-Attas et al., 2000; Meng and Lowell, 1997). SFKs are also involved in cytokine production downstream of TLRs as chemical inhibition of SFKs or knock-down of Hck in human macrophages suppresses TLR-triggered cytokine production (Smolinska et al., 2008; Smolinska et al., 2011). SFKs are dispensible, however, for certain PRR-induced responses such as C3-opsonized phagocytosis of zymosan and TLR-dependent NF-kB activation or MAPK signalling (Fitzer-Attas et al., 2000; Meng and Lowell, 1997; Smolinska et al., 2011). In all, SFKs are involved in a variety of macrophage functions including adhesion and response to microbes, albeit the effects of different SFKs can be opposing.

The contribution of SFKs is dictated by their capacity to regulate signalling through receptors, such as immunoreceptors and integrins, or

cytoplasmic proteins (reviewed in Berton et al., 2005). SFK-dependent phosphorylation of ITAM or ITIM motifs, either contained within the cytoplasmic portion of immune receptors or of a receptor-associated signalling adaptor, is triggered upon ligand binding to the receptor. In general, phosphorylation of ITAM motifs leads to the recruitment and activation of Syk kinase, which subsequently triggers the activation of multiple signalling pathways, whereas phosphorylation of ITIM motifs leads to the recruitment of the phosphatases SHP-1 or SHP-2, which dampens tyrosine kinase signalling cascades. For example, Lyn has been shown to trigger inhibitory signalling downstream of many ITIM-containing receptors (Pereira and Lowell, 2003).

SFKs have also been shown to regulate non-ITAM- or –ITIM-containing receptors in macrophages, such as integrins (Berton et al., 2005; Miranti and Brugge, 2002; Suen et al., 1999). Reminiscent of what happens with immunoreceptors, SFKs and Syk are recruited to the cytoplasmic portion of integrins where Syk is activated in an SFK-dependent manner (Arias-Salgado et al., 2003). In addition, SFKs are responsible for the phosphorylation of many other cytoplasmic proteins, including adaptor molecules (e.g. Cbl), cytoskeletal-associated proteins (e.g. Paxillin, talin, vinculin), kinases [e.g. FAK, Pyk2, phospholipase C-γ (PLC-γ), MAPK] and DNA binding proteins (e.g. STAT) downstream of a variety of receptors (Dikic et al., 1996; Korade-Mirnics and Corey, 2000; Roskoski, 2005). For example, SFKs Hck and Fgr are necessary for the phosphorylation of paxillin and Pyk2 upon integrin stimulation, as well as for the normal localization of paxillin during macrophage adhesion (Suen et al., 1999).

SFKs possess domains termed Src homology (SH) domains, which can also be found in other PTKs. The Src homology 1 (SH1) domain is responsible for catalytic activity while the Src homology 2 (SH2) and Src homology 3 (SH3) domains promote protein-protein interactions (Bolen and Brugge, 1997; Pawson and Gish, 1992). The SH2 domain, which consists of approximately 100 amino acids, binds to phosphotyrosine-containing peptide motifs. The SH3 domain is smaller (40 to 70 amino acid residues) and allows for binding to proline-rich peptide sequences of a target protein.

The kinase activity of SFKs is regulated through two highly conserved key tyrosine sites: the positive autophosphorylation regulatory site (Y416 on Src) and the negative phosphorylation regulatory site (Y527 on Src) (Roskoski, 2005). The positive regulatory site is found in the activation loop of the kinase domain and its intermolecular autophosphorylation is necessary for catalytic activity. The negative regulatory site is phosphorylated by carboxy-terminal Src kinase (Csk), which is ubiquitously expressed, or Csk homology kinase [Chk; also known as megakaryocyte-associated kinase (MATK)], which is expressed in hematopoietic and neuronal cells, and results in decreased kinase activity (Avraham et al., 1995a; Davidson et al., 1997; Hirao et al., 1997; Okada and Nakagawa, 1989). Therefore, dephosphorylation of the negative regulatory site is necessary to achieve optimal activity. This can be mediated by a number of phosphatases, such as protein tyrosine phosphatase 1B (PTP1B), which is ubiquitously expressed, and CD45, which is expressed in all hematopoietic cells with the exception of red blood cells and their immediate progenitors (Roskoski, 2005).

CD45 can dephosphorylate both of the regulatory tyrosine sites. In accordance, the absence of CD45 in T-cells has been shown to lead to hyperphosphorylation of Lck at both the negative and positive regulatory sites (D'Oro and Ashwell, 1999; Mustelin and Altman, 1989; Ostergaard et al., 1989). In macrophages, the absence of CD45 also leads to the hyperphosphorylation of Hck and Lyn, but not Fgr (Roach et al., 1997). The hyperphosphorylation of these kinases can lead to either a reduction or an increase in kinase activity depending on the cell type; this is thought to be dependent on the balance between the phosphorylation of these sites by CD45 (D'Oro and Ashwell, 1999; and reviewed in Hermiston et al., 2003). Thus, SFK activity in immune cells is intricately linked to CD45.

1.4.2 Focal Adhesion Kinase Family

FAK and Pyk2 (also known as RAFTK, CAK-β, CADTK and FAK2) are the two members of the focal adhesion kinase family. This family of kinases is involved in integrin-mediated cell adhesion and motility (Avraham et al., 2000; Schlaepfer et al., 1999). FAK is ubiquitously expressed whereas Pyk2 expression is restricted mostly to hematopoietic and neuronal cells. They share approximately 45% amino acid identity and 65% similarity (Avraham et al., 1995b; Herzog et al., 1996; Lev et al., 1995; Sasaki et al., 1995; Yu et al., 1996). These proteins have similar domain structure with an N-terminal FERM (Protein 4.1, Ezrin, Radixin, Moesin) domain, a centrally located kinase domain, two proline-rich regions in the C-terminus, and a FAT (focal adhesion targeting) domain within the extreme C-terminus (Figure 1.3) (Avraham et al., 2000;

Ostergaard and Lysechko, 2005; Schlaepfer et al., 1999). The FERM domain is involved in the regulation of Pyk2 and FAK activity (Cooper et al., 2003; Lietha et al., 2007; Riggs et al., 2011). The proline-rich regions provide docking sites for SH3-containing proteins such as $p130^{Cas}$, a protein involved in motility, proliferation and survival (Avraham et al., 2000). The FAT domain is necessary for interaction with the cytoskeletal-associated protein paxillin (Lulo et al., 2009; Shen and Schaller, 1999).

Alternative RNA splicing of Pyk2 and FAK gives rise to multiple isoforms of these proteins. The deletion of a 42 a.a. sequence in the proline-rich region of Pyk2 by alternative splicing gives rise to Pyk2-H, an isoform expressed in hematopoietic cells (Dikic et al., 1998; Li et al., 1998; Xiong et al., 1998). The reason why certain cell types express one form or the other, or both, is not currently known. Pyk2-H possesses similar catalytic activity to full-length Pyk2 and is also capable of association with paxillin (Dikic, Dikic et al. 1998). Both Pyk2 and FAK possess non-catalytic isoforms that constitute the C-terminal portion of the full-length version, which are termed Pyk2-related non- kinase (PRNK) and FAK-related non-kinase (FRNK), respectively (Schaller et al., 1993; Xiong et al., 1998). Both these isoforms are targeted to focal adhesions and are thought to function as endogenous dominant negatives inhibitors of their full-length counterparts by competing for binding partners (Schaller et al., 1993; Xiong et al., 1998). However, expression of FRNK is limited, mostly in smooth muscle cells, and PRNK protein expression remains to be confirmed (Nolan et al., 1999; Taylor et al., 2001; Xiong et al., 1998).

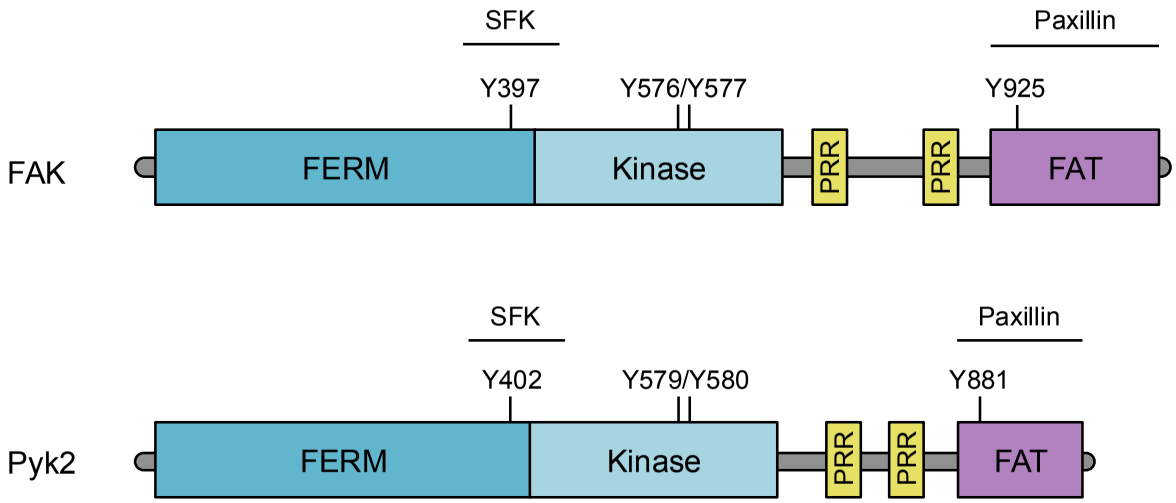

Figure 1.3. Schematic representation of the structure of focal adhesion kinase family members FAK and Pyk2. The N-terminal domain of FAK and Pyk2 is comprised of the FERM domain, which regulates kinase activity. This domain also contains the autophosphorylation site (Tyrosine 397 in FAK and Tyrosine 402 in Pyk2), which provides a docking site for SFKs. Both proteins possess a centrally located kinase domain which requires phosphorylation at Tyrosines 576 and 577 in FAK and Tyrosines 579 and 580 in Pyk2 by Src family kinases. The C-terminus of both proteins contains proline-rich regions (PRR), which can mediate interaction with SH3-containing proteins, and the FAT domain, which is responsible for localization of these proteins to focal complexes and allows for interaction with paxillin.

Pyk2 and FAK have four conserved tyrosine residues that can become phosphorylated upon activation (Figure 1.3). Tyrosine 402 of Pyk2 (Y397 in FAK) is located in the linker between the FERM and kinase domains and serves as an autophosphorylation site (Dikic et al., 1996; Felsch et al., 1998; Li et al., 1999; Park et al., 2004; Schlaepfer et al., 1999). Two tyrosine residues, Y579/Y580 in Pyk2 (Y576/Y577 in FAK), are located within the activation loop of the catalytic domain and function to enhance catalytic activity upon phosphorylation (Li et al., 1999; Park et al., 2004; Schlaepfer et al., 1999). The fourth tyrosine residue, Y881 in Pyk2 and Y925 in FAK, is located in the C-terminus and has been implicated in recruiting the adaptor protein Grb2 upon phosphorylation (Felsch et al., 1998; Schlaepfer et al., 1999). The current model for activation of Pyk2 and FAK is that upon stimulation it first becomes autophosphorylated, providing a docking site for SFK (Avraham et al., 2000; Dikic et al., 1996; Felsch et al., 1998). The recruited SFK then phosphorylates additional tyrosine residues within Pyk2 and FAK, thus enhancing catalytic activity and providing new docking sites for SH2 domain-containing proteins (Ostergaard and Lysechko, 2005; Park et al., 2004; Schlaepfer et al., 1999).

Pyk2 is highly expressed in macrophages and is essential for their function: it is involved in macrophage adhesion, migration and polarization in response to integrin engagement or microbial stimuli (Duong and Rodan, 2000; Hatch et al., 1998). The importance of Pyk2 in macrophage adhesion has been highlighted by the use of Pyk2 KO mice. Macrophages isolated from these mice were unable to polarize and migrate during chemotaxis *in vitro*, showed defects in the contractile

capacity of lamellipodia, had impaired F-actin localization and displayed decreased integrin-activated phosphoinositide 3-kinase (PI3K) activity (Okigaki et al., 2003). These macrophages were also unable to migrate towards inflammatory sites *in vivo*. Pyk2 has also been shown to localize to macrophage podosomes, where is co-localizes with the $\alpha_M\beta_2$ integrin, as well as the cytoskeletal-associated proteins paxillin, vinculin and talin, and is phosphorylated upon $\alpha_M\beta_2$ engagement (Duong and Rodan, 2000). Pyk2 is also involved in TLR-dependent signalling: it is phosphorylated downstream of LPS and its direct interaction with the TLR signal adaptor MyD88 increases NF-kB activation and cytokine secretion (Hatch et al., 1998; Hazeki et al., 2003; Xi et al., 2010).

FAK expression in macrophages is variable: it is undetected in M-CSF-treated human macrophages but is present in murine bone-marrow-derived macrophages (BMDM) and murine macrophage cell lines (De Nichilo and Yamada, 1996; Kume et al., 1997; Rovida et al., 2005). The importance of FAK in macrophage function is thus difficult to assess and likely dependent on the macrophage source. One study demonstrated that human macrophages that lack FAK expression do not exhibit defects in focal complexes and FAK overexpression in these cells does not disturb morphology or adhesion (De Nichilo et al., 1999). Other studies have demonstrated a role for FAK in macrophage function. For example, expression of a dominant negative form of FAK or conditional deletion of FAK from murine macrophages leads to decreased adhesion, cell spreading and motility (Owen et al., 2007a; Rovida et al., 2005).

FAK has also been shown to be necessary for macrophage chemotaxis or motility upon TLR stimulation (Abshire et al., 2011; Maa et al., 2011).

The investigation of the roles of Pyk2 and FAK in macrophage function is further complicated by the possibility that these kinases might possess similar functions, as they share high sequence similarity and possess many of the same binding partners. Indeed, decreased expression of FAK or Pyk2 leads to reduced macrophage motility but the combined loss of both does not have a greater effect than the absence of either alone, indicating that they are likely to function in a similar fashion (Owen et al., 2007a). Conversely, another study has shown that FAK and Pyk2 mediate phagocytosis of the bacteria *Yersinia pseudotuberculosis* by two separate, non-redundant, pathways that are triggered by the stimulation of distinct integrins by individual bacterial proteins (Owen et al., 2007b). Therefore, it is possible that these kinases are activated by distinct stimuli but still result in a similar outcome in terms of macrophage function. The differences in functions of FAK and Pyk2 may also reside in how these kinases are regulated. Additional studies in the mechanisms that regulate the activation of these kinases in macrophages are required to dissect the precise role of each in macrophage signalling pathways and functions.

1.4.3 Paxillin

Paxillin was one of the first proteins shown to be part of the integrin adhesome (Turner et al., 1990). It is a 68 kDa protein that does not possess kinase activity, but acts as a scaffold for the coordination of protein signalling at sites of

adhesion (reviewed in Deakin and Turner, 2008). The structure of paxillin reflects this role, as it contains multiple protein interacting domains (Figure 1.4). The N-terminus of paxillin contains five leucine- and aspartate-rich motifs (termed LD1 through 5) that contain the consensus sequence LDXLLXXL, except for LD3 in which the LD is substituted by a valine and glutamic acid (VE) (reviewed in Tumbarello et al., 2002). These motifs are necessary for localization of paxillin to the actin cytoskeleton and the MTOC, as well as for association with multiple cytoskeletal-associated proteins such as FAK, Pyk2 and vinculin, and the actin-binding protein actopaxin (Lulo et al., 2009; Robertson and Ostergaard, 2011; Shen and Schaller, 1999; Tumbarello et al., 2002). A proline-rich region is found between LD1 and LD2 and is necessary for interaction with SH3 domain-containing proteins such as Src (Weng et al., 1993). The C-terminus of paxillin contains four lin-11, isl-1, mec-3 (LIM) domains, which are double zinc-finger motifs that mediate protein-protein interactions (ex. PTP-PEST). LIM2 and LIM3 have also been shown to be important for localization of paxillin to focal adhesions (Brown et al., 1996). Phosphorylation of paxillin at several key residues has been shown to regulate its function, its localization as well as its protein interactions. Phosphorylation at Y31 and Y118 by FAK following cell adhesion allows for interaction with SH2-containing proteins such as Crk and SFKs (Schaller and Parsons, 1995). Tyrosine phosphorylation of paxillin by FAK and/or Pyk2, as well as JNK-mediated phosphorylation of S178, has been shown to regulate cell motility in lymphoid and cancer cells

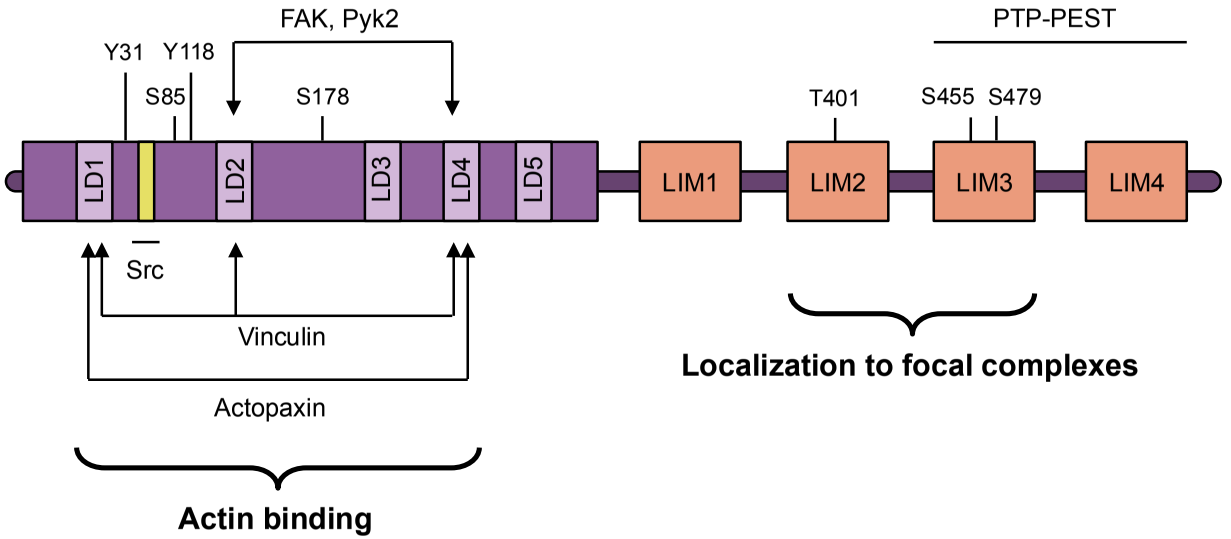

Figure 1.4. Schematic structure of the scaffold protein paxillin. The N-terminal portion of paxillin contains five LD domains, of which LD2 and 4 mediate interaction with FAK and Pyk2, LD1, 2 and 4 mediate interaction with vinculin and LD1 and 4 allow for interaction with actopaxin. As such, these domains are important for paxillin association with the actin cytoskeleton. A proline-rich region (in yellow) is also contained within the N-terminal half and allows for binding of SH3-containing proteins such as Src. The C-terminal portion of paxillin is comprised of four LIM domains, which target paxillin to focal complexes and allow for interaction with PTP-PEST. Paxillin is tyrosine phosphorylated at residues 31 and 118, which allow for interaction with SH2-containing proteins such as SFKs. Serine 85 is phosphorylated by MAPK regulates interaction with Pyk2. Serine 178 is phosphorylated by JNK and regulates cell motility. Several serine and threonine residues are phosphorylated in the LIM domains and regulate paxillin localization at focal adhesions.

(Huang et al., 2003; Huang et al., 2008; Li and Earp, 1997; Petit et al., 2000; Romanova et al., 2004). Phosphorylation of S85 by MAPK has been shown to regulate interaction with Pyk2, as phosphorylation at this site reduces Pyk2 binding in neuronal cells (Huang et al., 2004a). Phosphorylation of serine/threonine residues in LIM2 (T401) and LIM3 (S455, S479) regulate paxillin localization to focal adhesions (Brown et al., 1998). In all, the many binding domains and phosphorylation sites contained in paxillin allow for tightly coordinated signalling events downstream of cellular adhesion. Although some of these protein interactions and phosphorylation events have been characterized, the paxillin domains involved in the interaction with many other proteins, and the function of additional phosphorylation sites remain to be determined.

As the role of paxillin in cell adhesion and motility has been established in a variety of cell types, it is not surprising that it has similar functions in macrophages. For example, paxillin has been shown to localize to focal contacts in macrophages (Allen et al., 1997; De Nichilo and Yamada, 1996). It is also serine/threonine-phosphorylated downstream of integrin-mediated adhesion of macrophages (De Nichilo and Yamada, 1996; Hirano and Kanno, 1999). Thus, even though the role of paxillin in macrophage adhesion can be predicted through the study of other cell types and subsequently confirmed in these cells, paxillin interaction with hematopoietic-specific cytoskeletal-associated proteins requires further investigation. For example, as paxillin is known to associate with Pyk2, and Pyk2 is also involved in macrophage adhesion, it would be of interest to

determine whether these proteins interact at focal complexes of macrophages to regulate adhesion and motility; however, this has not yet been examined.

In addition to macrophage adhesion, paxillin has been shown to be involved in signalling pathways related to macrophage responses to microbes. Indeed, paxillin is phosphorylated downstream of FcRs and CR3 during phagocytosis (Allen and Aderem, 1996; Greenberg et al., 1994). Its distribution during both types of phagocytosis, however, is distinct as it is contained in punctate foci rich in actin and vinculin below the phagosome during CR3-mediated phagocytosis while it is uniformly distributed during FcR-mediated phagocytosis. Moreover, FcR-mediated phagocytosis, but not CR3-mediated phagocytosis, is depedent on tyrosine kinases. Interestingly, Pyk2 and Hck are also phosphorylated downstream of FcR-mediated phagocytosis (Kedzierska et al., 2001). Paxillin is also tyrosine phosphorylated alongside Pyk2, downstream of TLR-stimulation of monocytes and macrophages (Achuthan et al., 2006; Hazeki et al., 2003; Kleveta et al., 2012; Williams and Ridley, 2000). These events are correlated with rapid induction of cell adhesion and spreading. Moreover, paxillin is required for macrophage chemotaxis towards CSF-1 (Abshire et al., 2011). Thus, paxillin may function to link macrophage responses to microbes to modulation of the cytoskeleton; however, how paxillin functions to tie these pathways has not been fully elucidated.

1.4.4 CD45

CD45 (also named common leukocyte antigen and protein tyrosine phosphatase, receptor type, C or PTPRC) is a receptor PTP expressed on the surface of almost all hematopoietic cells (reviewed in Hermiston et al., 2003; and Saunders and Johnson, 2010). It is highly expressed on these cells and can make up to about 10% of total cell surface proteins. The extracellular domain comprised of three fibronectin type III (FNIII) repeats and a cysteine-rich domain that are extensively N-linked glycosylated, as well as three alternatively spliced exons (A, B and C) in the N-terminus that encode multiple O-linked glycosylation sites (Figure 1.5). Alternative splicing of these exons can theoretically give rise to eight isoforms, five of which have been confirmed at the protein level (McNeill et al., 2004); the smallest isoform of CD45 (i.e. CD45RO) lacks the A, B and C exons whereas the largest isoform (i.e. CD45RABC) contains all three exons. Consequently, the molecular weight of CD45, in addition to its overall glycosylation and negative charge, significantly varies (180-240 kDa). The expression of the different CD45 isoforms, as well as their level of expression, is dependent on cell type, maturation stage and activation status (reviewed in Trowbridge and Thomas, 1994). For example, the monocyte precursor expresses different CD45 isoforms (i.e. CD45RA, CD45RB) than resting monocytes and macrophages (CD45RO), and isoform expression further changes upon activation of these cells (CD45RO, CD45RA). The significance of this change in expression is not known but is conserved between species.

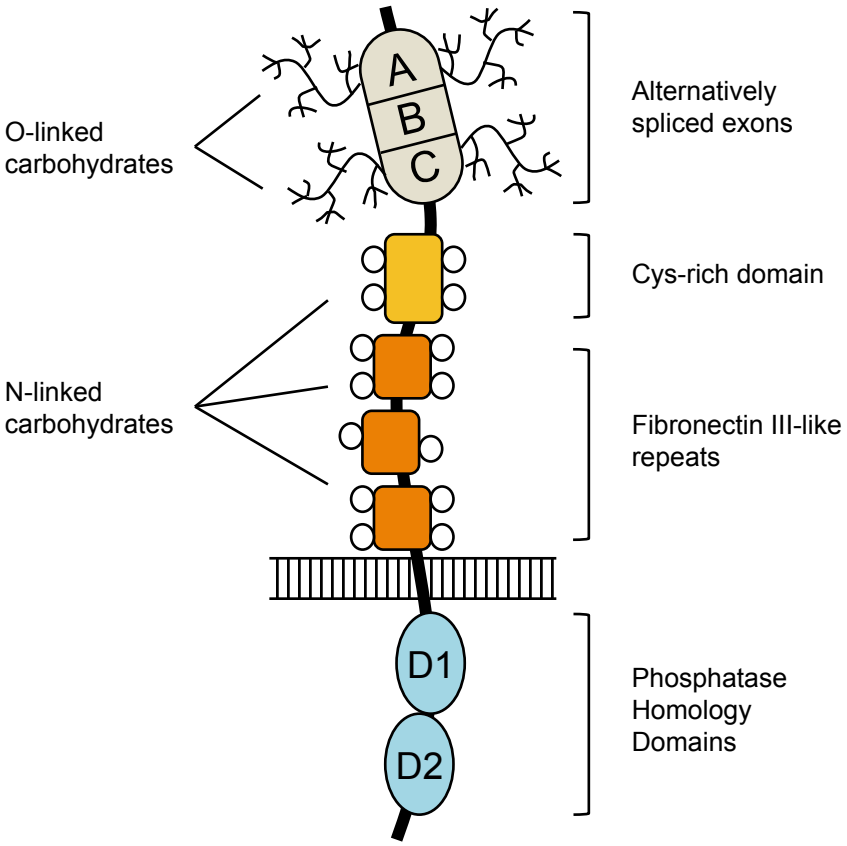

Figure 1.5. Schematic presentation of the structure of CD45. The external portion of CD45 contains three fibronectin type III domains and a cysteine-rich domain which allow for N-linked glycosylation. The differential splicing of exons encoding for the membrane-distal part of the external domain gives rise to up to eight isoforms of which the structure of CD45RABC is depicted here. CD45 contains only one transmembrane domain. The cytoplasmic portion of CD45 contains two PTP domains (D1 and D2), of which only the membrane proximal one (D1) is enzymatically active.

The cytoplasmic domain of CD45 contains two PTP homology domains named domain 1 (D1) and domain 2 (D2), of which only D1 possesses catalytic activity (Figure 1.5). As mentioned previously, SFKs are known substrates for CD45, and both the negative and positive regulatory sites of SFKs can be dephosphorylated by CD45. The regulation of Lck by CD45 has been extensively described (Saunders and Johnson, 2010). In macrophages, Hck and Lyn, but not Fgr, have been shown to be regulated by CD45, therefore highlighting preference of CD45 for certain SFK substrates (Roach et al., 1997). The Janus kinase (JAK) family has also been shown to be substrates for CD45 (Irie-Sasaki et al., 2001). This is supported by *in vitro* studies in which recombinant CD45 could dephosphorylate JAK2 (Fleming et al., 2004; Irie-Sasaki et al., 2001; Yamada et al., 2002). Accordingly, the absence of CD45 in T-cells and B-cells leads to hyperphosphorylation of these kinases (Fleming et al., 2004; Irie-Sasaki et al., 2001; Yamada et al., 2002). Identification of other substrates for CD45 has been difficult as many of the hyperphosphorylated proteins in CD45-deficient cells are regulated by SFKs, therefore the regulation of these proteins by CD45 may be indirect and need to be confirmed by *in vitro* studies.

Many models have been proposed for the regulation of CD45 activity and have been reviewed extensively in Hermiston, Xu and Weiss (2003) and Saunders and Johnson (2010). The main models propose that CD45 activity is regulated by ligands, expression levels or its subcellular localization. The regulation of CD45 by ligands is supported by the fact that binding of antibodies to distinct parts of the extracellular domain of CD45 induce changes in CD45 activity (Gruber et al.,

1992; Hamann et al., 1996; Liles et al., 1995; Lorenz et al., 1993; Pfau et al., 2000; Yu et al., 2002). No specific ligand for CD45, however, has been identified; all of the ligands identified up to date interact with CD45 through lectin binding of CD45 carbohydrates. These include Galectin-1 and -3, glucosidase II, MR and macrophage galactose lectin (Baldwin et al., 2000; Martinez-Pomares et al., 2006; Perillo et al., 1995; Stillman et al., 2006; van Vliet et al., 2006; Walzel et al., 1999). The functional significance of these interactions has still not been elucidated although some groups have proposed that these interactions may have a role in apoptosis of lymphocytes or stimulation of T-cells (Perillo et al., 1995; Stillman et al., 2006; van Vliet et al., 2006).

The level of expression of CD45 on the surface of cells has also been proposed as a mechanism to regulate CD45 activity. This model is supported by a study by McNeill et al (2007) that demonstrated that expression of low levels of CD45 in CD45-deficient T-cells decreased TCR signalling whereas intermediate levels of CD45 led to a hyperactivation of T-cells (McNeill et al., 2007). The regulation of CD45 activity through its localization is supported by the fact that CD45 is excluded from sites of active signalling. For example, upon T-cell engagement with APCs, CD45 is initially found at the immunological synapse where it can prime SFKs but is then rapidly excluded where it could inhibit integrin activation and ITAM signalling (Freiberg et al., 2002; Johnson et al., 2000). It is finally later recruited as a possible means to downregulate signalling. Similarly, CD45 is excluded at the phagocytic synapse on macrophages during Dectin-1-mediated phagocytosis of fungal particles to allow for productive

signalling (Goodridge et al., 2011). All things considered, the precise mechanism(s) by which CD45 activity is regulated remains largely hypothetical and needs to be further investigated.

The function of CD45 in T- and B-cells has been extensively studied and these studies have revealed a major role for CD45 in the regulation of signalling in these cells. For example, through the use of CD45-deficient mice, CD45 has been found to regulate B- and T-cell receptor signalling (Justement et al., 1991; Koretzky et al., 1991; Weaver et al., 1991). Moreover, CD45-deficient mice show impaired T-cell thymic development and defects in B-cell proliferation (Kishihara et al., 1993). A role for CD45 in regulating T-cell adhesion has also been demonstrated (Li et al., 2001; Wong et al., 2011). It is also necessary for cytokine and chemokine secretion by NK cells stimulated through the C-type lectin receptor Ly49D (Hesslein et al., 2006; Huntington et al., 2005; Mason et al., 2006). Cross-linking of CD45 on the surface of neutrophils has implicatied it in the regulation of FcγRIIIb signal transduction, cytokine secretion and expression of CR3 (Hoffmeyer et al., 1995; Yu et al., 2002). Through the few studies that have investigated the role of CD45 in macrophages, CD45 has been shown to regulate their adhesion as well as their responses to PAMPs. One study has shown that CD45-deficient macrophages are unable to maintain integrin-mediated attachment (Roach et al., 1997). Cross-linking of CD45 on macrophages has also highlighted its role in the regulation of arachidonate acid release, respiratory burst and cytokine production upon stimulation with LPS (Gruber et al., 1992; Liles et al., 1995; Pfau et al., 2000). The molecular mechanisms downstream of CD45 in

these processes, however, remain undefined. Additional studies will be required to establish a clear role for CD45 in macrophages as well as to identify the signalling pathways controlled by CD45 necessary for macrophage function.

Several cytoskeletal-associated proteins have been shown to be regulated by CD45, such as Pyk2, FAK and paxillin (Fernandis et al., 2003; Li et al., 2001; Wong et al., 2011). For example, CD45-deficient T-cells displayed increased tyrosine phosphorylation of Pyk2 upon CD44-mediated cell spreading (Li et al., 2001; Wong et al., 2011). Moreover, phosphorylation of Pyk2 in these cells was dependent on SFKs and actin polymerization. On the other hand, a study by Fernandis et al. (2003) found that Pyk2, FAK and paxillin phosphorylation was decreased in CD45-deficient T-cells during chemotaxis compared to CD45 positive cells. These studies suggest that regulation of cytoskeletal-associated proteins by CD45 may vary depending on the stimuli. Further investigation is needed in order to determine whether CD45 regulates these proteins in macrophages and whether this regulation accounts for the observed differences in adhesion between CD45-deficient and WT macrophages.

A number of T- or B-cell-associated diseases have been linked with CD45 disregulation. For example, mutations leading to CD45 inactivation have been observed in patients with T-cell acute lymphoblastic leukemia and this correlates with increased activity of JAK and Lck and underlines the role of CD45 as a tumor suppressor (Porcu et al., 2012). Abnormal expression of CD45 isoforms by T-cells has been observed in patients with rheumatoid arthritis and adult T-cell leukemia (Mamoune et al., 2000; Suzuki et al., 1998). Altered localization of

CD45 during BCR signalling in B-cells from patients with systemic lupus erythematosus was demonstrated and may explain the defective intracellular signalling, hyperactivity and autoantibody production observed in the course of this disease (Flores-Borja et al., 2007). Increased expression of CD45 by microglia has been observed in patients with Alzheimer's disease and in transgenic mouse models of Alzheimer's (Maier et al., 2008; Masliah et al., 1991; Wilcock et al., 2001). In this context, CD45 increased expression is favourable as it functions to downregulate the production of proinflammatory cytokines that contribute to the disease (Tan et al., 2000a; Tan et al., 2000b). Ultimately, CD45 presents an interesting therapeutic target for many diseases and additional investigation of its role in cell types such as macrophages may present additional therapeutic opportunities in diseases associated with defective macrophage responses.

1.5 Study objectives

In this study, I investigated the roles of signalling proteins involved in the regulation of macrophage adhesion, motility and function. More specifically, I addressed the following questions:

i. *How are the intracellular distribution of Pyk2 and its association with paxillin regulated in macrophages?*
ii. *By which mechanisms does CD45 regulate macrophage adhesion, morphology and motility?*
iii. *Is CD45 involved in macrophage responses to fungal stimuli?*

1.6 Hypotheses

As CD45 is a key regulator of SFK activity (Roskoski, 2005), it is reasonable to predict that the pathways leading to CD45-mediated regulation of macrophage adhesion may involve proteins controlled by SFKs. The tyrosine kinase Pyk2 requires SFKs for activation and is involved in macrophage adhesion, migration and polarization (Duong and Rodan, 2000; Okigaki et al., 2003). Paxillin is a scaffold protein that coordinates cell signalling at focal complexes and is phosphorylated by Pyk2 and SFKs (Deakin and Turner, 2008). In T-cells, tyrosine phosphorylation of both these proteins has been shown to be disregulated in the absence of CD45 (Fernandis et al., 2003; Wong et al., 2011). Therefore, I hypothesize that these cytoskeletal-associated associated proteins are downstream of CD45 in macrophages. Moreover, Pyk2 and paxillin have been shown to associate at specific intracellular sites to regulate cell adhesion (Duong and Rodan, 2000; Pixley et al., 2001). I further hypothesize that their association is tightly regulated in macrophages, possibly through post-translational modifications. Finally, as SFKs regulate many macrophage responses to PAMPs (Fitzer-Attas et al., 2000; Meng and Lowell, 1997), I expect CD45 to regulate certain aspects of the macrophage response to stimuli such as the fungal stimuli zymosan.

CHAPTER TWO: MATERIAL AND METHODS

2.1 Antibodies

The generation of the F245 and F298 polyclonal antibodies to Pyk2 have been described previously (Berg and Ostergaard, 1997; Ma et al., 1997). The monoclonal antibody to phosphotyrosine was purified from the PY72.10.5 hybridoma and is described elsewhere (Ostergaard et al, 1998). Antibodies to paxillin (clone 349, clone 165), Pyk2/CAKβ (clone 11) and dynamin were obtained from BD Biosciences (Mississauga, ON). Anti-actin was purchased from Sigma-Aldrich (Mississauga, ON). Anti-Erk antibody was purchased from Invitrogen (Camarillo, CA). Anti-calpain and anti-SUMO-1 antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-active caspase-3, anti-Syk and antibodies to phosphorylated Syk were purchased from Cell Signaling Technology (Danvas, MA). Anti-GAPDH was obtained from Meridian Life Science (Saco, ME). Anti-F4/80-PE, anti-F4/80-FITC, anti-CD69-PE and corresponding isotypes controls were bought from eBiosciences (San Diego, CA). Anti-CD11b-PE, anti-CD45-APC and matching fluorochrome-conjugated isotype controls were acquired from BD Biosciences (Mississauga, ON). FITC-conjugated anti-CD40 was purchased from BioLegend (San Diego, CA). AlexaFluor 647-conjugated anti-Dectin-1, matching isotype control and unconjugated anti-Dectin-2 were purchased from AbCam (Cambridge, MA). Rabbit anti-mouse antibody, anti-mouse IgG-HRP, FITC-conjugated anti-rat and Rhodamin-conjugated goat anti-mouse were procured from Jackson

ImmunoResearch Laboratories (West Grove, PA). Donkey anti-rabbit conjugated to AlexaFluor 488 was purchased from Invitrogen (Camarillo, CA).

2.2 Reagents

Protein A sepharose beads were acquired from Amersham Biosciences (Piscataway, NJ). Phalloidin conjugated to fluorescein was bought from Invitrogen (Burlington, ON). Annexin V conjugated to AlexaFluor 647 was purchased from eBiosciences (San Diego, CA). Protein A conjugated to HRP was obtained from Pierce (Rockford, IL). U73122, U73433 and Cl-II were purchased from Calbiochem (San Diego, CA). Staurosporine, ALLN, lidocain, thioglycollate and N-ethylmalemeide (NEM) were obtained from Sigma-Aldrich (Mississauga, ON). The FAK and Pyk2 inhibitor PF431396 was purchased from Symansis (Shanghai, China). FITC-conjugated zymosan and unlabelled zymosan were purchased in its reconstituted form from Invitrogen (Camarillo, CA). Pam_2Csk_4 was purchased from InvivoGen (San Diego, CA). Murine GM-CSF was purchased from Peprotech (Rocky Hill, NJ). FBS was acquired from PAA Laboratories (Etobicoke, ON). The protease inhibitor cocktail tablets were acquired from Roche (Indianapolis, IN). ECM-coated plates were obtained from BD Biosciences (Mississauga, ON). ELISA kits for the detection of TNF-α and IL-10 were purchased from eBiosciences (San Diego, CA). The B-cell magnetic cell separation kit was obtained from Stem Cell Technologies (Vancouver, BC).

2.3 Preparation of PAMPs

Preparation of zymosan prior to cell stimulation was done either by sonication of zymosan purchased in suspension (Invitrogen, Camarillo, CA) or by reconstitution of zymosan powder (Sigma-Aldrich, Mississauga, ON) in PBS followed by sonication for 30 seconds. Oxidized zymosan was prepared as described by Hida et al. (2006). Briefly, reconstituted zymosan was incubated overnight at 4°C followed by centrifugation for 5 minutes at 5000 x g. The supernatant was aspirated and the insoluble fraction was suspended in 1 ml of PBS and sonicated for 30 seconds. LPS and Pam_2Csk_4 were reconstituted in DMSO.

2.4 Cell lines

The RAW 264.7 macrophage cell line was purchased from Sigma-Aldrich (Mississauga, ON) and maintained in RPMI with 10% FCS and 100U/ml penicillin/streptomycin. NIH 3T3 cells were obtained from Dr. Jim C. Stone (University of Alberta, Edmonton, AB) and were maintained in DMEM with 8% FCS. The CHO cell line expressing GM-CSF was obtained from Dr. Kevin Kane (University of Alberta, Edmonton, AB).

2.5 Mice

B6.129ptprc$^{tm1\text{-}holmes}$ (CD45Δexon9, CD45-/-) (reviewed in Ehlers, 2000) and C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129ptprc$^{tm1\text{-}holmes}$ mice were crossed with C57BL/6J for several

generations and genotyped to derive a CD45 KO colony and a CD45 WT colony. Mice were bred and housed in viral-antigen-free mouse facilities (Heath Sciences Lab Animal Services, University of Alberta).

2.6 Splenocyte isolation

Spleens were isolated from CD45 KO or WT 12 week-old female mice. Spleens were homogenized and cells were washed twice with RPMI. Erythrocytes were lysed by resuspending the cell pellet in a hypotonic solution of ammonium chloride for 5 minutes followed by a wash with RPMI supplemented with 10% FCS. Remaining splenocytes were counted and stained for flow cytometry analysis of F4/80 expression.

For isolation of splenic B-cells, spleens were isolated, homogenized and treated as above, followed by positive selection of B-cells using the EasySep magnet system. B-cells were then counted and lysed at 1 x 10^7 cells per ml in lysis buffer (1% NP-40, 10mM Tris, 5mM EDTA, 150mM NaCl, 1mM orthovanadate, protease inhibitor cocktail) for 20 minutes at 4°C. Lysates were then used for immunoprecipitation assays as described below.

2.7 Culture of bone-marrow-derived macrophages

Bone marrow was obtained by flushing tibiae and femurs of 12 to 15 week-old mice with PBS. Bone marrow precursor cells were cultured in bone marrow media (RPMI, 10% FCS, 2mM L-Glutamine, 100U/ml penicillin, 100U/ml streptomycin, 0.053mM β-mercaptoethanol) supplemented with 20% of filtered

culture medium from GM-CSF-producing CHO cells (gift from the Dr. K.P. Kane, University of Alberta, Edmonton, AB). Progenitor cells were plated at 2 x 10^7 cells per 20 cm diameter tissue-culture-treated dish or 4 x 10^6 cells per 10 cm diameter tissue-culture-treated dish in media. Fresh media was added at day 3 and cells were cultured for a total of 7 days. Cells were then used for experimentation or lysed in at 10^7 cells/ml of lysis buffer (1% NP-40, 10 mM Tris, 5 mM EDTA, 150 mM NaCl, 1 mM orthovanadate and protease inhibitors) for 20 minutes on ice followed by centrifugation at 13 000 x g for 3 minutes to pellet out the nuclei. Post-nuclear lysates were used for immunoprecipitation or loaded onto SDS-PAGE gels for subsequent Western blotting.

2.8 Isolation of thioglycollate-elicited peritoneal macrophages

An autoclaved solution of 2% thioglycollate was aged for at least three months before use. Two week-old mice were injected intraperitoneally with 1ml of thioglycollate. At day 4, mice were sacrificed and cells were harvested by intraperitoneum lavage. Cells were assessed for F4/80 expression by flow cytometry or lysed in lysis buffer for subsequent Western blotting.

2.9 Flow cytometry

For flow cytometry, 10^6 cells were resuspended in 100 μl of PBS containing 2% BSA and 1% FCS and incubated with the indicated antibodies. For assessment of macrophage marker expression, cells were incubated with fluorochrome-conjugated anti-F4/80, anti-CD11b or corresponding isotype

controls. BMDM activation was evaluated by staining zymosan- or LPS-stimulated cells with fluorochrome-conjugated anti-CD69, anti-CD40 or corresponding isotype controls. For other antibodies, cells were incubated with fluorochrome-conjugated antibodies, unconjugated primary antibody followed by incubation with a fluorochrome-conjugated antibody or corresponding isotype controls. Cells were incubated 30 to 45 minutes at 4°C in the dark followed by three washes with washing buffer (PBS, 1% FCS). In the case of unconjugated antibodies, cells were incubated with a conjugated secondary antibody for an additional 30 to 45 minutes followed by three washes. After washes, cells were either fixed with 4% formaldehyde or resuspended in PBS and analysed using the FACS Calibur flow cytometry system (BD Biosciences). Phagocytosis of zymosan was measured by flow cytometry as follows: 10^6 BMDM were harvested and washed, then incubated 5, 15 or 30 minutes at 37°C with 5 x 10^6 FITC-conjugated zymosan particles in PBS. Phagocytosis was terminated by washing cells with PBS supplemented with 2mM EDTA and 4 mg/ml of lidocaine hydrochloride at the end of each time point. As controls, BMDM were incubated with fluorescein-conjugated zymosan particles for 30 minutes at 37°C in the presence of 2mM EDTA and 4mg/ml of lidocaine, or 30 minutes at 4°C in the absence of the inhibitor. Each flow cytometry experiment was executed a minimum of three times unless otherwise indicated in figure legend.

2.10 Adhesion assays and cell treatment

To assess cell spreading in culture, adhered macrophages from day 7 cultures were washed and pictures were taken from three independent cultures. To assess the effects of inhibitors on cell spreading, day 7 BMDM were washed in-plate and BMDM media was replaced serum-poor media (RPMI, 0.5% FCS) with the indicated amounts of inhibitors (U73122, ALLN, PF431396) or control (carrier or inert control analog). Cells were incubated for 4 hours at 37°C. Quantification of cell spreading was done using the ImageJ software version 1.43u (http://rsb.info.nih.gov.login.ezproxy.library.ualberta.ca/ij). Cells were then lysed directly on the plate with 1X RSB and loaded on 8.5% SDS-PAGE gel for subsequent Western Blotting. To assess the capacity of BMDM to maintain adhesion over 24h, cells were harvested, washed and 1 x 10^6 cells were replated in tissue-culture-treated or extracellular matrix-coated wells from 6 well plates in serum-poor media (RPMI, 0.5% FCS) for 24 hours at 37°C. After incubation, media and unadhered cells were aspirated and remaining adhered cells were directly lysed in 1X RSB and samples were boiled. As an input control, 1 x 10^6 cells were lysed directly with 1X RSB and boiled instead of replating into wells. Samples were loaded onto 8.5% SDS-PAGE gel for subsequent Western Blotting. Adhesion assays were done a minimum of three times for each treatment.

For BMDM stimulation with GM-CSF, cells were harvested and cells were replated in 12-well plates (10^6 cells/well) overnight in normal growth serum without GM-CSF. Media was replaced with 37°C RPMI containing 25ng/ml of recombinant GM-CSF (Peprotech, Rocky Hill, NJ) for the indicated amount of

time, at 37°C. After incubation, the supernatant was aspirated, cells were lysed directly in the wells with 1X RSB and lysates were boiled for 3 minutes.

For analysis of SUMOylated proteins, RAW 264.7 cells were treated for 10 mintues with 10mM of NEM in PBS on ice, followed by lysis in lysis buffer containing 10mM of NEM. Subsequent immunoprecipitations were also performed in the presence of NEM. SDS-PAGE and Western blot were done as described in 2.12.

For BMDM stimulation with PAMPs, cells were harvested, counted and incubated for various times with the indicated amounts of zymosan, LPS or Pam_2Csk_4. Cells were then washed and lysed in 1X RSB, followed by SDS-PAGE and Western Blot. For cytokine secretion analysis, supernatants were collected and analysed by ELISA as per the manufacturer's protocol.

2.11 Immunoprecipitation

For immunoprecipitation, cells were harvested, washed with PBS and lysed at 1 x 10^7 cells/ml in lysis buffer for 20 minutes at 4°C. Post-nuclear lysates of $1x10^7$ RAW 264.7, BMDM, B-cells or AB.1 T-cells were incubated with anti-Pyk2 antisera, anti-paxillin or other indicated antibody for 15 minutes, followed by incubation rabbit anti-mouse IgG for 15 minutes (if required) and Protein A Sepharose beads (30µl of 50% slurry) for 1.5 hours. Incubations were done at 4°C on a rotator. Beads were pelleted and washed three times with lysis buffer before resuspension in 1X RSB and boiled for 3 minutes. For sequential Pyk2 immunoprecipitates, post-nuclear lysates were subjected to immunoprecipitation

with the first Pyk2 antiserum (either F298 or F245) for 12 h at 4 °C at which time the beads were pelleted and saved as the first immunoprecipitate, and the supernatant was transferred to a new tube. Beads from the first immunoprecipitation were washed three times with lysis buffer, resuspended in 1X RSB, and boiled for 3 min. The supernatant from the first immunoprecipitation was then subjected to a second round of immunoprecpitation with the second Pyk2 antiserum (either F298 or F245) for 12 h at 4 °C. Beads were washed and resuspended as described above, and the procedure repeated as indicated.

2.12 Alkaline phosphatase treatment

For alkaline phosphatase treatment, Pyk2 immunoprecipitates from RAW 264.7 cells were prepared as described above. Beads were resuspended in 70 μl of water and 10 μl of 10X reaction buffer (provided with enzyme) was added along with 20 U of calf intestinal alkaline phosphatase (CIAP) or dilution buffer. Reactions were allowed to proceed at 37°C for 3 hours with occasional agitation. After 3 hours, beads were pelleted, washed twice with lysis buffer, resuspended in 1X RSB buffer, and boiled for 3 minutes.

2.13 SDS-PAGE and Western blotting

Total cell lysates or immunoprecipitates were loaded onto 8.5% SDS-PAGE gels followed by transfer to polyvinylidene difluoride (PVDF) membranes. Western blots were performed using the indicated primary and appropriate HRP-

coupled secondary antibodies and visualized by ECL (PerkinElmer Life Science Products, Boston, MA). When sequential Western blots were performed on the same membrane, the membrane was stripped in buffer containing β-mercaptoethanol, SDS and Tris-HCl (pH 6.7) at 56°C in between each blot. The order of the blots in the figures is representative of the order in which the membranes were probed with the indicated antibodies. Quantification of Western blot bands was performed using the ImageJ software, version 1.43u (http://rsb.info.nih.gov/ij/).

2.14 Transfection of Pyk2-H

Full-length Pyk2-H was amplified, ligated in the pC1-Neo vector and sequenced by Tara Lysechko. NIH 3T3 cells were mock transfected or transfected with 2 μg of Pyk2-H DNA using Effectene (QIAGEN, Mississauga, ON). Cells were lysed 48 hours later with 1% NP-40 lysis buffer as described above.

2.15 RT-PCR of Pyk2

RNA was extracted from RAW 264.7 cells lysed in Trizol reagent (Invitrogen, Carlsbad, CA). Pyk2 products were generated by direct amplification of mRNA with the Superscript III One-Step RT-PCR System containing a mixture of SuperScript III Reverse Transcriptase and Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). A negative control for contaminating genomic DNA was done by substituting the Reverse Transcriptase and Platinum *Taq* DNA

polymerase mixture by Taq DNA polymerase only (QIAGEN, Mississauga, ON). A positive control for Pyk2-H product amplification was done by replacing RAW 264.7 mRNA with DNA from the Pyk2-H/pC1-Neo plasmid. The following primers were used to amplify Pyk2 products containing the alternative splice region: 5'-CAA GAA AGG AAT GCT CGC TAC-3' (forward) and 5' TAT ATC TAG ATC ACT CTG CAG G 3' (Reverse). This resulted in a products corresponding either to the full-length Pyk2 (829 bp) or to the Pyk2-H isoform (703 bp). The RT-PCR products were then run on a 1% agarose gel in 1X TBE buffer and visualized using Ethidium Bromide under UV light.

2.16 Confocal microscopy and live cell imaging

For confocal microscopy of RAW 264.7 cells, cells were incubated overnight in media on sterile coverslips placed in 6-well plates to allow for adhesion. The coverslips were then washed once with PBS containing 1% FCS. The cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, and coverslips were washed twice with 1% FCS in PBS. The cells were permeabilized with 0.2% NP-40 in PBS for 5 minutes at room temperature and the coverslips were subsequently washed three times with 1% FCS in PBS. Cells were incubated in blocking buffer (1%FCS, 1% normal goat serum or 1% normal donkey serum in PBS) for 30 minutes at room temperature. Coverslips were then incubated with each primary and fluorochrome-conjugated secondary antibody diluted in 1% FCS in PBS for 45-60 minutes at room temperature in a dark chamber. The coverslips were washed three times with PBS for 5 minutes

after each antibody incubation. Coverslips were mounted on glass slides using mounting medium (PBS, 20% glycerol, 0.1% Mowiol, 0.1% propyl gallate). Samples were analyzed on a Zeiss LSM 710 confocal microscope with the 63X/1.40 oil objective (Imaging Centre, Cross Cancer Institute). Z-stacks and multiple sections of each cell were taken to confirm proper colocalization of proteins observed but only single sections are shown in results.

For staining of BMDM, cells were harvested, washed and adhered onto Poly-L-lysine-coated coverslips for 15 minutes at room temperature. The cells were then fixed, permeabilized and stained with primary and secondary antibodies as described above. For live cell imaging, day 7 BMDM were harvested, washed and incubated in wells for 2 hours prior to imaging to allow for adherence to coverslips. Cells were tracked for 30 to 45 minutes on a Olympus IX-81 Motorised microscope equipped with a 37°C, 5% CO_2 chamber (Cell Imaging Centre, Faculty of Medicine Core Imaging Facility). Cell movement was analysed using the ImageJ software (Manual Tracking and Chemotaxis Tool plug-ins for ImageJ).

2.17 Statistical analysis

Statistical analysis of results was done using a two-tailed Student *t* test with the Prism software (GraphPad Software Inc).

CHAPTER THREE: PYK2 EXISTS AS TWO BIOCHEMICAL AND SPATIALLY DISTINCT POPULATIONS IN MACROPHAGES

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3.1 Introduction

Tyrosine phosphorylation-mediated signalling is key in conveying intracellular signals triggered by such events as cell adhesion and migration. This mechanism of signal transduction is mediated by the activity of protein tyrosine kinases (PTKs). It is therefore not surprising to find many PTKs at sites of cellular adhesion, where integrin receptors relay information on the extracellular environment to the cell through the activity of these kinases. FAK, a ubiquitously expressed PTK, functions to synchronize these events at sites of adhesion (Schaller, 2010). Pyk2 is a non-receptor tyrosine kinase expressed in numerous cell types but highly expressed in hematopoietic and neuronal cells. It is not entirely clear how Pyk2 kinase activity is regulated. Structural studies have revealed that FAK undergoes autoinhibition by binding of the FERM domain to the kinase domain (Cooper et al., 2003; Lietha et al., 2007). The mechanism(s) that relieve the autoinhibition are not known but may include protein binding, phosphorylation or sumoylation (Kadare et al., 2003). Given the high degree of sequence similarity between FAK and Pyk2, it is likely that Pyk2 would be

[1] Authors contributed equally to this work
[2] Data presented in this chapter was generated by this author

similarly regulated, although strong support for this molecular inhibition is currently lacking. Further studies are thus required to understand the contribution, if any, of the FERM domain to Pyk2 regulation.

The regions of highest homology between FAK and Pyk2 are found within the kinase domain (~60%) and the FAT domain (~61%) (Avraham et al., 1995b; Herzog et al., 1996; Lev et al., 1995; Sasaki et al., 1995; Schaller and Sasaki, 1997). High homology within the FAT domain has led to speculation that Pyk2 could bind the cytoskeletal adapter protein paxillin as it had been shown that this region was responsible for FAK binding to paxillin (Hildebrand et al., 1995; Tachibana et al., 1995; Turner and Miller, 1994). Indeed, earlier immunoprecipitation studies have shown that paxillin constitutively interacts with Pyk2 (Hiregowdara et al., 1997; Li and Earp, 1997; Ostergaard et al., 1998; Salgia et al., 1996). The interaction was later confirmed to occur specifically within the FAT domain of Pyk2 as fusion proteins that were lacking part of the FAT domain failed to bind paxillin (Xiong et al., 1998). Resolution of the crystal structure of the FAT domain of Pyk2 has since shown that it shares a high degree of similarity to the FAK FAT domain and that it interacts with paxillin in a similar manner to FAK (Hayashi et al., 2002; Lulo et al., 2009). What regulates Pyk2 interaction with paxillin, as well as the cellular localization of this interaction remain, however, poorly defined.

In this chapter, I investigated the characteristics of Pyk2 in macrophages through the use of antibodies that are specific to two distinct regions of Pyk2. I found that such antibodies identify biochemically and spatially distinct Pyk2

populations in macrophages. One population of Pyk2 constitutively associated with paxillin while the other displayed hyperphosphorylation on serine and/or threonine residues. This suggests that Pyk2 is found in multiple conformational states that depend on serine and/or threonine phosphorylation of the molecule. I further examined the colocalization of Pyk2 and paxillin and found that the population of Pyk2 that strongly associates with paxillin does so at the MTOC of hematopoietic cells. These results indicate that a reservoir of inactive Pyk2, in association with paxillin, localizes at the microtubule organizing centre (MTOC). Finally, molecular mass differences were observed between the two populations, which are not due to phosphorylation but possibly as the result of SUMOylation of Pyk2. Taken together, these results provide a better understanding of the regulation of Pyk2 in regards to its binding partners and post-translational modifications, and link this regulation to its intracellular location.

3.2 Results

3.2.1 Pyk2 antibodies specific for the C-terminal domain, but not the N-terminal domain, can coimmunoprecipitate paxillin

Our laboratory has generated two polyclonal antibodies directed against regions in the NT and CT domain of Pyk2 (Ma et al., 1997). The F298 and the F245 antibodies were produced in rabbits by injection of a peptide corresponding to amino acids 2-12 and 720-862 of Pyk2, respectively (Berg and Ostergaard, 1997; Ma et al., 1997). The F245 antigen spans the proline rich regions of Pyk2 and the alternatively spliced variant lacking amino acids 738-780 (Pyk-H) (Figure

3.1a). This splice variant has been shown to be preferentially expressed in hematopoietic cells (Dikic et al., 1998). When I compared the ability of these Abs to immunoprecipitate Pyk2 in RAW 264.7 cells, I found that only the F245 Ab was able to co-immunoprecipitate paxillin with Pyk2 (Figure 3.1b). Minimal association of paxillin with Pyk2 could be detected with the F298 Ab upon longer exposure of the immunoblot, however, this association was significantly lower than that obtained with the F245 Ab (Figures 3.1b, 3.2 and St-Pierre et al. (2011)). Moreover, Pyk2 recovered with either antibody displayed similar total tyrosine phosphorylation levels (Figure 3.1b).

Similar to what was observed with RAW 264.7 macrophages, AB.1 T-cell clones also showed paxillin co-immunoprecipitation with the F245 antibody, and not the F298 antibody (St-Pierre et al., 2011). This association was constitutive, as stimulation through the TCR did not affect paxillin coimmunoprecipitation with the F245 antisera. Thus, only Pyk2 pulled down with the F245 antisera associates with paxillin and appears to do so independently of the tyrosine phosphorylation status of Pyk2.

3.2.2 F298 and F245 antisera recognize distinct but overlapping Pyk2 populations

To further investigate how F245 and F298 differ in their ability to recognize Pyk2, I performed serial immunodepletion experiments. Three immunodepletion steps were done with RAW 264.7 cell lysates with F245 antisera; however, this

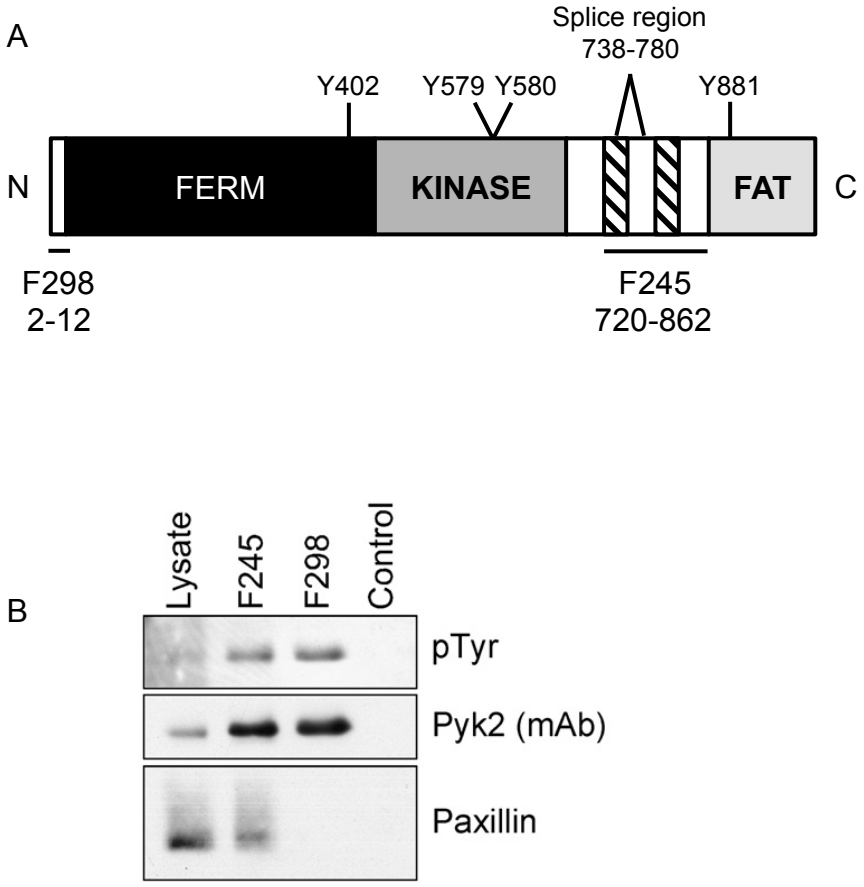

Figure 3.1. Coimmunoprecipitation of paxillin with Pyk2 depends on the anti-Pyk2 antibody used for immunoprecipitation. (A) Diagram of the domain structure of Pyk2 indicating the antibody epitopes. (B) F245 and F298 immunoprecipitates were generated from RAW 264.7 lysates followed by immunoblotting with anti-phosphotyrosine, monoclonal anti-Pyk2 and anti-paxillin antibodies. A sample of the cell lysate used for immunoprecipitation is shown in the first lane and a control immunoprecipitate containing protein A beads with no antibody is shown in the last lane. Representative of a minimum of three independent experiments.

was insufficient to deplete all F245-immunoreactive Pyk2 (Figure. 3.2). In contrast, with the same lysate, F298-immunoreactive Pyk2 was readily depleted and F245 was able to recover Pyk2 with a faster relative migration. The F245 antibody recognized two distinct migrating species in the Pyk2 immunoprecipitates while the F298 preferentially recognized the more slowly migrating species. Both species have been confirmed to be Pyk2 by mass spectrometry analysis. Additional immunoblots with two different commercially sourced anti-Pyk2 antibodies (C-19 and the Pyk2 mAb) confirmed that F245 recovered a more rapidly migrating form of Pyk2 after F298 depletion while the N-19 antiserum, similar to F298 antiserum, did not strongly detect the more rapidly migrating F298-depleted Pyk2 (Figure 3.2). Together, these results suggest that F298 and F245 bind overlapping but distinct populations of Pyk2, and that F245 selectively captures a population of paxillin-bound Pyk2.

3.2.3 Pyk2 recognized by F245 localizes, along with paxillin, at the microtubule-organizing centre in macrophages

The existence of two biochemical populations of Pyk2 in both T cells and macrophages was demonstrated. Whether such distinct populations of Pyk2 differ in their cellular localization was next tested. To investigate this possiblity, I examined the localization of both populations of Pyk2 and paxillin by confocal microscopy in RAW 264.7 macrophages. The results showed that F245-immunoreactive Pyk2 colocalized with α-tubulin, at the MTOC, and along microtubules (Figure 3.3). This is consistent with the localization of F245-

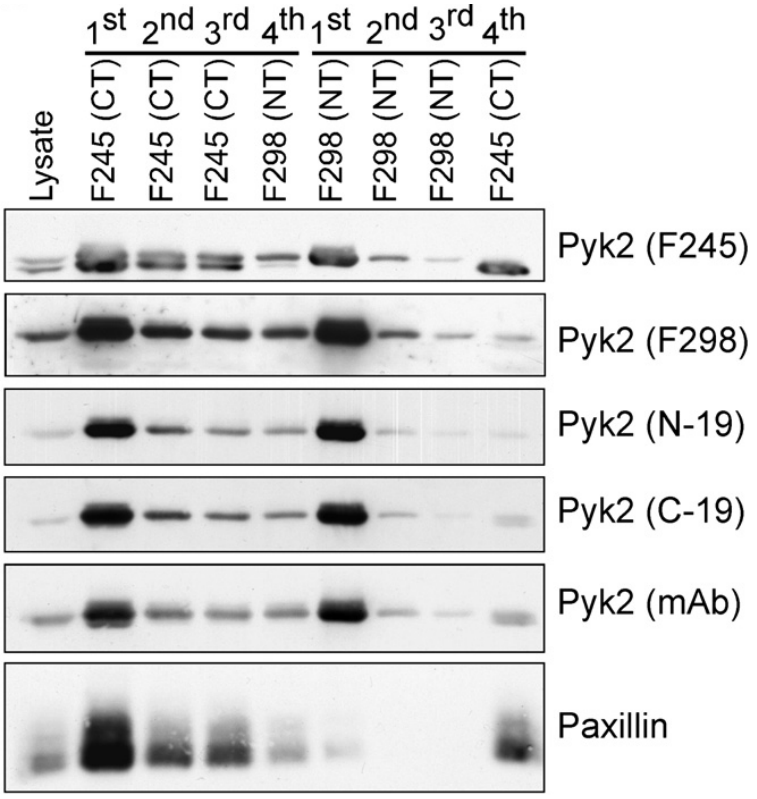

Figure 3.2. The F245 and F298 antisera recognize overlapping and distinct subsets of Pyk2. Sequential Pyk2 immunoprecipitations were performed on cell lysates from RAW 264.7 macrophages. After immunoprecipitation using the first indicated anti-Pyk2 antibody (1st), the supernatant was transferred to a new tube and subjected to a second and third round of immunoprecipitation with the same antibody (2nd, 3rd). After three rounds of depletion with the first antibody, the supernatant was subjected to an immunoprecipitation with the second antibody (4th). Each sequential immunoprecipitation was subjected to electrophoresis and probed by immunoblotting with the indicated antibody. Experiment shown is representative of three independent experiments.

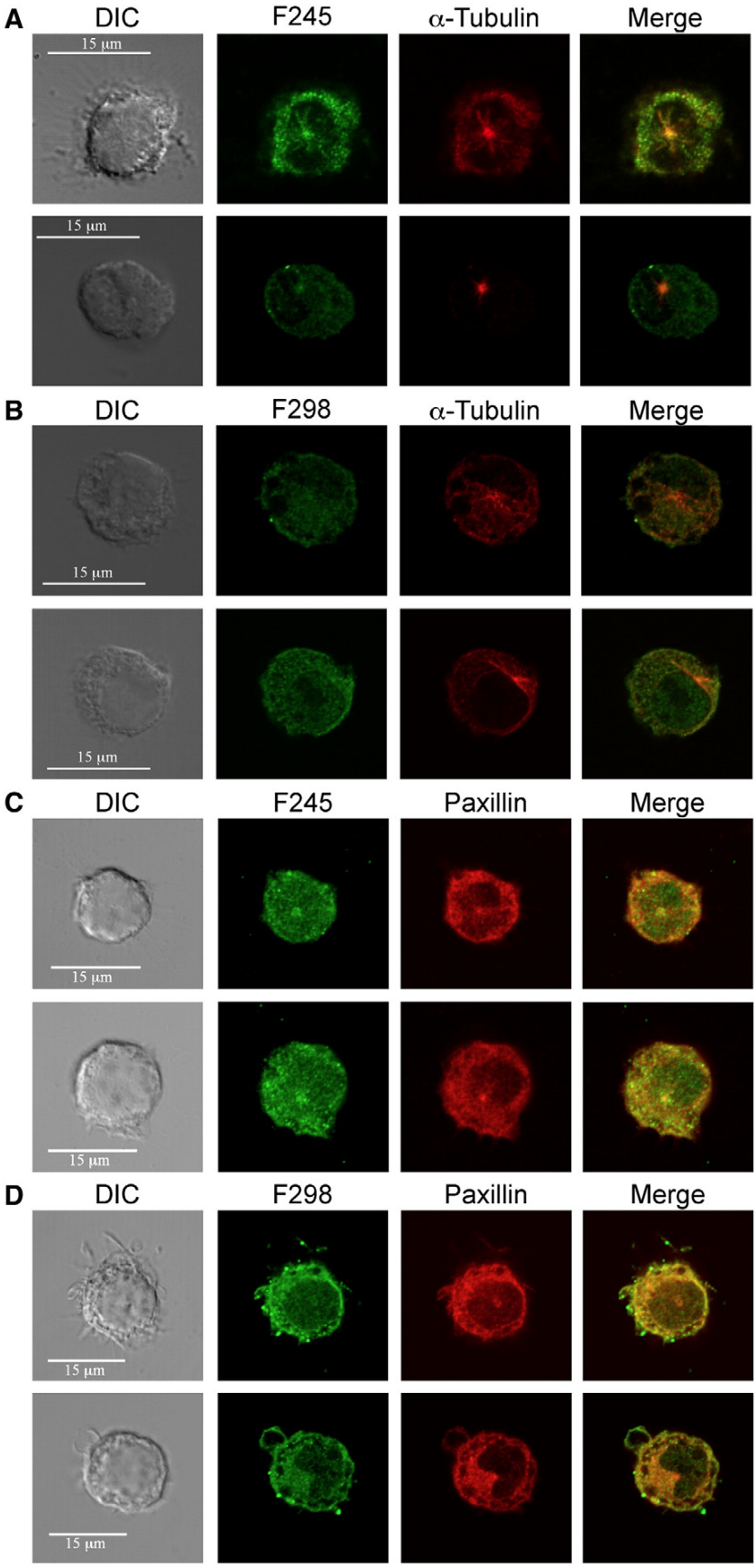
A
DIC
F245
α-Tubulin
Merge
15 μm
15 μm
B
DIC
F298
α-Tubulin
Merge
15 μm
15 μm
C
DIC
F245
Paxillin
Merge
15 μm
15 μm
D
DIC
F298
Paxillin
Merge
15 μm
15 μm

Figure 3.3 Pyk2 recognized by the F245 antibody, but not the F298 antibody, colocalizes with paxillin in the region of the MTOC in macrophages. Unstimulated RAW 264.7 cells were fixed, permeabilized and immunostained with (A) F245 (green) and anti-α-tubulin (red), (B) F298 (green) and anti-α-tubulin (red), (C) F245 (green) and anti-paxillin (red) or (D), F298 (green) and anti-paxillin (red) then visualized by confocal microscopy. Images are representative of three independent experiments.

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immunoreactive Pyk2 in T-cells (St-Pierre et al., 2011). In contrast, the cellular distribution of F298-immunoreactive Pyk2 did not reveal any association with MTOC or with α-tubulin, but appeared to be slightly enriched at the membrane (Figure 3.3). Supporting the biochemical data obtained with macrophages, the F245-immunoreactive Pyk2 was found to be enriched alongside paxillin, in the region of the MTOC, whereas the F298-immunoreactive Pyk2 did not strongly colocalize with paxillin (Figure 3.3). Both of these pools of Pyk2 showed overlapping localization in the cytoplasm and the nucleus. Overall, the staining patterns of both antibodies in macrophages were similar to those observed in T cells.

3.2.4 F245 preferentially recognizes hypophosphorylated Pyk2

The immunodepletion experiments indicated that F245 and F298 might recognize distinct but overlapping molecular species. To further characterize the potential differential reactivity of these two antibodies, I compared their ability to bind Pyk2 recovered with either antiserum. I performed Pyk2 immunoprecipitations with both antibodies in RAW 264.7 cell lysates. One set of immunoprecipitates was then probed with F298 and the other with F245. The results showed that, while the F298 antiserum was capable of immunoblotting

Pyk2 captured with either antibody, recognition by the F245 antiserum was limited to F245-immunoprecipitated Pyk2 (Figure 3.4a). A weak but detectable signal could be obtained upon over-exposure of F245 immunoblots from F298-immunoprecipiated Pyk2, suggesting that this is not an all or none recognition but rather a preferential recognition (Figure 3.2). This was also demonstrated in bone-marrow-derived macrophages (BMDM) (Figure 3.4b), splenic B-cells (Figure 3.4c) and AB.1 T-cell clones (Figure 3.4d). In addition, I was able to distinguish different migrating species of Pyk2. The molecular species that is strongly detected with the Pyk2 monoclonal antibody (mAb) and F298 corresponds to the faintly detected F245 band on the F245 blot (indicated by the arrowhead). This demonstrates that although F245 does not recognize Pyk2 immunoprecipitated with F298, F245 immunoprecipitates a form of Pyk2 that is strongly recognized by F298, however it possess distinct migration characteristics on SDS-PAGE (Figure 3.4a).

To determine whether the differences in immunoblotting and relative gel mobility of Pyk2 recovered with these antibodies could be attributed to differences in the phosphorylation status of Pyk2, I first assessed whether dephosphorylated F298 recovered Pyk2 from macrophages could be recognized by the F245 antibody by immunoblotting. To accomplish this, F298 immunoprecipitates were prepared from RAW 264.7 macrophages and treated with calf intestinal alkaline phosphatase (CIAP) to remove phosphate groups from the protein, followed by immunoblot with the F245 antiserum. Our results showed that treatment of the F298 immunoprecipitate with CIAP allowed for

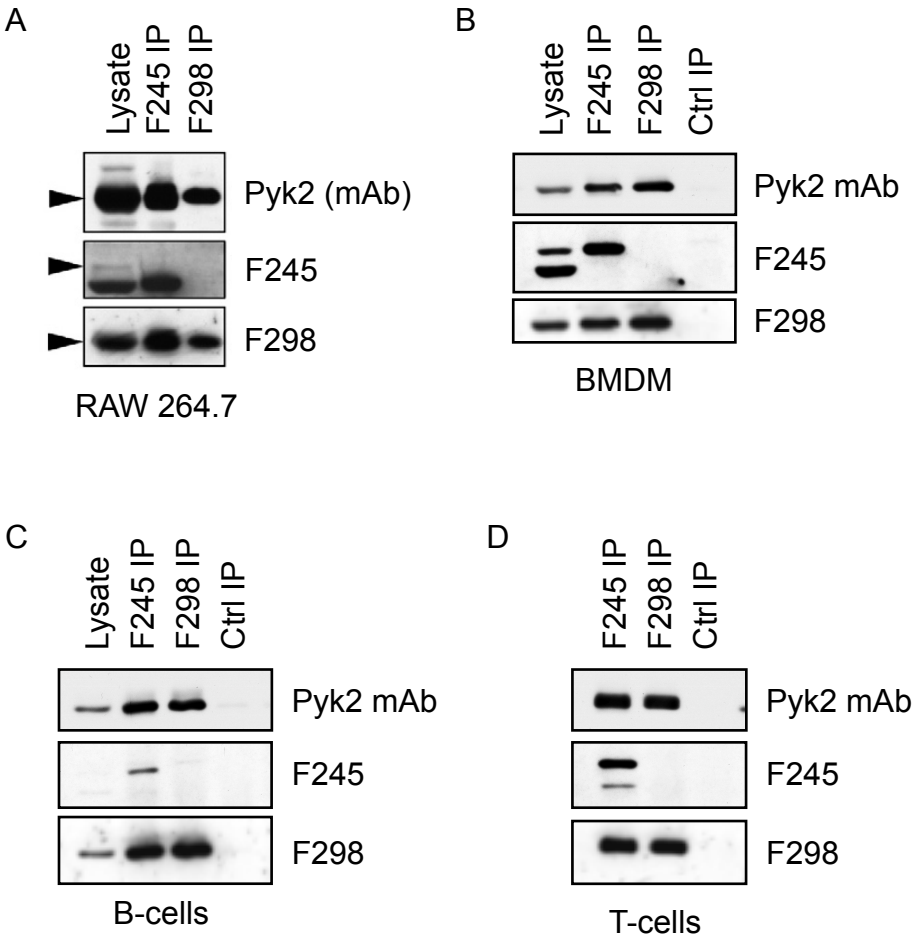


Figure 3.4. F245 does not recognize Pyk2 pulled down with F298 antisera. Pyk2 was immunoprecipitated with either F245 or F298 antisera from RAW 264.7 cell lysates (A), BMDM (B), splenic B-cells (C) or AB.1 T-cell clones (D). Lysates were sequentially probed with the Pyk2 monoclonal antibody (mAb) and F245 and F298 antisera. The position of the arrowhead indicates the same position on the gel in each immunoblot. Western blots shown are representative of at least three (A, B) or two (C, D) independent experiments.

increased recognition by the F245 antibody in RAW 264.7 cells (Figure 3.5a), as well as in AB.1 T-cell clones (St-Pierre et al., 2011). An increase in recognition of the slower migrating form of Pyk2 in the dephosphorylated F245 immunoprecipitate was also observed upon Western blot with F245 antisera (Figure 3.5a). This implies that phosphorylation is a determinant for discrimination of recognition of the slower migrating form of Pyk2 by immunoblotting with these two antibodies. On the other hand, dephosphorylation of Pyk2 did not affect the mobility of Pyk2, as detected by F298 immunoblotting. This indicates that the mobility differences in the Pyk2 captured with the two antibodies may not be due to differences in phosphorylation, but rather some other post-translational or post-transcriptional modification of Pyk2. However, it is possible that interaction of Pyk2 with other proteins protects Pyk2 from CIAP cleavage. Also noteworthy is that the faint bands detected above Pyk2 in the F298 and F245 immunoblots are a result of non-specific secondary reagent binding of proteins bound to the sepharose beads as they have been observed in sepharose bead control samples.

Pyk2 tyrosine-phosphorylation occurs at four major sites, one of which (Y881) is proximal to the F245 epitope (Figure 3.1a). Whether phosphorylation at Y881 interferes with F245 recognition is, however, unlikely because Y881 is phosphorylated in Pyk2 when pulled down with the F245 antibody (St-Pierre et al., 2011). I thus examined whether Pyk2 captured by either antibody displayed differences in serine or threonine phosphorylation, as many potential serine and

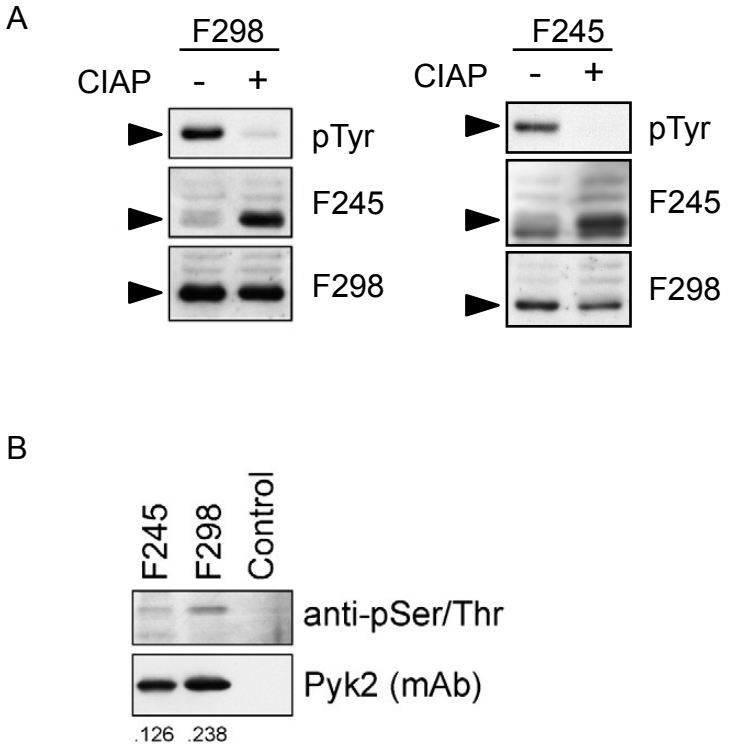


Figure 3.5. F245 preferentially recognizes hypophosphorylated Pyk2. (A) Pyk2 immunoprecipitates from lysates of 10^6 RAW 264.7 cells were subjected to treatment with CIAP. Membranes were probed, in order, with anti-phosphotyrosine, F245 and F298. Representative of three independent experiments. (B) Pyk2 immunoprecipitates were assessed for serine/threonine phosphorylation by Western blot. Quantification of band intensity, represented as a ratio of the phosphoserine/threonine to the anti-Pyk2 band intensity, is shown below the lanes and is representative of three independent experiments.

threonine phosphorylation sites are located within the F245 epitope region. To test this possibility, F245 and F298 immunoprecipitates were probed with an anti-phosphoserine/threonine antibody. The results showed that F298-captured Pyk2 displayed higher overall serine/threonine phosphorylation levels when compared to F245-captured Pyk2 ($p < 0.0066$) (Figure 3.5b). Overall, these results suggest that yet uncharacterized phosphorylation sites within the F245 epitope region interfere with binding of the F298 antiserum. They also imply that serine/threonine hyperphosphorylated Pyk2 is in a conformational state that is preferentially detected by the F298 antiserum.

3.2.5 Higher apparent molecular mass of Pyk2 is not due to isoform expression

Alternative splicing of exon 23 of the *Pyk2* gene gives rise to the Pyk2-H isoform, which is preferentially expressed in hematopoietic cells (Dikic et al., 1998; Li et al., 1998; Xiong et al., 1998). This isoform harbours a 42 a.a. deletion in the proline-rich regions (Figure 3.1a) and therefore migrates slightly faster on SDS-PAGE gel relative to full-length Pyk2. I thus examined whether differences in molecular mass displayed by Pyk2 immunoprecipitation in RAW 264.7 cells was due to the expression of these isoforms. For this purpose, RT-PCR was performed on mRNA from RAW 264.7 cells using primers encompassing the alternative splicing region of Pyk2. Amplification of this sequence with DNA from a Pyk2-H-encoding plasmid was used as a positive control. A negative control for contaminating genomic DNA was also performed by replacing the

reverse transcriptase with *Taq* DNA polymerase. I found that RAW 264.7 cells expressed mRNA encoding both full-length Pyk2 (Pyk2 FL) and Pyk2-H (Figure 3.6a). To determine whether these isoforms are differentially recognized by the F298 and F245 antibodies, I used NIH 3T3 cells, which express very low levels of Pyk2 recognized by F298 but do express detectable levels of the faster migrating form of Pyk2 recognized by F245 (Figure 3.6b). I first transfected these cells with an expression vector encoding the hematopoietic isoform of Pyk2 (Pyk2-H) and conducted comparative immunoprecipitation analysis with both F245 and F298 antisera. Interestingly, I found that when Pyk2-H is transfected into the cells, there is an appearance of the more slowly migrating form of Pyk2 that is recognized by F298, similar to what was observed in RAW 264.7 cells (Figure 3.6b). As FAK is highly expressed in these cells, I also confirmed that neither antibody immunoprecipitated FAK non-specifically (Figure 3.6b). Taken together, these results suggest that the slower migration of Pyk2-H was more likely due to post-translational modifications.

3.2.6 Paxillin associates with the more slowly migrating species of Pyk2

I next examined which of the slow and fast migrating species recognized by the F245 antisera associated with paxillin. Using NIH 3T3 cells transfected with the Pyk-2H-encoding plasmid, I found that paxillin preferentially associated with the more slowly migrating exogenous Pyk2-H isoform (Figure 3.7a). It is possible, however, that the faster migrating Pyk2 species in F245 immunoprecipitates is capable of association with paxillin as paxillin co-

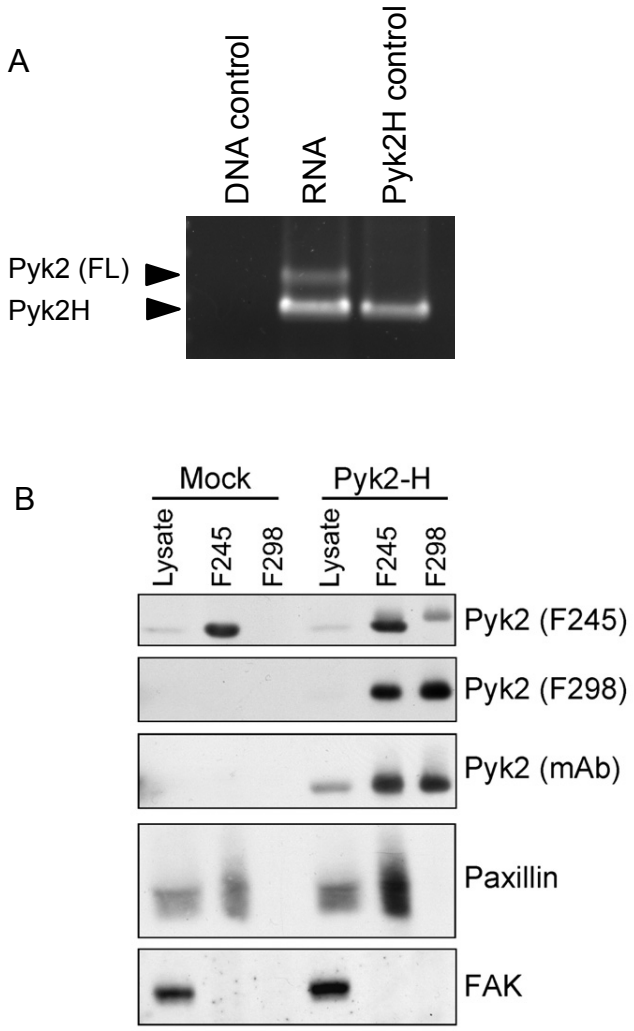


Figure 3.6 Differences in Pyk2 apparent molecular mass are not due to expression of the two different isoforms. (A) The Pyk2 splicing region was amplified by one-step RT-PCR from RNA extracted from RAW 264.7 macrophages. Amplification of this region from a Pyk2-H-encoding plasmid was used as a positive control. A control for the presence of genomic DNA was also included. (B) NIH 3T3 cells were mock transfected or transfected with the Pyk2-H isoform. Lysates were prepared and Pyk2 immunoprecipitated with either F245 or F298 and probed sequentially with the indicated antibodies. Figures shown are representative of three independent experiments.

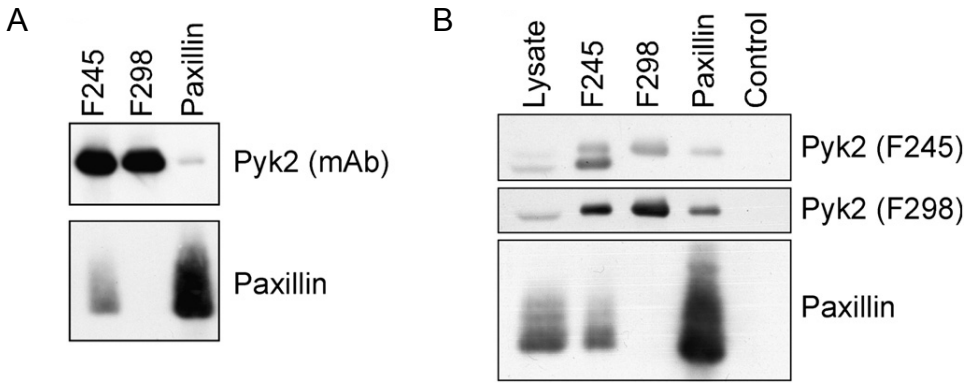


Figure 3.7. Paxillin associates with a single slower migrating Pyk2 molecular species. (A) Paxillin and Pyk2 were immunoprecipitated from lysates of Pyk2-H-transfected NIH 3T3 cells and immunoblotted with the indicated antibodies. (B) RAW 264.7 cells were lysed and immunoprecipitations performed with F245, F298, paxillin or with secondary antibody alone and probed sequentially with the indicated antibody. Western blots are representative of three independent experiments.

immunoprecipitation was observed in mock-transfected NIH 3T3 cells. Alternatively, the levels of Pyk2 co-immunoprecipitated with paxillin may be too low to be detected by the F245 Ab. Nonetheless, paxillin appears to preferentially interact with the slower migrating species as its recovery is significantly increased upon transfection of NIH 3T3 cells with Pyk2-H (Figure 3.6b), and it is the only species detected in paxillin immunoprecipitates (Figure 3.7). To confirm that paxillin preferentially associates with the more slowly migrating species of Pyk2 in macrophages, I then performed paxillin immunoprecipitates from RAW 264.7 cells. Pyk2, as detected with either F245 or F298, was co-immunoprecipitated with paxillin (Figure 3.7b). As with transfected NIH 3T3 cells, I could detect only the more slowly migrating form of Pyk2 in association with paxillin (Figure 3.7b). This implies that there are distinct populations of Pyk2 found within the most slowly migrating Pyk2, possibly as a result of phosphorylation, which determine the capacity of Pyk2 to associate with paxillin.

3.2.7 SUMO-1 differentially associates with Pyk2 pulled down with F245 and F298 antisera

I further investigated the cause of the differences in apparent molecular mass of Pyk2 pulled down with the two antisera. As phosphorylation and isoform expression were ruled out, I next examined whether such differences were related to SUMOylation of Pyk-2. SUMO proteins are small ubiquitin-like proteins that covalently attach to proteins via a lysine residue. SUMOylation of FAK has been demonstrated *in vitro* and in cells transfected with FAK, SUMO-1 and PIAS1

(Kadare et al., 2003). SUMO-1 attachment to FAK occurs at Lysine 152 in the FERM domain and leads to increased autophosphorylation of FAK. To address this possibility, I first assessed whether Pyk2 could be immunoprecipitated in RAW 264.7 cell lysates with anti-SUMO-1. As shown in Figure 3.8, the slower migrating form of Pyk2 was pulled down with anti-SUMO-1, indicating that this protein may be directly SUMOylated. A yet unidentified 80 kDa protein was also co-immunoprecipitated with the SUMO-1 Ab, as well as with the F298 antisera (Figure 3.8a). I next assessed whether stabilization of SUMOylated proteins increased Pyk2 pull-down with anti-SUMO-1 using NEM, which inhibits de-SUMOylation of proteins by irreversibly binding to SUMO cysteine proteases (Park-Sarge and Sarge, 2009). Our results showed that, not only was the amount of Pyk2 pulled down with anti-SUMO-1 significantly increased in the presence of NEM, but that the apparent molecular mass of Pyk2 was slightly increased upon treatment with NEM (Figure 3.8b, c). This suggested that Pyk2 interaction with SUMO-1 was enhanced by the stabilization of SUMOylated proteins. Further confirmation of the direct SUMOylation of Pyk2, as well as the site of SUMOylation on Pyk2, are currently being investigated in our laboratory.

Dynamin is a 100 kDa GTPase involved in endocytosis that localizes at sites of adhesion (Ezratty et al., 2005). It has also been shown to interact with both Pyk2 as well as with the SUMOylation machinery, although the function of these interactions remains largely unknown (Bruzzaniti et al., 2009; Mishra et al., 2004). In fact, dynamin is capable of direct interaction with

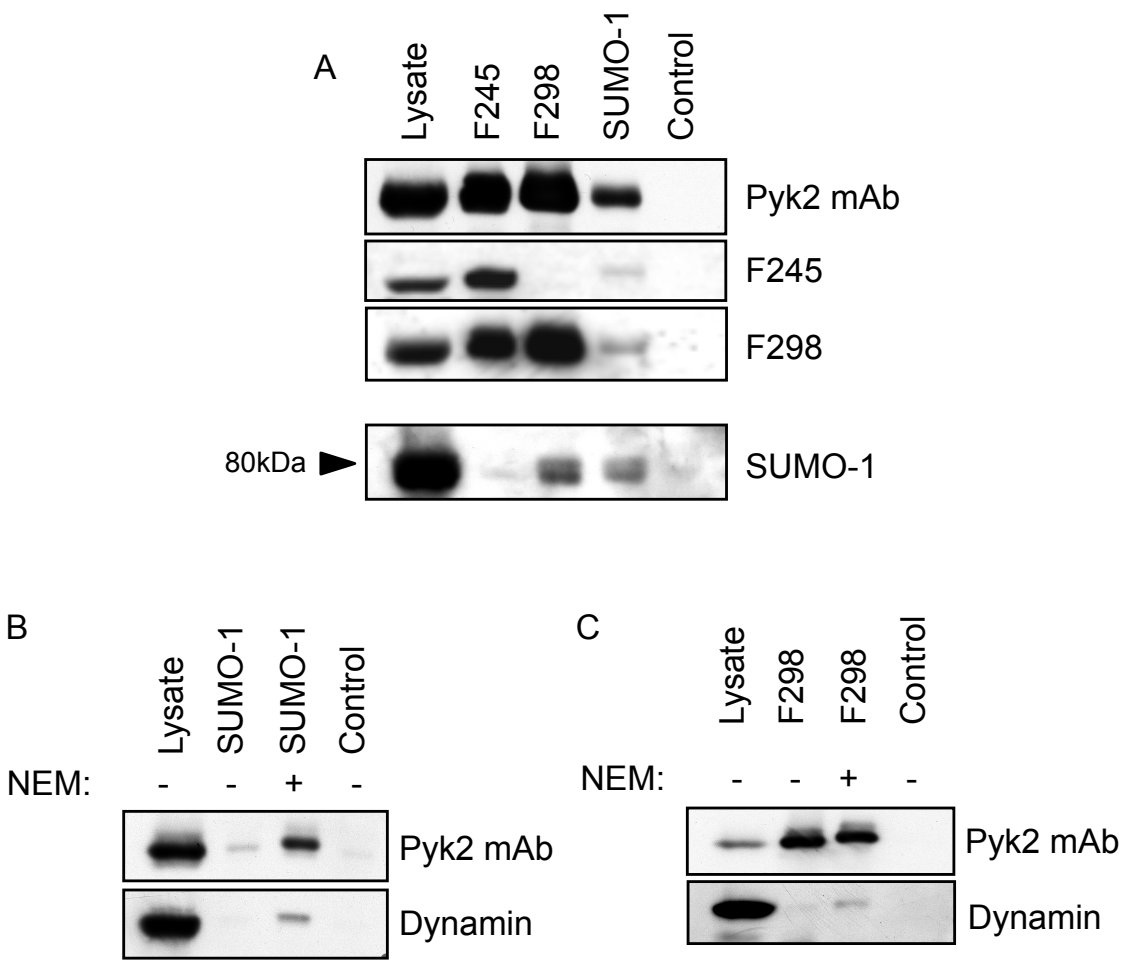


Figure 3.8. The slower migrating species of Pyk2 interacts with SUMO-1, dynamin and a SUMOylated 80 kDa protein. (A) Pyk2 and SUMO immunoprecipitates were performed using RAW 264.7 cell lysates and immunoblotted with anti-Pyk2 (Pyk2 mAb, F245, F298) or anti-SUMO-1. RAW 264.7 cells were treated with NEM, or left untreated, followed by cell lysis and immunoprecipitation with anti-SUMO-1 (B) or anti-Pyk2 (C). Immunoprecipitates were blotted with anti-Pyk2 and anti-Dynamin. Control lanes represent IP controls with isotype controls and sepharose beads. Western blots shown above are representative of three independent experiments.

Figures 3.6b, c were done in collaboration with Jessica Beatty.

the SUMO protein although it is not itself directly SUMOylated. I hypothesized that F298-reactive Pyk2 population, which localizes at the cell membrane and displays a higher molecular mass forms a complex with dynamin and SUMO-1. To test this hypothesis, I immunoblotted for dynamin in SUMO-1 and F298 immunoprecipitates. I found that dynamin did indeed associate with both SUMO-1 and Pyk2 upon treatment with NEM (Figure 3.8b, c). This complex may also contain the 80 kDa protein previously detected by SUMO-1 immunoblot (Figure 3.8a). Further characterization of this complex may provide insight in the function of the F298-reactive population of Pyk2.

3.3 Discussion

In this chapter, two populations of Pyk2 were identified within macrophages, based on their biochemical features and intracellular localization. As demonstrated by the immunodepletion experiments, these populations bind unique, but overlapping, pools of Pyk2 in macrophages. One population, as identified with the F245 antiserum, constitutively associated with paxillin, colocalized with paxillin at the MTOC and displayed a lower apparent molecular mass. The second population, as identified with the F298 antiserum, did not associate with paxillin, localized near the plasma membrane, displayed increased serine/threonine phosphorylation and higher apparent molecular mass, and associated with dynamin and an unidentified 80 kDa protein. I also showed that the difference in migration on SDS-PAGE between the two populations was not due to phosphorylation or expression of the different isoforms but may be due to

SUMOylation of Pyk2 recovered with the F298 antisera. Together, these results demonstrate that Pyk2 localized at specific areas within the cell, displays differential binding partners and post-translational modifications. These features may in turn reflect distinct functions of Pyk2 within macrophages.

The most striking difference initially noted between the two populations regarded their association with paxillin. Interestingly, the previous studies that demonstrated that Pyk2 could associate with paxillin used antibodies that recognized a GST fusion protein consisting of the C-terminus (a.a. 861-1009) or the proline-rich regions (a.a. 680-860) of Pyk2 (Hiregowdara et al., 1997; Li and Earp, 1997; Salgia et al., 1996). At present, it is not clear why F298-reactive Pyk2 does not bind paxillin as strongly as Pyk2 captured with the F245 antisera. The F298 binds to the N-terminus of Pyk2 while paxillin binds to the C-terminal FAT domain of Pyk2, so it is unlikely that F298 binding directly blocks paxillin association or that paxillin directly blocks accessibility to the F298 epitope on Pyk2. It is however, logical to hypothesize that F298 recognizes a conformation of Pyk2 that is not associated with paxillin. Both functional and structural studies of FAK have revealed that the FERM domain of FAK binds to the kinase domain forming an autoinhibitory conformation (Cooper et al., 2003; Lietha et al., 2007). While this has not been directly shown for Pyk2, given the high level of homology between the two kinases, it is probable that Pyk2 would also form a closed, inhibited form of the molecule. I propose that when Pyk2 is in the autoinhibited conformation, the F298 epitope is not accessible, preventing immunoprecipitation with the F298 antiserum while still allowing binding to F245

and paxillin. Upon stimulation, a fraction of the Pyk2 molecules become tyrosine phosphorylated, thereby favouring an open structure that allows for binding to F298, while preserving F245 and paxillin binding. This could represent the small fraction of F298-captured Pyk2 that is able to bind to paxillin. To maintain an open and active confirmation, Pyk2 may then become phosphorylated on a number of serine and/or threonine residues that both keeps the molecule from closing and disrupts the interaction with paxillin. This phosphorylation would also reduce F245 binding by immunblotting and immunoprecipitation. I suggest that the differential binding of these antibodies provides us with important clues regarding the regulation of Pyk2.

Although both antisera immunoprecipitated tyrosine-phosphorylated Pyk2, they showed differences in their recognition of serine/threonine-phosphorylated Pyk2. These data are consistent with those previously demonstrated in T-cells showing that paxillin association with Pyk2 is constitutive and does not depend on tyrosine phosphorylation of Pyk2 (Ostergaard et al., 1998). Even the phosphorylation of Y881, which is located near the paxillin binding region, has been shown not to disrupt paxillin binding (Lulo et al., 2009). When blotting for serine/threonine phosphorylation, Pyk2 captured by the F298 antibody showed higher levels phosphorylation compared to F245-immunoprecipitated Pyk2. Moreover, in immunoblots performed using lysates from T cells or macrophages, F245 is less capable of recognizing Pyk2 recovered with the F298 antibody, unless Pyk2 was dephosphorylated. Therefore, the F298 recognized population of Pyk2 is likely phosphorylated at sites within, or near, the F245 epitope, though

not necessarily exclusively so. Given that the presence of an alternatively-spliced sequence within the region of Pyk2 was used for immunization, and because the CTL clones expressed exclusively the Pyk2-H isoform (Lysechko, 2007), there is actually a relatively small region of Pyk2 that is potentially bound by the antibody in T cells. Using *in silico* analysis, two potential tyrosine phosphorylation sites (Y722 and Y849), two potential serine phosphorylation sites (S788 and S789) and one potential threonine phosphorylation site (T842) were predicted in this region. This region of FAK has also been shown through mass spectrometry mapping studies to be rich in *bona fide* serine/threonine phosphorylation sites (Grigera et al., 2005). Based on the data I have obtained, it is likely that F298 recognizes Pyk2 that is phosphorylated on a site(s) that prevents recognition by F245.

I found that F245-recovered Pyk2 from macrophages exhibited distinct molecular species. More specifically, I found that F298-captured Pyk2 consistently migrated at a higher molecular mass compared to the major F245 population. These distinct molecular species were not attributed to differential phosphorylation patterns, as treatment with CIAP did not affect migration of these proteins. One cannot eliminate, however, the possible presence of a very specific, CIAP resistant site(s) causing a mobility shift. Alternatively, I considered the possibility that these different molecular species might reflect the presence of two known isoforms of Pyk2. This possibility is unlikely as examination of the protein bands by mass spectrometry indicated that both proteins were derived from Pyk2-H. Moreover, Pyk2-H also shows slower migration on SDS-PAGE when transfected in NIH 3T3 cells. Rather, I believe that the smaller protein from

the macrophages might be a cleavage product of Pyk2 that likely occurs at the N-terminus of the protein, thus precluding F298 recognition, or that reflects the existence of a novel variant of Pyk2 lacking all or part of the N-terminus.

As FAK has been shown to undergo SUMOylation, I examined whether SUMO attachment to Pyk2 accounted for the apparent differences in the molecular mass (Kadare et al., 2003). I found that the slower migrating form of Pyk2 was coimmunprecipitated with SUMO-1 in RAW 264.7 cells. Moreover, this slower migrating species of Pyk2 complexed with dynamin and a yet unidentified 80 kDa protein. It is possible that SUMOylation of Pyk2 may provide a bridge for Pyk2 interaction with these proteins. Whether Pyk2 is directly SUMOylated and at which site, however, remains to be confirmed. In addition, investigations in the binding partners of the F298 population might lead to the characterization of the function of this population. At present, as the significance of FAK SUMOylation and the association of dynamin with the SUMO machinery has not been determined, it is too early to speculate on what this function may be.

In addition to the biochemical differences discussed above, differential localization of both populations of Pyk2 were observed within immune cells. I have shown that the F245 antiserum, which immunoprecipitated paxillin-bound Pyk2, was enriched in the region of the MTOC along with paxillin. In contrast, Pyk2 identified by the F298 antiserum appeared to be enriched at the membrane. Such colocalization of Pyk2, paxillin and the MTOC has been previously described in NK cells (Sancho et al., 2000). I have shown that only the

population of Pyk2 associated with paxillin also colocalized with paxillin at the MTOC, suggesting that paxillin might be responsible for its MTOC localization. However, the function that Pyk2 and paxillin might be performing at the MTOC is still unclear.

In summary, the results presented here provide us with a better understanding of Pyk2 regulation through its post-translational modifications and association with binding partners in relation to its intracellular location. Additional studies will be needed to determine the specific serine/threonine sites that are differentially phosphorylated between the two populations identified, as well as whether these populations harbour functional differences.

CHAPTER FOUR: A ROLE FOR CD45 IN MACROPHAGE ADHESION AND MIGRATION THROUGH THE REGULATION OF PAXILLIN EXPRESSION

4.1 Introduction

CD45 is a transmembrane PTP expressed on cells of hematopoietic origin involved in the regulation of leukocyte adhesion and motility (Shivtiel et al., 2008; Shivtiel et al., 2011; Windhagen et al., 2007). The absence of CD45 from macrophages has been shown to lead to the disregulation of macrophage adhesion as these cells exhibited increased detachment from tissue-culture-treated dishes (Roach et al., 1997). In CD45 KO macrophages, the SFKs Hck and Lyn, but not Fgr, were shown to be hyperphosphorylated and hyperactive (Li et al., 2001; Roach et al., 1997; Roach et al., 1998; Shivtiel et al., 2008). Given the role of SFKs in the regulation of cell adhesion, it is possible that they are implicated in this process through the phosphorylation of substrates involved in cell adhesion. Opposingly, because of the fact that hyperphosphorylation of SFKs in CD45-deficient macrophages does not correlate with whether macrophages are in an adhesive state or not, it can also be argued that CD45 could control signalling pathways independent of SFK regulation. Further investigation is critically needed to better determine the contribution of CD45 in the regulation of the molecular mechanisms involved in macrophage adhesion and motility.

In this chapter, I have examined in detail the role of CD45 in cell adhesion and motility, focusing on the mechanisms by which CD45 regulates these

functions in macrophages. I provide evidence that CD45 functions to balance signalling and protein expression to regulate macrophage adhesion turnover, morphology and motility. This new regulatory mechanism provides further insight in the complexity of interactions between phosphatases and the macrophage cytoskeleton.

4.2 Results

4.2.1 Normal maturation of CD45 KO BMDM in the presence of GM-CSF

M-CSF and GM-CSF are cytokines routinely used to drive the maturation of BMDM *in vitro*. However, it has been shown that both of these cytokines generate morphologically and phenotypically distinct populations of macrophages. GM-CSF-derived BMDM constitute a more heterogeneous population of macrophages with broader cell diameter and increased cell spreading compared to the smaller and more uniform M-CSF-derived BMDM (Falk et al., 1988; Pelegrin and Surprenant, 2009). Functional differences between GM-CSF- and M-CSF-derived BMDM have also been reported. For example, because they secrete high levels of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-12p70 and IL-23, upon stimulation with LPS, GM-CSF BMDM are generally considered pro-inflammatory macrophages (Brissette et al., 1995; Fleetwood et al., 2007). In contrast, M-CSF-derived BMDM are known to secrete higher levels of anti-inflammatory cytokines such as IL-10 and CCL2 upon LPS stimulation (Fleetwood et al., 2007). M-CSF BMDM have also been shown to secrete higher levels of IFN-α/β following infection with Vesicular Stromatitis Virus (Falk and

Vogel, 1990; Fleetwood et al., 2009). In light of these phenotypical differences, GM-CSF- and M-CSF-derived BMDM are considered to reflect human M1 and M2 cell phenotypes. While previous studies on BMDM from CD45 KO mice have used M-CSF to mature bone marrow progenitor cells into macrophages (Roach et al., 1997; Zhu et al., 2008), I have chosen to mature bone marrow progenitor cells with GM-CSF as they show increased cell spreading in culture, thereby facilitating the detection of differences in adhesion between CD45 KO and WT BMDM. I will also take this opportunity to determine whether the defects in adhesion previously reported occur in both types of macrophages or whether they are restricted to M-CSF-derived macrophages.

Because there have been no studies on GM-CSF-derived CD45 KO BMDM, it was crucial to first establish whether progenitor cells from CD45 KO mice undergo normal maturation in the presence of GM-CSF. For this purpose, bone marrow progenitor cells from WT and CD45 KO mice were harvested and incubated for 7 days in the presence of GM-CSF. The cell surface expression levels of two common macrophages markers, F4/80 and CD11b, were then compared by flow cytometry (Murray and Wynn, 2011). F4/80 is a 125 kDa transmembrane protein expressed on the majority of mature macrophages (Leenen et al., 1994). CD11b (Mac-1, integrin α_M) is a 165 kDa transmembrane protein expressed on the surface of monocytes, macrophages, granulocytes, activated lymphocytes and a subset of NK cells (Larson and Springer, 1990; Leenen et al., 1994). CD11b heterodimerizes with the β_2 integrin chain and is involved in mediating cell adhesion and migration (Larson and Springer, 1990). It is the main

integrin mediating macrophage adhesion to tissue-culture-treated plates (Roach et al., 1997). The results showed that both of these macrophage markers are expressed at similar levels at day 7 on both WT and CD45 KO BMDM, indicating that absence of CD45 does not affect macrophage maturation *in vitro* in the presence of GM-CSF (Figure 4.1). Accordingly, GM-CSF-derived BMDM were used for all studies described in this chapter, notably because CD45 KO BMDM were found to mature normally in the presence of GM-CSF. These cells could thus be used to determine whether similar adhesion defects are observed for these cells as previously reported for M-CSF-derived CD45 KO BMDM (Roach et al., 1997). Additionally, GM-CSF is more relevant than M-CSF with regards to pro-inflammatory macrophage function, which will be the focus of the following chapter.

4.2.2 CD45 KO BMDM show decreased maintenance of adhesion, altered morphology and decreased movement in culture

Roach et al. (1997) have reported that M-CSF-derived CD45 KO BMDM exhibit defects in their capacity to maintain adhesion in the absence of serum. Whether this phenotype also occurs during integrin-mediated cellular adhesion of GM-CSF-derived CD45 BMDM is currently unknown. To address this question, I have examined whether these cells were able to maintain attachment to various

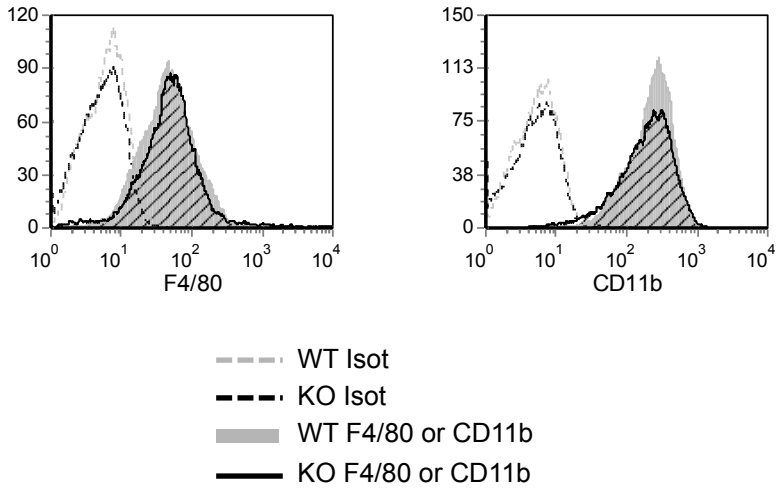


Figure 4.1. Normal maturation of CD45 KO BMDM with GM-CSF. Day 7 BMDM from WT (grey, filled) or CD45 KO (black line) were harvested, washed and stained with PE-conjugated anti-CD11b, anti-F4/80 or isotype control (WT in dashed grey line, KO in dashed black line). No differences in staining were observed between WT and CD45 KO BMDM. Representative of a minimum of three independent experiments.

ECM substrates coated on a solid support in the absence of serum for more than 24 hours. TC-treated wells were used as a non-ECM substrate, although attachment of macrophages to TC-treated plastic is mediated through CD11b (Roach et al., 1997). For this experiment, 10^6 day 7 CD45 KO BMDM or WT cells were harvested and left to readhere onto wells coated with various ECM (FN, Lam, Col I, Col, IV) or non-ECM substrates (TC-treated plastic). After 24 hours, unadhered cells were removed by washes with PBS. The remaining adherent cells were then assessed by light microscopy or lysed directly on the plates followed by SDS-PAGE and processed for Western blot analysis (Figure 4.2a, b). The results showed that in the absence of serum, WT cells were able to readhere to many of the substrates, albeit a reduced adherence to type IV collagen was observed. In contrast, CD45 KO BMDM were unable to maintain attachment to all substrates tested. The input lanes are representative of 10^6 cells lysed directly after the harvest of day 7 BMDM and before replating. Because CD45 KO BMDM express WT levels of CD11b (Figure 4.1), this suggests that differences in adhesion are independent of integrin expression. All together, these results demonstrate that the defects in adhesion previously observed with M-CSF-derived CD45 KO BMDM are also observed in GM-CSF-derived CD45 KO cells. Furthermore, this adhesion defect extended to all substrates examined.

It is well known that both cell spreading and cell adhesion both rely on the stable formation of focal contacts. Cell locomotion, on the other hand, relies on

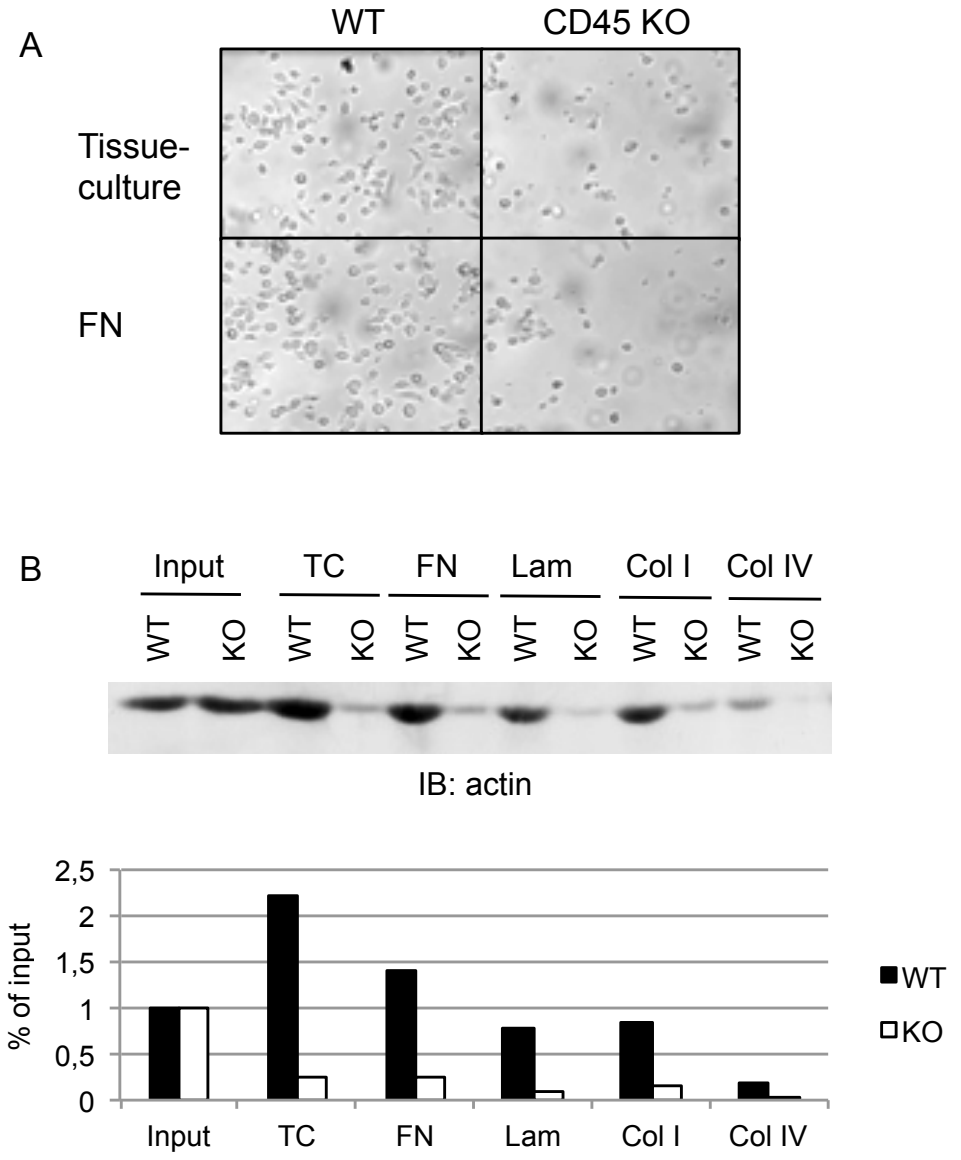
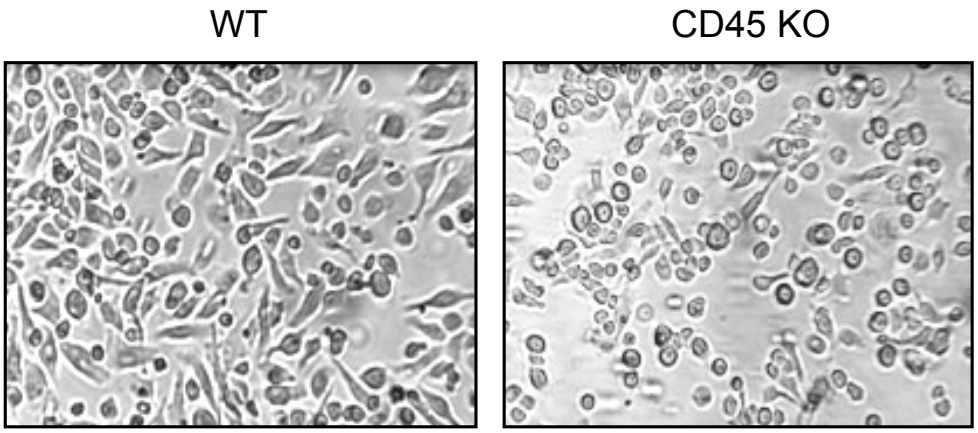


Figure 4.2. CD45 KO BMDM cannot maintain attachment to various extracellular matrices. Day 7 BMDM from WT or CD45 KO mice were harvested and incubated 24 hours without serum on various surfaces, after which unadhered cells were washed away and adhered cells were lysed directly in plate. (A) Light microscopy pictures of adhered cells after 24 hours. (B) Lysates were loaded on an SDS-PAGE gel and relative amounts of adhered cells are represented by a Western Blot for actin. The input lanes represent the amount of actin of 10^6 cells. Quantification of actin amounts are represented below the Western blot and are relative to the amount of actin in the input control. Quantification was done with the Image J software. Representative of two independent experiments.

mechanisms that coordinate the assembly and disassembly of focal complexes. I have thus examined if these processes were affected by the absence of CD45 in macrophages. For this purpose, cell cultures were examined for morphological changes (i.e. cell spreading) by light microscopy at day 7. I found statistically significant differences in the morphology of macrophages derived from CD45 KO mice compared to WT (Figure 4.3a, b). Although CD45 KO BMDM were able to adhere to plastic surfaces during differentiation and cell culture, they showed reduced spreading and stretching when compared to WT BMDM. While the majority of WT cells showed spreading, less than half the cells in the CD45 KO BMDM culture displayed a spread phenotype. Cell rounding of CD45 KO BMDM was similar to the phenotype reported in studies where adhesive cell types were shown to be unable to maintain stable focal adhesions (Allen et al., 1997; Carragher et al., 2001; Carragher et al., 1999). The altered morphology of CD45 KO might therefore be indicative of defects in the stability of adhesion complexes.

Live-cell imaging was then used to study cell motility of WT or CD45 KO BMDM in culture. For this purpose, BMDM were harvested at day 7 of culture and replated on TC-treated cell chambers for one hour prior to imaging. Cell movement was tracked for a period of 30 minutes and analysed with the Chemotaxis Tool plugin of the ImageJ software. The results showed that CD45 KO macrophages showed significantly less movement in culture compared to WT macrophages, as shown by measurements of cell velocity (Figure 4.3 c, d). In the case of CD45 KO cells, although these cells were able to form the extensions

A



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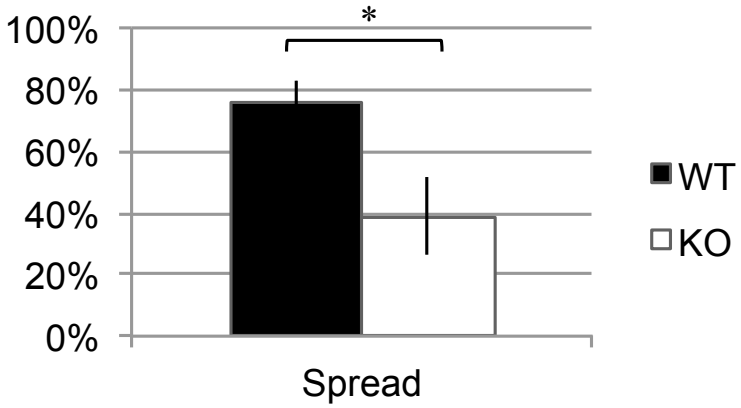

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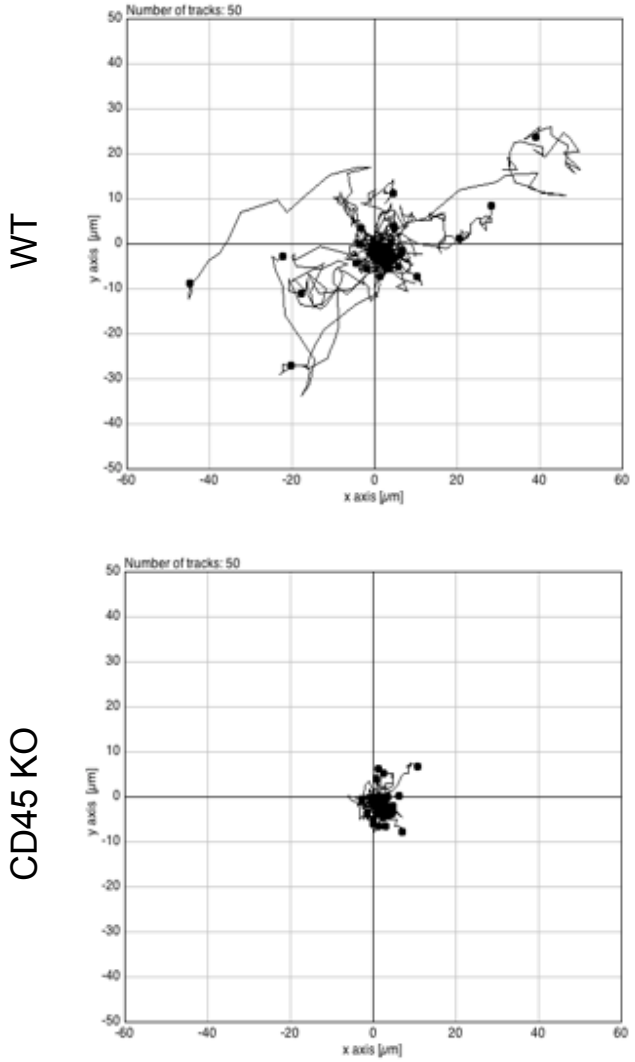


D

Cell type	Average Velocity (µm/min)	
WT (*n* = 99)	0.862	p<0.0001
KO (*n* = 88)	0.325	

Figure 4.3. CD45 KO BMDM exhibit decreased cell spreading and motility compared to WT BMDM. (A) Morphology of day 7 WT and CD45 KO BMDM in culture. (B) Quantification of cell spreading from three independent cultures of WT (black) and CD45 KO (white) BMDM. The difference in the number of spread cells between WT and CD45 KO BMDM is statistically significant ($p<0.00001$). Example of cell tracking of WT and CD45 KO BMDM on tissue-culture-treated wells for 30 minutes (C) and their average velocity (D). Difference in cell velocity is statistically significant ($p<0.0001$). Representative of three independent experiments.

necessary for crawling, they appeared to be unable to move in a particular direction, staying at the same position throughout the time-lapse video analysis.

Such reduced motility of CD45 KO macrophages may be due to several defects. First, this could be indicative of a high rate of focal complex turnover, where the disassembly of the complex occurs too rapidly for the establishment of a leading edge necessary for crawling. The decrease in cell motility could also be due to higher stability of focal structures already in place, preventing assembly of new focal structures at the leading edge of the cell. Alternatively, the high stability of established focal complexes could prevent the cells from detaching from the trailing edge of the cell. In this latter scenario, however, the cells should have displayed a stretched out morphology instead of the rounded morphology observed in Figure 4.2.

Altogether, the adherence, morphology and cell motility defects observed in CD45 KO BMDM strongly suggest that disregulation of focal contact and/or focal complex stability occurs in the absence of CD45. Because adhesive structures rely on many of the same cytoskeletal and cytoskeletal-associated proteins and are regulated by similar mechanisms, it is thus likely that CD45 regulates proteins involved in both functions.

4.2.3 CD45 KO BMDM show altered basal tyrosine phosphorylation levels

Adhesion complex assembly and disassembly is regulated by tyrosine phosphorylation, most notably via SFKs, which are known substrates of CD45. The dysregulation of SFKs in the absence of CD45 has been reported in many

immune cell types, including macrophages and T-cells (Roach et al., 1997; Shenoi et al., 1999). In fact, it is thought that CD45-deficient T-cells and macrophages exhibit defects in adhesion as a result of disregulated SFK-dependent integrin signalling. Since many proteins involved in cell adhesion are subjected to regulation by tyrosine phosphorylation and the CD45 PTP, I hypothesized that the adhesion defects observed in CD45 KO macrophages are a consequence of the dysregulation of proteins normally regulated by tyrosine phosphorylation, such as SFKs. To test this, I first compared the overall tyrosine phosphorylation patterns of cellular lysate of CD45 KO and WT BMDM (Figure 4.4). For this purpose, cellular lysates of 10^6 day 7 BMDM from WT or CD45 KO BMDM were loaded onto an SDS-PAGE gel and probed with anti-phosphotyrosine, or anti-GAPDH as a loading control. A similar analysis was carried out using 10^6 cells from thioglycollate (TG)-ellicited peritoneal macrophages collected from WT or CD45 KO mice. First, although BMDM and TG-ellicited peritoneal macrophages do exhibit some minor differences in the phosphorylation of proteins, they nonetheless exhibit certain key similarities in their phosphorylation patterns, represented by asterisks in Fig 4.4. These major similarities in tyrosine phosphorylation reinforce the fact that that phenotypes observed in BMDM are representative of *in vivo* macrophages. Secondly, the results showed that both types of macrophages from CD45 KO mice exhibited phosphorylation patterns that are distinct from WT macrophages (Figure 4.4). Although most proteins are unaffected by the loss of CD45, the tyrosine phosphorylation of a specific subset of proteins is altered in CD45 KO cells when compared to WT. In the 116-140

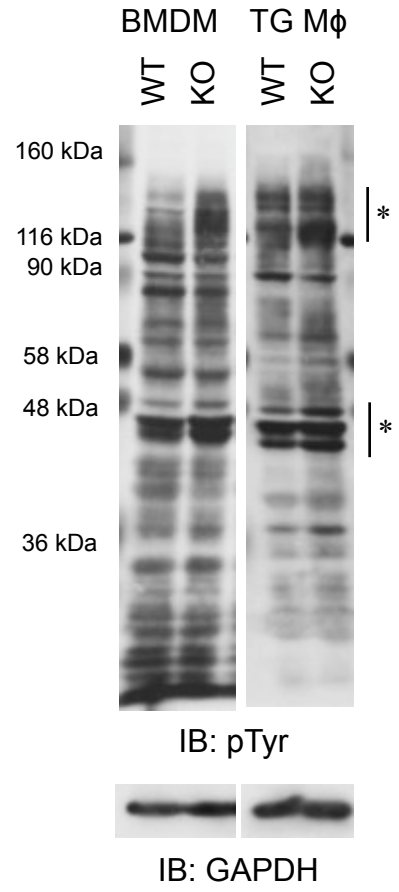


Figure 4.4. CD45 KO macrophages exhibit distinct phosphorylation patterns in cell lysates compared to WT. Lysates from day 7 cultures of BMDM and thioglycollate-elicited (TG) macrophages were probed with anti-phosphotyrosine. Anti-GAPDH was used as a loading control. Western blot is representative of two (TG Mϕ) or more than three (BMDM) experiments.

kDa molecular weight range, lysates from CD45 KO macrophages displayed hyperphosphorylation of multiple proteins when compared to the WT lysates. This was also true of proteins in the 50 to 60 kDa range. It is important to note that the molecular weight standards used tend to migrate more slowly than predicted. This range includes SFKs, which are normally hyperphosphorylated in the absence of CD45 (D'Oro and Ashwell, 1999; Mustelin and Altman, 1989; Ostergaard et al., 1989; Roach et al., 1997). There are likely additional differentially regulated proteins in CD45 KO macrophages, however they are not present in sufficient amounts to be detected in cell lysates. Given the adhesion and motility defects displayed by CD45 KO macrophages, these differentially phosphorylated proteins may include proteins involved in those functions.

Although the identity of these proteins has not been confirmed, speculations can be made as to which proteins could be differentially tyrosine phosphorylated in the absence of CD45 based on results obtained in other cell types. T-cells treated with anti-CD45 have been shown to trigger tyrosine phosphorylation of three major cytoskeletal-associated proteins: Pyk2, FAK and paxillin (Allen et al., 1997; Berg et al., 1998; Ostergaard et al., 1998). Pyk2 also exhibits increased tyrosine phosphorylation in CD45-deficient T-cells upon treatment with anti-CD44, and this correlates with SFK hyperactivity in these cells (Li et al., 2001; Wong et al., 2011). Phosphorylation of FAK in T-cells is also negatively regulated by CD45, albeit to a lesser degree than Pyk2 (Li et al., 2001). Pyk2 was immunoprecipitated from CD45 KO and WT BMDM, however no tyrosine phosphorylation was detected in either sample. This is likely the

consequence of basal levels of phosphorylation of this protein is too low to be detected in the absence of stimulation. Therefore, no conclusions can be made with respect to Pyk2 phosphorylation in CD45 KO or WT macrophages.

In conclusion, the observed differential phosphotyrosine patterns between WT and CD45 KO macrophages provide evidence that CD45 regulates many proteins through tyrosine phosphorylation. Moreover, this disregulation of tyrosine phosphorylation is not compensated by other PTPs therefore the regulation of these proteins by CD45 is not redundant.

4.2.4 CD45 KO BMDM show decreased expression of paxillin

Paxillin is a 68 kDa scaffold protein located at sites of adhesion and is tyrosine phosphorylated by SFK, FAK and Pyk2. It has also been shown to be tyrosine phosphorylated upon treatment with cross-linking with an anti-CD45 Ab in T-cells (Ostergaard et al., 1998). I hypothesized that in CD45 KO cells, which harbour hyperactivated SFKs, paxillin would exhibit increased tyrosine phosphorylation. To assess the effects of CD45 on paxillin, cellular lysates from day 7 WT or CD45 KO BMDM were immunoprecipitated and immunoblotted with anti-paxillin (Figure 4.5a). Such analysis, however, was complicated by the fact that CD45 KO BMDM exhibited a significant decrease in paxillin protein levels (Figure 4.5a). This reduced expression level of paxillin was consistently observed and statistically significant ($p<0.000001$) from that of WT BMDM, as measured by quantitative Western blot analyses of results obtained from five independent experiments (Figure 4.5b).

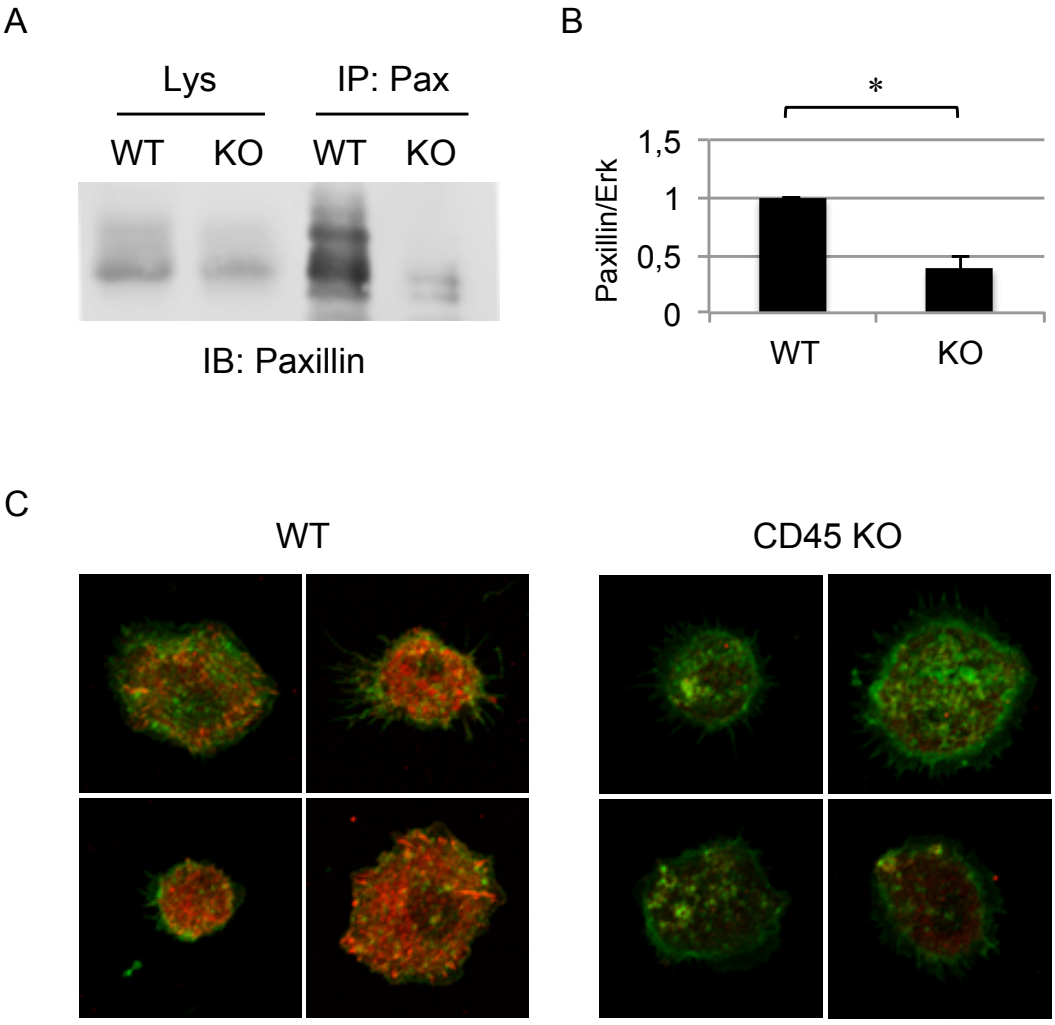


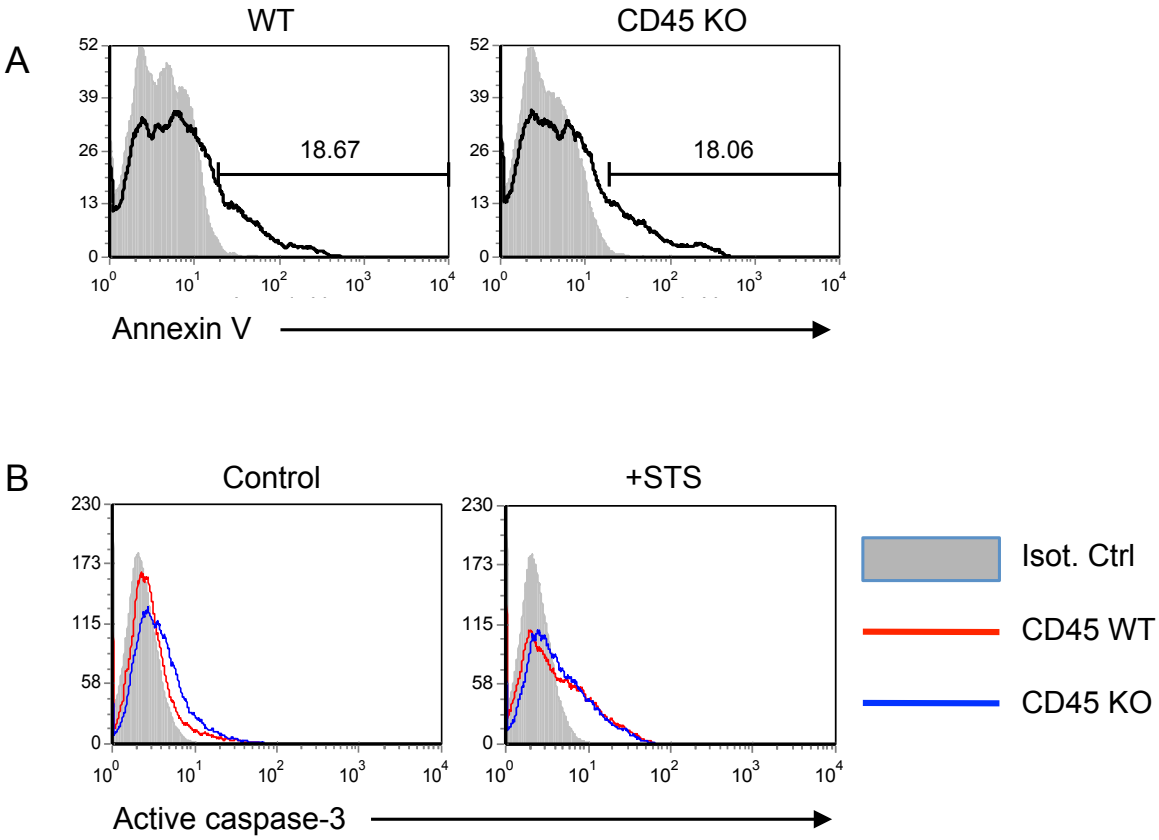
Figure 4.5. Paxillin expression is decreased in CD45 KO BMDM. (A) Paxillin was immunoprecipitated from lysates of 10^7 day 7 WT or CD45 KO BMDM cells, followed by SDS-PAGE and Western blot with anti-paxillin. Paxillin levels were decreased in both immunoprecipitates and total lysates of CD45 KO BMDM compared to WT. Lysate control represents 4 x 10^5 cells. (B) Quantification of paxillin in Western blots of BMDM lysates as represented by a ratio of paxillin in relation to the loading control (Erk). Represented is the average ratio obtained from five independent experiments and the difference is statistically significant ($p<0.000001$). (C) Immunofluorescence staining of paxillin (red) and actin (green) in day 7 CD45 KO and WT BMDM. Confocal images are representative of two independent experiments.

Such decreased paxillin expression in CD45 KO BMDM was also observed by confocal microscopy (Figure 4.5c). In WT BMDM, paxillin was mostly localized to sites of adhesion, however diffuse staining of paxillin was also found in the cytoplasm. In contrast, in CD45 KO BMDM, paxillin staining was substantially weaker, although low amounts of paxillin could still be detected at sites of adhesion and in the cytoplasm (Figure 4.5c). Such decrease in expression of paxillin was specific because no differences in actin expression, or localization, were observed between WT and CD45 KO BMDM (Figure 4.5c).

4.2.5 Decreased expression of paxillin in CD45 KO BMDM is not due to cleavage by caspases

Proteolytic cleavage by caspases has been shown to regulate of the turnover of several cytoskeletal-associated proteins. Such a regulatory mechanism is essential for rapid dismantlement of adhesion complexes needed for adhesion turnover. In apoptotic cells, cleavage of paxillin by caspases leads to cell rounding and detachment (Chay et al., 2002; Harrington et al., 2001; Shim et al., 2001). A number of studies have also showed that CD45 is a regulator of apoptosis (reviewed in Dupere-Minier et al., 2010). For example, several studies have shown that cross-linking of CD45 can induce cell death in eosinophils, T- and B-cells (Blaylock et al., 1999; Ferguson and Ostergaard, 2010; Klaus et al., 1996; Lesage et al., 1997; Ogimoto et al., 1994). Moreover, mice lacking CD45 display decreased thymocyte survival compared to WT (Ferguson and Ostergaard, 2010). As CD45 KO BMDM display a similar phenotype to apoptotic cells in

regards to cell rounding and detachment, I hypothesized that the defects in adhesion in CD45 KO BMDM resulted from apoptosis and increased caspase activation and paxillin degradation. To test this hypothesis, day 7 BMDM from CD45 KO and WT mice were assessed for Annexin V staining and activation of caspase-3 by flow cytometry and Western blot respectively (Figure 4.6a, b, c). No significant differences were observed in Annexin V or anti-active caspase-3 staining between resting CD45 KO and WT BMDM. Thus, CD45 KO cells did not appear to be undergoing higher basal levels of apoptosis. Treatment with staurosporine, a non-specific kinase inhibitor known to induce apoptosis, was used as a positive control for caspase-3 activation. Staurosporine treatment was used to induce apoptosis in BMDM cells to examine whether the triggering of apoptosis would lead to changes in morphology and to decreased paxillin expression in WT macrophages (Figure 4.6d, e). I found that a four-hour treatment with staurosporine induced hyperadhesion in both WT and CD45 KO BMDM without affecting paxillin expression (Figure 4.6d, e). Treatment with staurosporine also induced phosphorylation of paxillin in both cell types, as suggested by the shift in molecular mass. This shift in paxillin molecular mass has been reported previously and has been attributed to serine/threonine phosphorylation (Ku and Meier, 2000; Liu et al., 2002). These results are in apparent contradiction to those observed in the RAW 264.7 macrophage cell line, where staurosporine treatment induced cell rounding and paxillin degradation as measured by flow cytometry (Figure 4.7a, b). This indicates that although paxillin degradation can be observed in macrophages undergoing apoptosis, this


A
WT
CD45 KO
18.67
18.06
Annexin V
B
Control
+STS
Isot. Ctrl
CD45 WT
CD45 KO
Active caspase-3
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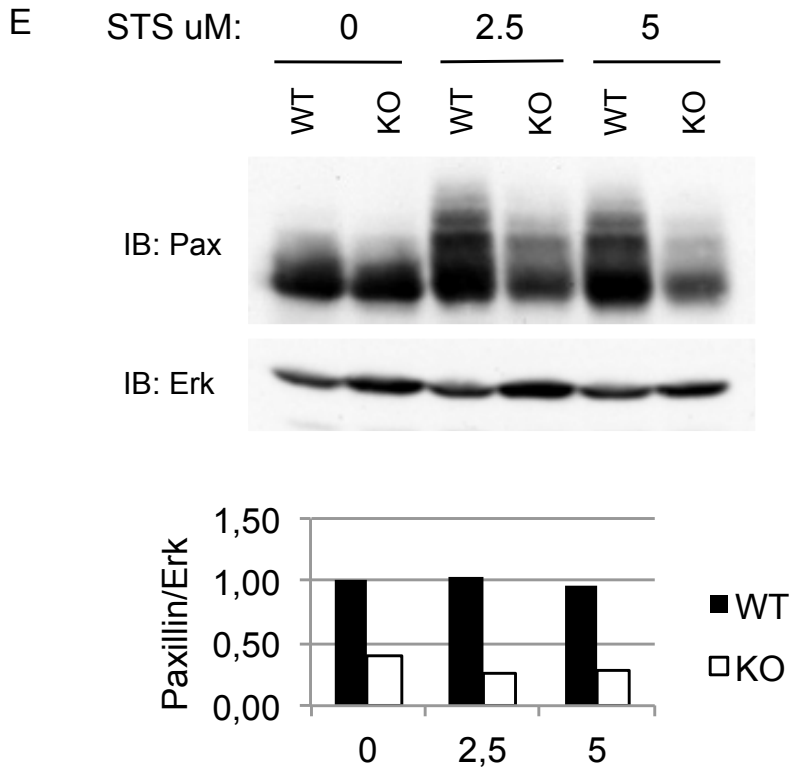

Figure 4.6. Decreased paxillin expression in CD45 KO BMDM is not linked to apoptosis. (A) Unstimulated day 7 WT or CD45 KO BMDM were stained with Annexin V (black line) or left unstained (grey, filled). Both cell types exhibited similar levels of basal Annexin V staining. (B,C) Day 7 WT or CD45 KO BMDM were treated with 5μM of staurosporine (STS) for 4 hours (right panel), or DMSO control (left panel), and stained with anti-active caspase-3. Both cell types displayed similar levels of active caspase-3 upon staurosporine treatment. Staurosporine treatment of CD45 KO and WT BMDM induced changes in cell morphology (D) but did not influence expression of paxillin, as measured by Western blot (E). Quantification of the density of the Western blot bands was done with the Image J software. Experiments shown in this figure are representative of of two (A, B, C) or three (D, E) independent experiments.

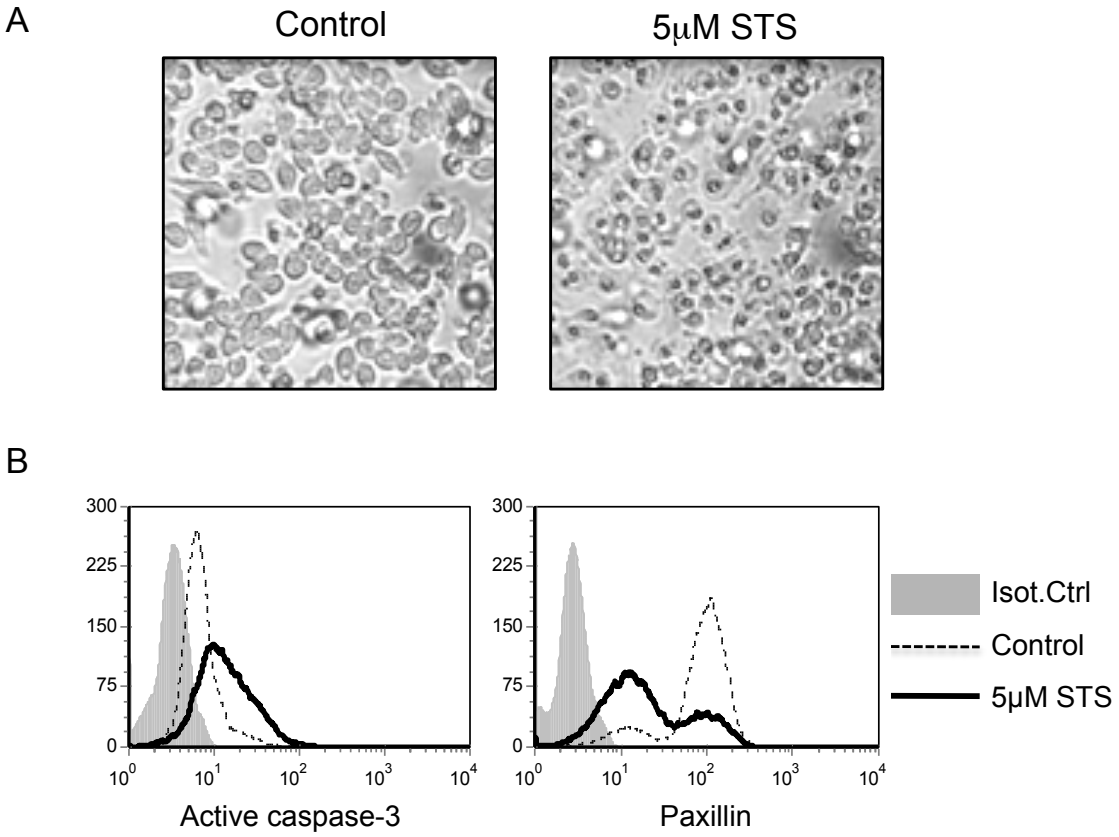

Figure 4.7. Decreased paxillin expression is observed in apoptotic RAW 264.7 macrophages. RAW 264.7 cells were treated with 5μM of staurosporine (STS) or DMSO as a negative control for 4 hours. (A) Morphology of the cells was assessed by light microscopy. (B) Expression of active caspase-3 and paxillin were examined by flow cytometry. Representative of two independent experiments.

observation in the RAW 264.7 murine leukaemic macrophage cell line may not be an accurate model of primary macrophages in terms of adhesion regulation.

Although apoptosis did not correlate with paxillin degradation in CD45 KO BMDM, it was still possible that low levels of caspase activity may lead to paxillin degradation in CD45 KO cells without triggering apoptosis *per se*. In fact, recent studies have shown that caspases have many nonapoptotic functions, including a role in the regulation of cell migration (Kuranaga and Miura, 2007). For example, caspase-8-deficient MEFs display defects in motility and lamellipodia formation (Helfer et al., 2006). Alternatively, it is possible that other caspases are also able to cleave paxillin in macrophages. Such a possibility, however, is unlikely since treatment of macrophages with CI-II, a pan caspase inhibitor, did not restore expression of paxillin in CD45 KO macrophages (Figure 4.8). It is still possible, however, that inability of CD45 KO BMDM cells to maintain adhesion may eventually lead to apoptosis, although this does not occur prior to paxillin degradation.

4.2.6 Treatment of CD45 KO BMDM with a calpain inhibitor restores paxillin expression

Calpains are calcium-dependent, non-lysosomal cysteine proteases that cleave a variety of proteins at sites of focal complexes, thereby favouring a rapid turnover of focal complexes (reviewed in Perrin and Huttenlocher, 2002). Interestingly, paxillin has been shown to be sensitive to proteolysis by calpain

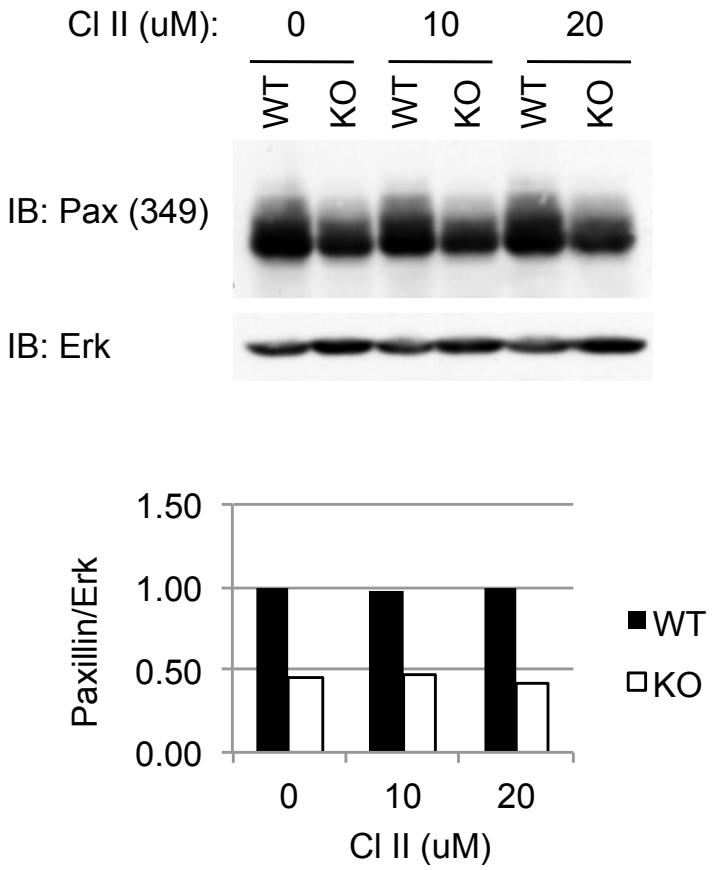

Figure 4.8. Inhibition of caspases does not restore paxillin expression in CD45 KO BMDM. Day 7 BMDM were treated with the indicated concentration of caspase inhibitor II (CI-II) for 4 hours and lysed. Paxillin expression was assessed by Western blot. Anti-Erk was used as a loading control. Quantification of the density of the Western blot bands was done with the Image J software. Representative of three independent experiments.

(Carragher et al., 1999; Liu and Schnellmann, 2003). Cleavage of paxillin occurs at serine 95 has been shown to negatively regulate focal adhesion disassembly and decrease cell migration (Cortesio et al., 2011). Other sites of calpain cleavage are likely present within paxillin, however, as the S95 point mutation only partially prevents cleavage of paxillin.

To assess whether paxillin was susceptible to proteolysis by calpain in BMDM, a calpain inhibitor, ALLN, was used. The results showed that a four-hour treatment of BMDM with ALLN restored paxillin expression in a dose-dependent manner (Figure 4.9a). Paxillin expression in WT BMDM also increased with ALLN treatment, indicating that paxillin is likely regulated by calpain cleavage in these cells as well.

Next, I examined whether the restoration of paxillin expression by ALLN treatment induced changes in macrophage morphology. CD45 KO and WT cells in culture were treated for four hours with ALLN and examined by light microscopy (Figure 4.9b). Treatment of macrophages with ALLN increased, but did not fully restore, cell spreading in CD45 KO in a dose-dependent manner. Cell spreading was slightly increased in WT cells as well. Therefore, the increase of paxillin protein levels in CD45 KO correlated with the increase in cell spreading and stretching.

A link between Src activation and calpain translation and activity has been reported. Carragher et al (2002) have shown that transformation of fibroblasts by v-Src leads to increased translation of calpain, which in turn leads to the degradation of calpastatin, an endogenous calpain inhibitor

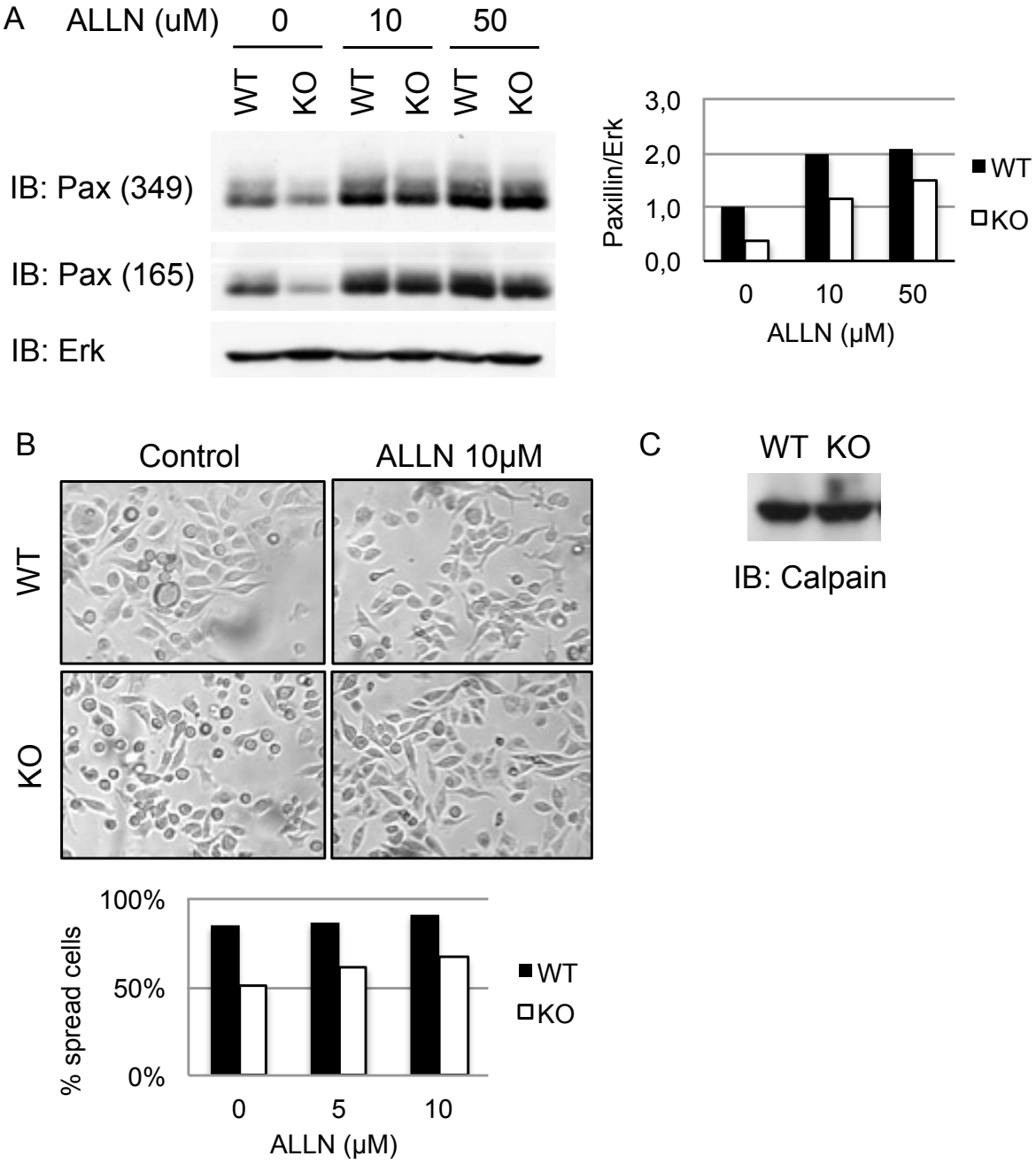

Figure 4.9. Inhibition of calpain restores paxillin expression and increases cell spreading. (A) Day 7 WT or CD45 KO BMDM cell lysates (10^6 cells) were treated with increasing amounts of calpain inhibitor ALLN for 4 hours. Final concentrations are indicated. Lysates of 10^6 cells were run on SDS-PAGE gel and immunoblotted with two anti-paxillin monoclonal antibodies (clones 349 and 165) and anti-Erk as a loading control. Quantification of paxillin expression, probed with antibody from clone 349, relative to Erk was done with the ImageJ software. (B) Light microscopy image and quantification of cell spreading of WT and CD45 KO BMDM upon treatment with 10μM of ALLN or DMSO (control) (C). All experiments shown are representative of three independent experiments.

This ultimately results in increased activity of calpains in these cells. Because SFK activity is increased in the absence of CD45, I verified whether this translated to changes in expression of calpain by Western Blot analysis. No differences in calpain expression, however, were detected between CD45 KO and WT BMDM, suggesting that in CD45 KO cells, degradation of paxillin is not due to increased expression of calpain (Figure 4.9c). The mechanisms by which CD45 might regulate calpain-mediated degradation of paxillin were further explored.

4.2.7 Paxillin expression in CD45 KO cells is not restored upon inhibition of PLC-γ

Calpains are known to be activated by intracellular calcium fluxes. Interestingly, CD45 has been shown to regulate intracellular levels of calcium through the regulation of PLC-γ in several types of immune cell types. In T-cells, for example, co-cross-linking of CD3 and CD45 results in decreased phosphorylation of PLC-γ and reduced mobilization of intracellular calcium (Kanner et al., 1992; Kiener and Mittler, 1989; Ledbetter et al., 1991; Ledbetter et al., 1988; Marvel et al., 1991). A study from Shivnan et al. (1992) has indicated, however, that this inhibitory effect of anti-CD45 cross-linking is non-specific and rather due to the inefficient CD3 cross-linking upon co-ligation. Nonetheless, CD45-deficient T-cells also exhibit defects in PLC-γ phosphorylation and calcium mobilization (Koretzky et al., 1992; Shiroo et al., 1992; Volarevic et al., 1992). In B-cells, monocytes and Langerhans cells, co-cross-linking of CD45 with

antibodies to stimulatory ligands inhibits calcium mobilization (Bieber et al., 1995; Corvaia et al., 1995; Rankin et al., 1993).

To examine whether paxillin degradation is a consequence of increased intracellular calcium levels in CD45 KO BMDM, I examined the role of PLC-γ, a known regulator of intracellular calcium fluxes. I hypothesized that PLC-γ disregulation in CD45 KO BMDM may affect intracellular levels of calcium, leading to activation of calpains and degradation of paxillin. To examine this, cells were treated with the PLC-γ inhibitor U73122, or its inactive analog, U73343, and examined paxillin expression by Western blot (Figure 4.10a). Paxillin expression, as well as cell morphology (Figure 4.10b), of CD45 KO BMDM were both largely unaffected by treatment with the PLC-γ inhibitor. These results demonstrate that CD45 regulation of paxillin degradation is not dependent on PLC-γ signalling.

4.2.8 Activation of the MAPK pathway does not affect paxillin expression

The MAPK pathway has been shown to be activated downstream of integrin-mediated adhesion and is critical for the regulation of adhesion turnover (Huang et al., 2004b; Klemke et al., 1997; Webb et al., 2004). Regulation of paxillin expression by the MAPK pathway could potentially occur through either the regulation of calpain activity or the regulation of paxillin phosphorylation. Thus, I examined the contribution of MAPK in the CD45-dependent regulation of paxillin expression.

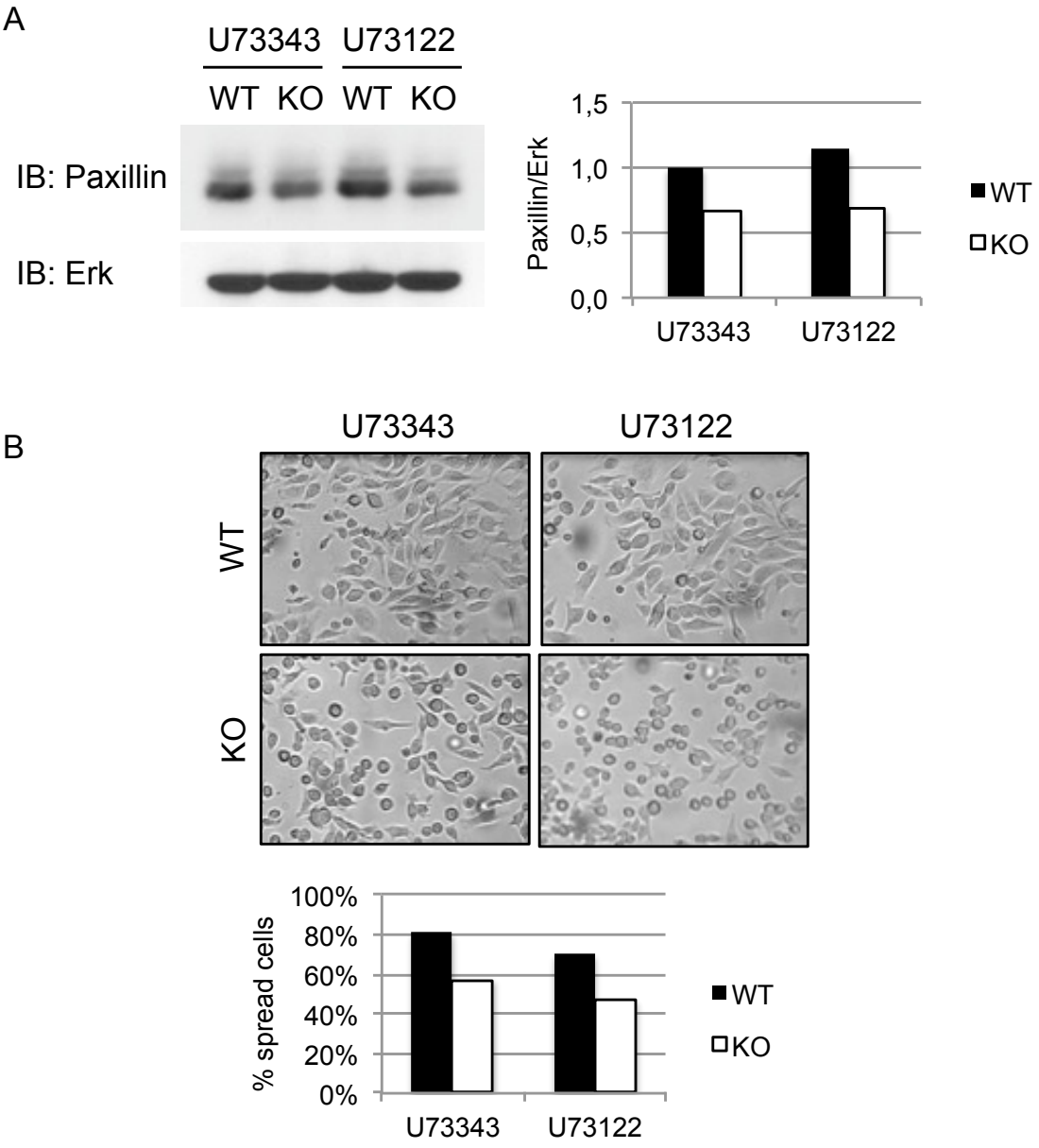

Figure 4.10. Inhibition of PLC-γ signalling does not restore paxillin expression and does not restore cell spreading in CD45 KO BMDM. (A) Day 7 WT or CD45 BMDM were treated for 4 hours with 1μM of the PLC-γ inhibitor U73122 or the inactive analog U73343 and lysed followed by immunoblot with anti-paxillin and anti-Erk. Quantification of paxillin protein levels relative to Erk was done with ImageJ software. Representative of three independent experiments. (B) Cell spreading of WT and CD45 KO BMDM after 4 hours of treatment with 1μM of U73122 or U73343. Quantification of cell spreading for each cell type and treatment. This data is representative of three independent experiments.

Signalling through the MAPK pathway and activation of calpain are both involved in the regulation of adhesion and motility. In addition, links between the two have been established. For example, signalling through Erk, but not PLC-γ, is required for m-calpain activation in EGF-induced fibroblast motility (Glading et al., 2000; Glading et al., 2001). This activation occurs through the direct phosphorylation of calpain by Erk and is thought to happen in the absence of intracellular calcium fluxes (Glading et al., 2004).

Another possible link between the MAPK pathway and the CD45-dependent regulation of paxillin expression is through paxillin phosphorylation. Phosphorylation of paxillin may alter paxillin conformation and expose calpain cleavage sites. Paxillin is serine phosphorylated by both Erk and JNK (Huang et al., 2004a; Huang et al., 2003; Ishibe et al., 2003; Ku and Meier, 2000; Liu et al., 2002). JNK phosphorylation of paxillin at serine 178 has been shown to regulate cell motility and morphology in different cell types (Huang et al., 2003). Serine phosphorylation of paxillin by Erk occurs at several residues and can regulate cell spreading and adhesion (Ku and Meier, 2000; Liu and Schnellmann, 2003). For example, signalling through growth factor receptors, such as hepatocyte growth factor receptor (HGFR), induces serine phosphorylation of paxillin by Erk, which leads to an increase in paxillin binding to FAK (Ishibe et al., 2003; Liu et al., 2002). Inversely, in some cells paxillin is required for adhesion-mediated Erk activation as it acts as a scaffold to recruit Erk to sites of cell adhesion where Erk is subsequently phosphorylated (Hagel et al., 2002). Thus, there is a strong

interdependency between paxillin and MAP kinases in the regulation of cellular adhesion.

CD45 has been shown to regulate the MAPK pathway both in a positive and negative manner (Bijian et al., 2007; Fernandis et al., 2003; Hesslein et al., 2006; Ogimoto et al., 2001). The net effect of CD45 on the MAPK pathway mostly depends on the cell type and experimental conditions. For example, CD45-deficient Jurkat T-cells or WT Jurkat T-cells treated with a CD45 inhibitor show decreased Erk signalling downstream of integrins (Bijian et al., 2007). CD45-deficient NK cells also show decreased phosphorylation of Erk and JNK downstream of various NK receptors compared to WT (Hesslein et al., 2006). On the other hand, in a chemotaxis assay, CD45-deficient Jurkat T-cells show an increase in the MAP kinase activity downstream of the chemokine receptor CXCR4 compared to WT (Fernandis et al., 2003). In light of these studies, I assessed whether the absence of CD45 in macrophages led to the disregulation of the MAPK pathway. I hypothesized that paxillin expression is influenced by the disregulation of the Erk pathway in CD45 KO BMDM. However, it is also possible the absence of paxillin in CD45 KO BMDM could lead to the disregulation of the MAPK pathway as its absence could prevent recruitment of Erk and JNK to sites of adhesion. Because signalling downstream of GM-CSF triggers both Erk and JNK phosphorylation, paxillin expression was examined following the addition of GM-CSF to GM-CSF-starved BMDM (de Groot et al., 1998; Li and Earp, 1997; McLeish et al., 1998; Suzuki et al., 1999). Serine and threonine phosphorylation of paxillin retards the migration of paxillin in SDS-

PAGE gels (Ku and Meier, 2000; Liu et al., 2002). The addition of GM-CSF to BMDM cells induces this mobility shift of paxillin on a SDS-PAGE gel in both WT and CD45 KO BMDM however no notable differences in paxillin expression were observed (Figure 4.11a). Moreover, few proteins were tyrosine phosphorylated upon the addition of GM-CSF, and none of these phosphorylated proteins detected were differentially regulated between WT and CD45 KO macrophages, at the exception of an unidentified protein(s) of approximately 40 kDa (Figure 4.11a). Therefore, tyrosine phosphorylation downstream of GM-CSF does not appear to be majorly regulated by CD45. This result is in line with the fact that CD45 KO macrophages mature normally *in vitro* with GM-CSF.

In Figure 4.11b, GM-CSF induced strong tyrosine phosphorylation of both Erk1/2 and JNK, with the strongest phosphorylation observed at 2 minutes post addition of GM-CSF. However, no significant differences were observed between WT and CD45 KO macrophages in terms of the strength and kinetics of tyrosine phosphorylation of these MAP kinases. As well, basal levels of phospho-Erk were similar in both cell types, demonstrating that this pathway is not affected by the absence of CD45 in unstimulated cells. This also eliminated the possibility of the GM-CSF-induced and hyperphosphorylated proteins of approximately 40 kDa observed in the CD45 KO cell lysates in Figure 4.11a were indeed Erk or JNK. These results are in accordance with a study from Suh et al. (2005) where treatment of macrophages with anti-CD45RO did not affect Erk and JNK signalling upon treatment with GM-CSF. Therefore, the decreased expression of paxillin in CD45 KO BMDM is not a consequence of the disregulation of its

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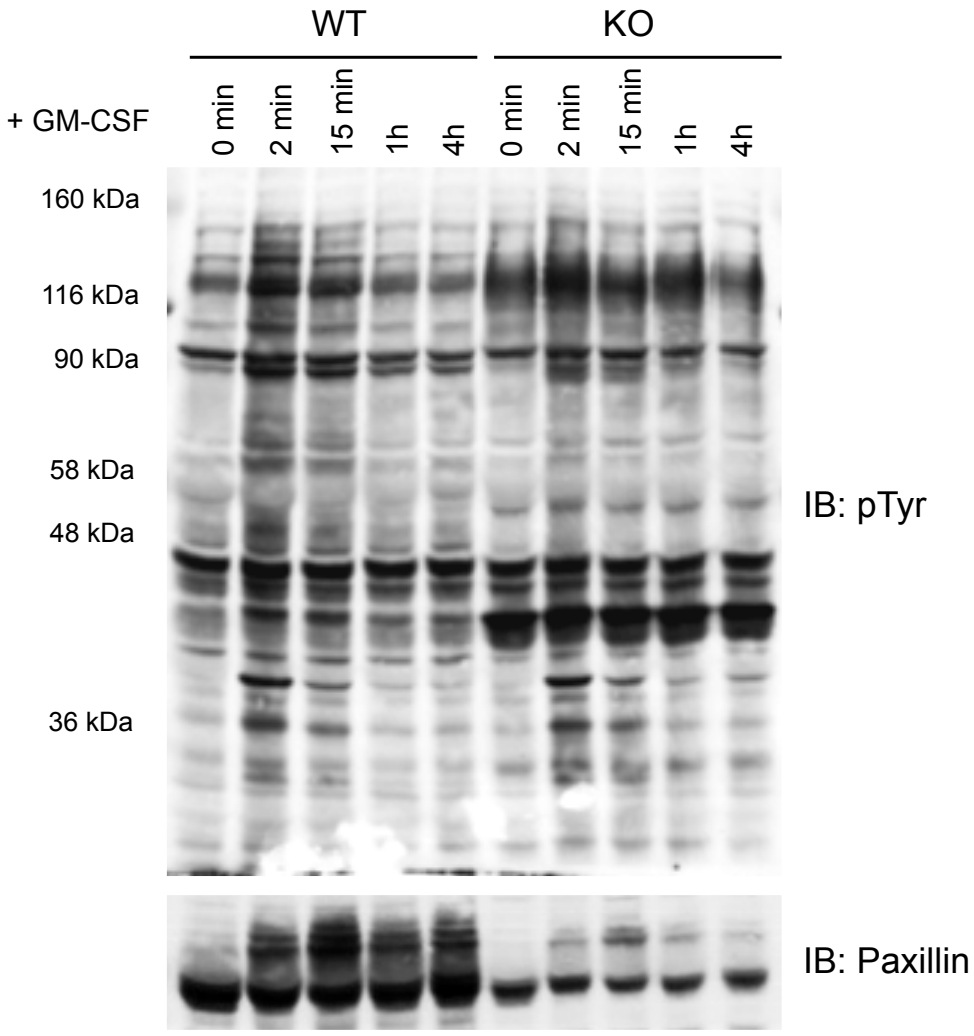
WT
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+ GM-CSF
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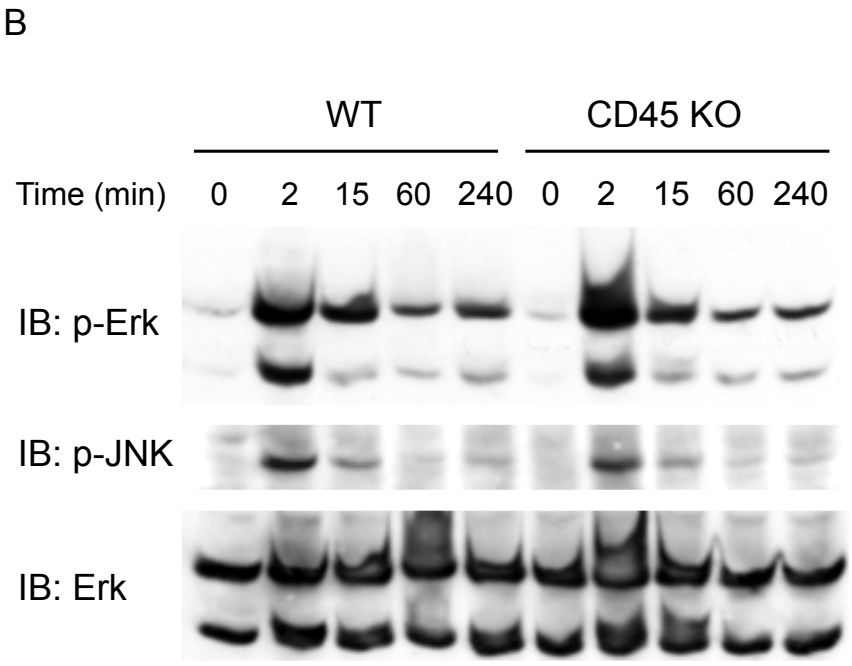

Figure 4.11. Signaling downstream of GM-CSF and the Erk/MAPK does not affect paxillin expression in CD45 KO BMDM. Day 7 BMDM were GM-CSF-starved for 24 hours prior to the addition of RPMI containing 25ng/ml of GM-CSF. Cells were lysed at the indicated times after the addition of GM-CSF. Lysates were probed with anti-phosphotyrosine and paxillin (A) or with anti-phospho-Erk, anti-phospho-JNK and total Erk (B). Stimulation downstream of the GM-CSF receptor, and through the MAPK pathway, triggered paxillin phosphorylation, as indicated by the molecular weight shift, but had no effect on the expression levels of paxillin (A). No differences in Erk and JNK phosphorylation were observed between WT and KO macrophages. Blots are representative of three independent experiments.

serine phosphorylation by MAP kinases. Moreover, its decreased expression does not affect MAPK signalling downstream of the GM-CSF receptor in CD45 KO BMDM. It is still possible however that this signalling pathway is impacted by CD45 downstream of other stimuli.

4.2.9 Paxillin expression and cell spreading is restored in CD45 KO macrophages upon inhibition of Pyk2/FAK

In normal adherent cells, calpain induces the limited proteolysis of cytoskeletal-associated proteins without leading to their complete degradation (Carragher et al., 2002; Cortesio et al., 2011; Franco et al., 2004). For example, in DCs, the inhibition of calpains by ALLN prevents cleavage of Pyk2, talin and WASP without affecting their overall protein levels (Calle et al., 2006). In the case of CD45 KO BMDM, however, calpain cleavage of paxillin leads to a decrease in its protein levels, indicating that there is amplification of this regulation mechanism to the point where there is depletion of paxillin. As calpain expression is not affected by the absence of CD45 (Figure 4.9), it is possible that cleavage of paxillin by calpains is related to the disregulation of paxillin itself rather that the disregulation of calpain activity. Post-translational modifications of paxillin could lead to its increased susceptibility to cleavage. Since I have already shown that serine/threonine phosphorylation of paxillin does not result in paxillin proteolysis in WT BMDM (Figure 4.11a), here, I hypothesized that tyrosine phosphorylation of paxillin may possibly contribute to its increased susceptibility to calpain cleavage. This hypothesis is supported by previous

studies showing that tyrosine phosphorylated paxillin has been shown to localize to focal complexes and focal adhesions, where it regulates adhesion turnover (Zaidel-Bar et al., 2007). The main kinases responsible for tyrosine phosphorylation of paxillin are Pyk2 and FAK (Turner, 1998, 2000). These kinases are directly phosphorylated and activated by SFKs, making them potential downstream targets of CD45 (Dikic et al., 1996; Korade-Mirnics and Corey, 2000). Moreover, upon CD44-mediated cell spreading of T-cells, Pyk2 and FAK exhibit increased phosphorylation in CD45 KO cells (Li et al., 2001; Wong et al., 2011). This increased Pyk2 and FAK phosphorylation is dependent on SFKs.

To examine the contribution of Pyk2 and FAK in paxillin proteolysis, I treated the cells for 4 hours with the Pyk2/FAK inhibitor PF431396. This inhibitor prevents ATP binding and kinase activity of both Pyk2 and FAK (Han et al., 2009). Upon inhibition of Pyk2 and FAK, paxillin expression was restored in CD45 KO BMDM (Figure 4.12a). Indeed, prior to treatment, paxillin expression was significantly different between WT and CD45 KO BMDM ($p<0.01$) whereas differences in paxillin expression between WT and CD45 BMDM after treatment with 5 μM of PF431396 were not significantly different. This indicates that tyrosine phosphorylation of paxillin by one, or both, of these kinases promotes its cleavage by calpains.

This experiment also provided an opportunity to test whether restoration in paxillin expression resulted in changes in macrophage morphology. For this purpose, I examined cell morphology of CD45 KO or WT BMDM by light

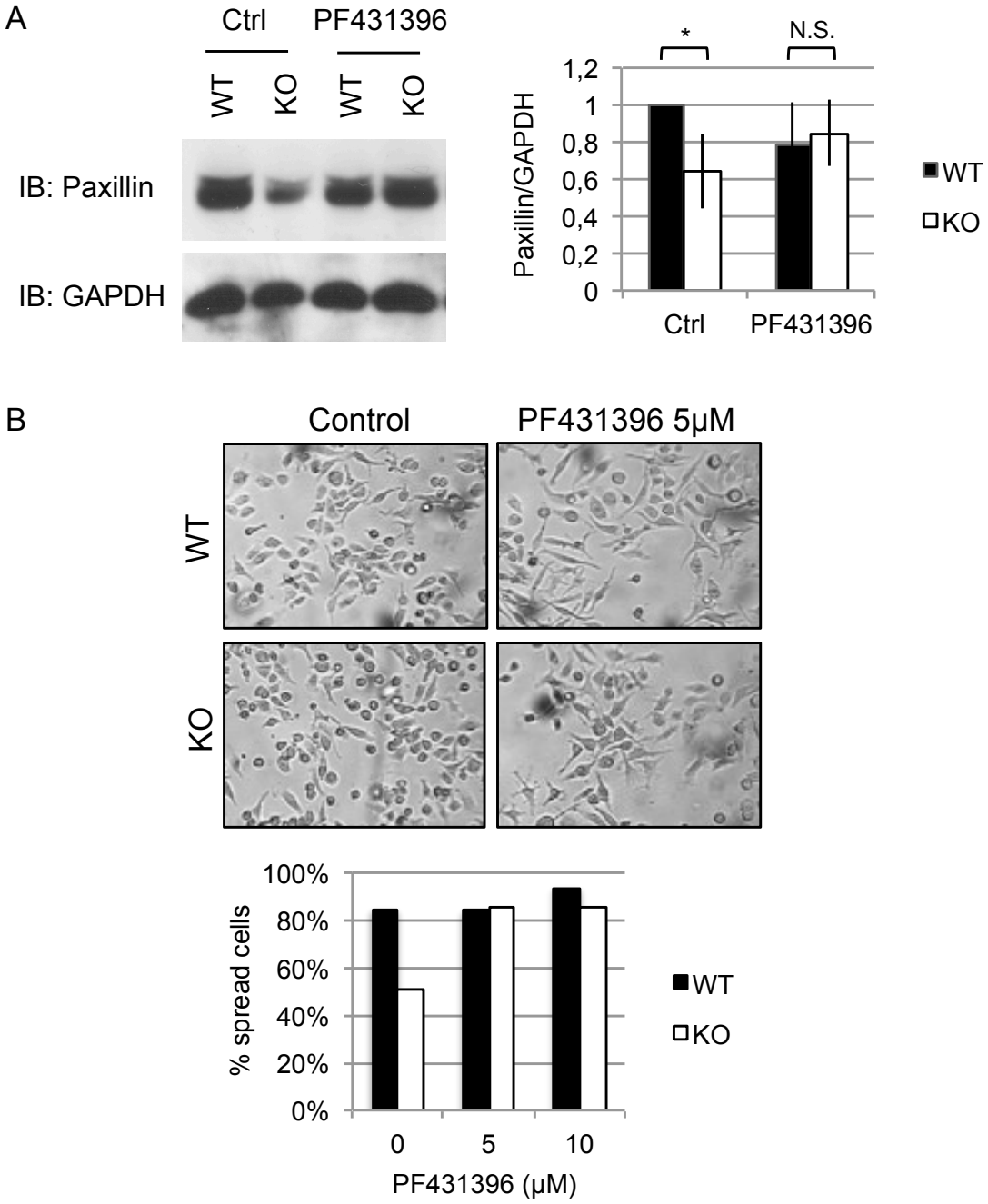

Figure 4.12. Inhibition of Pyk2 and FAK restores paxillin expression and cell spreading. (A) Day 7 WT or CD45 KO BMDM were treated with 5μM of the Pyk2/FAK inhibitor PF431496, or DMSO carrier, for 4 hours. Lysates were run on SDS-PAGE gel and immunoblotted with anti-paxillin and anti-GAPDH as a loading control. Quantification of paxillin in Western blots of BMDM lysates as represented by a ratio of paxillin in relation to the loading control (GAPDH). Represented is the average ratio obtained from three independent experiments. Differences in paxillin expression between DMSO-treated WT and CD45 KO BMDM are significant ($p<0.01$) but not between inhibitor-treated WT and CD45 KO BMDM. (B) Light microscopy and cell spreading quantification upon treatment with PF431396, or DMSO as control, for 4 hours. Data is representative of three independent experiments.

microscopy after a 4-hour treatment with increasing concentrations of PF431396 (Figure 4.12b). I found that even at low concentration, treatment with PF431396 restored a morphological phenotype in CD45 KO BMDM that was similar to that of WT macrophages. Such morphological changes correlated with the restoration of paxillin expression. The changes in morphology were more pronounced than what was observed with ALLN (Figure 4.9b). Whether this is due to the fact that CD45-regulation of Pyk2 and FAK directly contribute to defects in adhesion through a different pathway than calpain-mediated cleavage of paxillin remains to be determined.

4.2.10 CD45 KO mice exhibit high proportion of macrophages in the spleen

I next hypothesized that absence of CD45 in mice alters macrophage migration and localization. I have observed that CD45 KO mice exhibit enlarged spleens and higher cellularity (Figure 4.13a). This may be due to the significant increase in splenic B-cells observed in CD45 KO mice, despite their decrease in splenic T-cell numbers (Byth et al., 1996). However, there have been no reports concerning a possible effect of absence of CD45 on the numbers of macrophages in the spleen. To clarify this question, I have isolated splenocytes from 12 week-old CD45 KO or WT mice and stained them with anti-F4/80 for analysis by flow cytometry. My results showed that CD45 KO mice exhibited both higher frequency and higher total numbers of F4/80 positive splenocytes compared to WT mice (Figure 4.13b, c). Although the effect was not statistically significant due to the high variation, the general trend observed might be indicative of

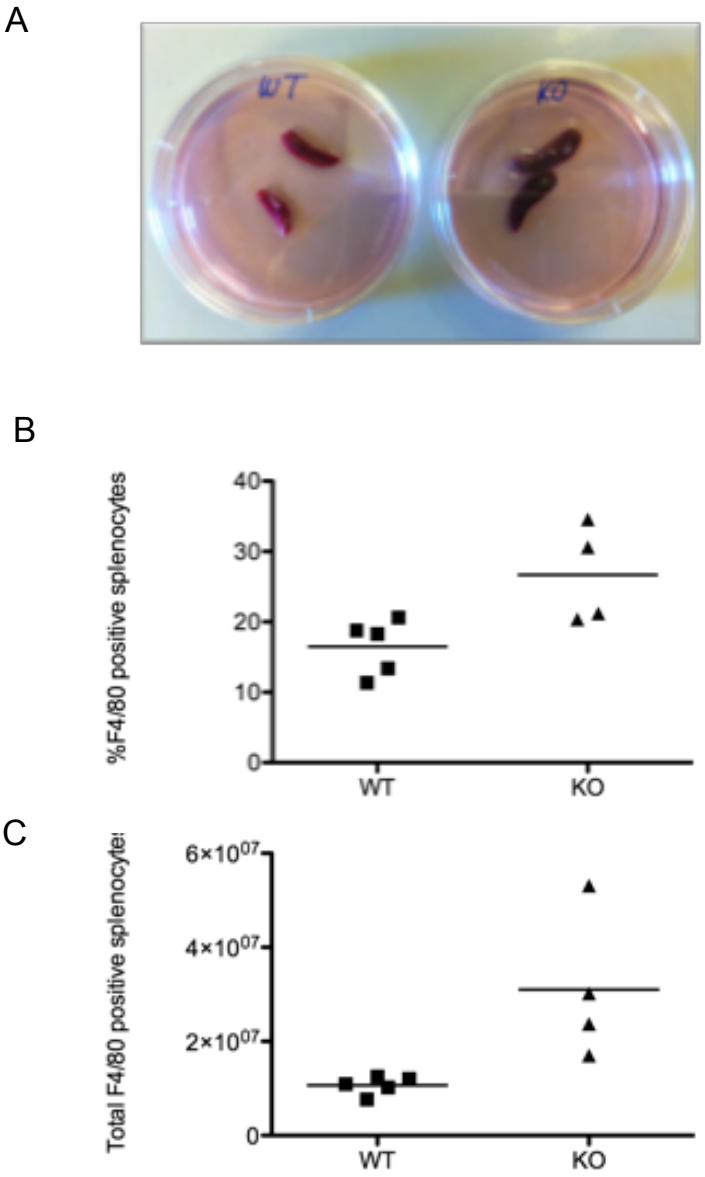

Figure 4.13. CD45 KO mice exhibit enlarged spleens and an increase in splenic macrophage numbers. (A) Spleens extracted from 12 week-old female WT or CD45 KO mice. Percentage of F4/80 positive cells (B) and total number of F4/80 positive cells (C) from splenocytes isolated from five 12 week-old WT or four 12 week-old CD45 KO female mice and stained with PE-conjugated anti-F4/80 or isotype control.

migratory defects *in vivo*. An alternative approach to the study of macrophage migratory defects *in vivo* will be needed to explore this further.

4.3 Discussion

In this chapter, I studied the detailed molecular mechanisms implicating CD45 in adhesion, cell morphology and cell motility in macrophages. More specifically, I found that 1) CD45 KO BMDM displayed a reduced ability to maintain integrin-mediated adhesion, 2) CD45 KO BMDM showed defects in cell spreading and motility, 3) The absence of CD45 led to an increase in calpain-mediated cleavage of paxillin, 4) The degradation of paxillin was inhibited with an inhibitor of Pyk2/FAK, and 5) The absence of CD45 in macrophages significantly altered the tyrosine phosphorylation status of several proteins, including SFKs. Together, these results provide evidence that in macrophages, tyrosine phosphorylation events downstream of CD45 play a central role in their adhesion and migration functions.

The inability of macrophages to maintain adhesion to ECM components has been previously demonstrated using *in vitro* M-CSF-derived CD45 KO BMDM (Roach et al., 1998). I have found a similar phenotype using GM-CSF-derived CD45 KO BMDM. Such defects were concomitant with changes in cell morphology and cell motility. Although such a phenotype is typical of cells undergoing apoptosis, no signs of apoptosis (such as elevated levels of AnnexinV staining and activation of caspase-3) were detected in cultured CD45 KO BMDM. These results are thus consistent with a model whereby CD45 regulates specific

signalling pathways that normally control the turnover of adhesion structures, whether during assembly or their disassembly, or both.

The differences in the tyrosine phosphorylation patterns that I observed between CD45 and WT cell populations are consistent with previous studies on the molecular mechanisms regulating adhesion. In order to uncover the mechanisms underlying CD45-dependent adhesion regulation, I first assessed differences in tyrosine phosphorylation (Figure 4.4). In addition to the hyperphosphorylation of SFKs in CD45 KO cells, which has been well established in the litterature, I have observed hyperphosphorylation of several proteins in the 116-140 kDa MW range. Although I have not determined the identity of these proteins, their identification and the role they play in CD45-regulated cell adhesion is an interesting avenue for future investigations, particularly so because several SFK-regulated cytoskeletal-associated proteins migrate in this MW range, including FAK (125 kDa), Pyk2 (112-116 kDa), p130Cas (130 kDa), and vinculin (145 kDa). Therefore, it is thus logical to predict that some of these proteins are potential downstream targets of CD45 during macrophage adhesion.

In the present work, I focussed on the cytoskeletal-associated protein paxillin as a potential downstream target of CD45. The rationale was based on the knowledge that paxillin is phosphorylated by SFKs and plays a key role in the assembly and turnover of adhesion structures. I initially hypothesized that paxillin would be hyperphosphorylated in CD45 KO BMDM. This hypothesis could not be tested, however, because paxillin expression was drastically reduced

in CD45 KO BMDM, making the detection of paxillin phosphorylation difficult. These results led to further investigation on the role of caspases and calpains, both of which have been shown to regulate adhesion complex turnover by cleaving cytoskeletal-associated proteins including paxillin. Using specific pharmacological inhibitors of calpain and caspases, I found that calpains, rather than caspases, were responsible for paxillin degradation in CD45 KO BMDM. Interestingly, restoration of paxillin expression also increased cell spreading in CD45 KO BMDM. Altogether, these results suggest the existence of a previously unidentified mechanism of CD45-dependent regulation of macrophage adhesion.

I then sought to identify by which means CD45 could promote paxillin degradation. I hypothesized that CD45 could regulate paxillin degradation either by the activation of cellular calpains, or through the regulation of paxillin itself. In the former scenario, for example, CD45 could regulate calpain activation through the control of intracellular calcium mobilization or through direct activation of calpains by Erk phosphorylation. In the latter scenario, phosphorylation of paxillin could lead to conformational changes in the protein, leading to exposed sites of calpain cleavage. This could occur either through serine/threonine phosphorylation which is mediated by MAP kinases, or through tyrosine phosphorylation mediated by Pyk2, FAK and SFKs. Both the MAPK and the tyrosine kinases Pyk2, FAK and SFKs have been shown to be regulated by CD45 and may be disregulated in the absence of this protein (Bijian et al., 2007; Deszo et al., 2001; Fernandis et al., 2003; Hesslein et al., 2006; Li et al., 2001; Ogimoto et al., 2001; Wong et al., 2011; Zhang et al., 2005). Our results

with inhibitors of PLC-γ, a known regulator of calcium mobilization showed, however, that paxillin degradation in CD45 KO cells is most likely independent of intracellular calcium levels. The fact that Erk phosphorylation in resting and GM-CSF-stimulated macrophages were similar in WT and CD45 KO BMDM, combined with our observation that expression levels of calpain were identical in WT and CD45 KO BMDM, further support the idea that CD45 regulation of paxillin expression is not linked to activation of calpains, but rather to paxillin itself. For this purpose, I first examined whether the triggering of the MAPK pathway in BMDM would lead to increased paxillin susceptibility to calpain cleavage in WT BMDM. Our results showed, however, that although paxillin displayed a mobility shift that is typical of serine/threonine phosphorylation, such a shift did not correlate with increased paxillin cleavage in WT cells and did not restore paxillin expression in CD45 KO BMDM.

Secondly, I assessed whether tyrosine phosphorylation of paxillin would increase its susceptibility to calpain cleavage. FAK is involved in the regulation of paxillin phosphorylation, and this step is critical for focal adhesion assembly (Richardson et al., 1997). This may occur due to direct phosphorylation of paxillin by FAK, or by bringing paxillin and Src in close proximity, leading to phosphorylation of paxillin by Src (Richardson et al., 1997; Thomas et al., 1999). Pyk2, a relative of FAK, can also associate with paxillin and is thought to regulate paxillin phosphorylation in a similar manner (Hiregowdara et al., 1997; Li and Earp, 1997; Salgia et al., 1996; Schaller and Sasaki, 1997). I hypothesized that these kinases were involved in the regulation of paxillin degradation through

tyrosine phosphorylation of paxillin. Upon treatment of BMDM with a Pyk2/FAK inhibitor, PF431396, paxillin expression and cell spreading were restored in CD45 KO BMDM. Since this inhibitor blocks the kinase activity of both Pyk2 and FAK, the ability of these kinases to phosphorylate substrate is therefore required (Han et al., 2009). This supports the hypothesis that tyrosine phosphorylation of paxillin by Pyk2 and/or FAK increases the susceptibility of paxillin to calpain-mediated cleavage. However, further experiments will be needed to confirm the status of paxillin phosphorylation prior to its degradation by calpains.

Interestingly, the major calpain cleavage site on paxillin (S95) is found in between the two major sites of tyrosine phosphorylation in paxillin (Y31 and Y118). It is thus possible that phosphorylation of paxillin at these sites promotes a conformation of the protein in which the calpain cleavage site is exposed. As calpains and phosphorylated paxillin are both found at sites of adhesion, proteolysis of paxillin at these sites may lead to focal complex disassembly and ultimately, to cell rounding and detachment. This model thus provides an additional mechanism by which Pyk2 and/or FAK regulate adhesion turnover.

Because Pyk2 and FAK play a key role in CD45-regulated paxillin degradation, it is likely that these kinases are hyperphosphorylated and hyperactivated in the absence of CD45. It has not been possible, however, to assess their phosphorylation status due to their low basal phosphorylation levels. At present, it is nevertheless logical to suggest that increased SFK activity in CD45 KO cells would lead to increased phosphorylation and activity of Pyk2

and/or FAK, both of which are direct substrates of SFKs. Increased activity of Pyk2 and/or FAK could lead to an increase in phosphorylation and subsequent proteolysis of paxillin. This is a likely scenario based on the following reasons: 1) Pyk2 and FAK are the major kinases responsible for tyrosine phosphorylation of paxillin (Hiregowdara et al., 1997; Li and Earp, 1997; Park et al., 2004; Salgia et al., 1996; Schaller and Sasaki, 1997), 2) their inhibition restores paxillin expression in CD45 KO BMDM (Figure 4.12a) and 3) their inhibition promotes cell spreading in CD45 KO BMDM (Figure 4.12b). Moreover, the MW of these kinases corresponds to the MW of hyperphosphorylated proteins in CD45 KO BMDM. However, further experiments will be needed to confirm this model.

I attempted to demonstrate that SFKs were also involved in the CD45-regulation of paxillin proteolysis. The use of PP2, however, generated inconsistent results with regards to restoration of paxillin expression. Moreover, treatment with PP2 leads to increased cell rounding in both WT and CD45 KO BMDM without affecting the ability of CD45 KO cells to spread upon contact with ECM proteins. Due to the fact that SFKs are upstream of many signalling pathways involved in cell adhesion, the contribution of their role in the regulation of calpain-mediated cleavage of paxillin may be difficult to determine experimentally. Nevertheless, there are many studies reporting the hyperphosphorylation and hyperactivation of SFKs in CD45-deficient immune cells and SFKs are responsible for phosphorylation of Pyk2, FAK and paxillin. It is thus reasonable to suggest that SFK disregulation in CD45 KO BMDM does contribute to the phenotype reported in this chapter. Additional experiments will

be needed to confirm whether SFKs provide the link between CD45 and Pyk2/FAK-mediated regulation of paxillin cleavage by calpains.

In summary, the results presented in this chapter have revealed a yet undescribed mechanism by which CD45 regulates macrophage adhesion, morphology and motility. This mechanism is based on the ability of CD45 to regulate calpain-mediated cleavage of paxillin in a Pyk2/FAK-dependent manner. Further understanding of this pathway will be important in the identification of methods to regulate macrophage adhesion and migration in disease.

CHAPTER FIVE: CD45-MEDIATED REGULATION OF THE MACROPHAGE RESPONSE TO FUNGAL STIMULI

5.1 Introduction

The ability of macrophages to recognize and react to pathogens is an important part of the innate immune response. Few studies have investigated the role of CD45 in the regulation of macrophage responses to pathogens in great detail. A study from Liles et al. (1995) has shown that the cross-linking of CD45 synergistically enhanced the respiratory burst and TNF-α secretion in monocytes stimulated with various stimuli such as LPS. A recent study from Goodridge et al. (2011) has reported that CD45-deficient BMDM exhibit decreased reactive oxygen species (ROS) and TNF-α production upon stimulation with whole glucan particles (WGP). Others have reported that CD45-deficient splenic macrophages show no differences in their ability to produce nitric oxide, present antigen upon challenge with the *L. monocytogenes* (Fujise et al., 1997). Phagocytosis of *L. monocytogenes* or WGP also seems unchanged in macrophages lacking CD45 (Fujise et al., 1997; Goodridge et al., 2011). As the response to microbes involves numerous receptors that trigger distinct signalling pathways, it is possible that CD45 regulates only a restricted number of these pathways. Therefore, the role of CD45 in the macrophage response to microbes requires further investigation.

Zymosan is a protein-carbohydrate mixture that has been widely used as a model fungal particle to study the inflammatory and phagocytic response of

macrophages to fungal stimuli. Its preparation was first described by Pillemer and Eckert in 1941 (Pillemer and Ecker, 1941). This method involves acid treatment, boiling and trypsinization of the single cell yeast *Saccharomyces cerevisiae* and generates particles consisting of the yeast cell wall, also known as "ghost" cells, of approximately 3μm in diameter that mostly consists of beta-glucans (β-1,3-glucans and β-1,6-glucans) and mannan (Di Carlo and Fiore, 1958; Pillemer and Ecker, 1941).

A variety of macrophage receptors have been shown to bind to zymosan, including Dectin-1, Dectin-2, the Toll like receptor heterodimer TLR2/6, mannose receptor and complement receptor 3 (CR3) (Berton and Gordon, 1983; Goodridge et al., 2009; McGreal et al., 2006; Roeder et al., 2004). Multiple studies have provided evidence that SFKs play a role in the regulation of downstream signalling and macrophage responses triggered by these PRRs. In the case of Dectin-1 and Dectin-2, this is expected as Dectin-1 possesses an ITAM in its cytoplasmic tail and Dectin-2 associates with an ITAM-containing molecule (Herre et al., 2004; Saijo and Iwakura, 2011). Many studies have confirmed SFK involvement in their downstream signalling. For example, PP2 pretreatment of monocytes or macrophages prior to zymosan-stimulation inhibits a variety of cell responses, including arachidonate release, Dectin-1-mediated superoxide production, and Dectin-1-mediated Syk phosphorylation (Elsori et al., 2011; Olsson and Sundler, 2007; Underhill et al., 2005). Macrophage responses upon Dectin-2 ligation, including cytokine secretion, ligand internalization and NF-κB

activation, require its association with FcRγ as well as SFK activity (Sato et al., 2006).

Although the regulation of TLR signalling by SFKs is controversial, some studies have found that inhibition of SFKs by PP2 or knockdown of Hck by siRNA inhibits the secretion of inflammatory cytokines upon treatment with TLR ligands (Smolinska et al., 2008; Smolinska et al., 2011). Moreover, stimulation of TLR2 and TLR4 have been shown to induce tyrosine phosphorylation of paxillin and Pyk2 in a SFK-dependent manner (Hazeki et al., 2003). Finally, CR3-dependent internalization of non-opsonized zymosan is dependent on Hck activity (Le Cabec et al., 2002). As CD45 is a key regulator of SFK activity, I hypothesized that the absence of CD45 from BMDM would lead to the disregulation of downstream signalling and macrophage responses to zymosan stimuli.

5.2 Results

5.2.1 CD45 KO cells are activated upon stimulation with zymosan and LPS

In order to study the role of CD45 in macrophage function upon stimulation with PAMPs, I examined whether CD45 KO BMDM can be activated upon treatment with fungal and bacterial stimuli. For this purpose, I treated WT and CD45 KO BMDM with 100μg/ml of unopsizined zymosan or 10μg/ml of LPS for 24 h at 37°C. Activation of BMDM was measured by comparing increased expression of CD69 and CD40 by flow cytometry (Alderson et al., 1993; Marzio et al., 1997). CD69 is a transmembrane C-type lectin and, although

its expression is constitutive on platelets and Langerhans cells, its expression is upregulated upon activation of lymphocytes, neutrophils and macrophages (Bieber et al., 1992; Gavioli et al., 1992; Gerosa et al., 1991; Lanier et al., 1988; Marzio et al., 1997; Testi et al., 1989; Testi et al., 1990). In macrophages, treatment with LPS, beta-glucans or a combination of TNF-α and IFN-γ leads to the upregulation of CD69 (Li et al., 2007; Marzio et al., 1997). CD40 is a costimulatory receptor of the TNF receptor family (reviewed in Schonbeck and Libby, 2001). It is constitutively expressed at low levels on macrophages and upregulated upon activation by stimuli including IFN-γ, LPS and zymosan (Alderson et al., 1993; Hoebe et al., 2003; Karumuthil-Melethil et al., 2008; Nguyen et al., 1998; Qin et al., 2005; Shibata et al., 2011). I found that treatment of BMDM with LPS or zymosan led to a substantial increase in the expression of both CD69 and CD40 (Figure 5.1). The initial levels of CD69 and increase in expression following stimulation is similar between both cell types, however some differences were observed in CD40 expression. Detection of CD40 levels on the surface of CD45 KO BMDM was below the levels detected in WT cells. Nonetheless, I can conclude that both markers are considerably upregulated upon stimulation with LPS and zymosan, which suggests that the absence of CD45 does not drastically impair activation of macrophages upon stimulation with the PAMPs LPS or zymosan.

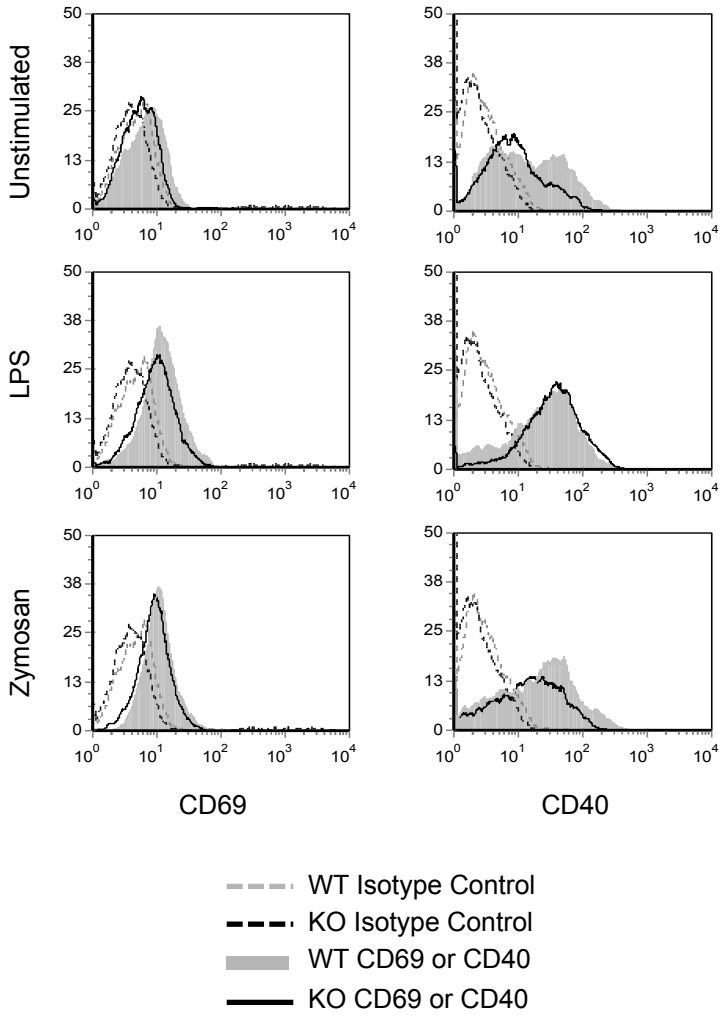

Figure 5.1. WT and CD45 KO BMDM upregulate activation markers CD69 and CD40 upon stimulation with LPS and zymosan. Day 7 BMDM from WT (grey, filled) or CD45 KO (black line) were left unstimulated or incubated 24 hours with 10μg/ml of LPS or 100μg/ml of zymosan. Cells were then stained with PE-conjugated anti-CD69, FITC-conjugated anti-CD40, or corresponding isotype controls. FACS plots shown are representative of two independent experiments.

5.2.2 The absence of CD45 does not affect the cell surface expression of PRRs Dectin-1 and Dectin-2

I next assessed whether the absence of CD45 would lead to variations in the cell-surface expression of PRRs responsible for macrophage activation by zymosan. For this purpose, I stained BMDM derived from WT or CD45 KO progenitor cells with anti-Dectin-1 or anti-Dectin-2 Abs and carried out flow cytometric analysis. These analyses showed that both PRR receptors were expressed at similar levels between WT and CD45 KO BMDM (Figure 5.2). This allowed for investigation in the functional differences between CD45 KO and WT BMDM stimulated with zymosan, as any differences observed in the response to zymosan between these cells are not to be attributable to differences in receptor expression.

5.2.3 The absence of CD45 leads to disregulation of tyrosine phosphorylation downstream of zymosan stimulation

Because many the PRRs known to bind zymosan have been shown to signal through SFKs, I hypothesized that the absence of CD45 would affect tyrosine phosphorylation downstream of zymosan stimulation (Elsori et al., 2011; Le Cabec et al., 2000; Olsson and Sundler, 2007; Sato et al., 2006; Smolinska et al., 2008; Smolinska et al., 2011; Underhill et al., 2005). To test this hypothesis, BMDM were incubated with 50μg/ml of unopsinized zymosan and lysed with 1X RSB. Equal amounts of protein lysates were subjected to SDS-PAGE gel electrophoresis and immunoblotted for phosphotyrosine. The results showed that

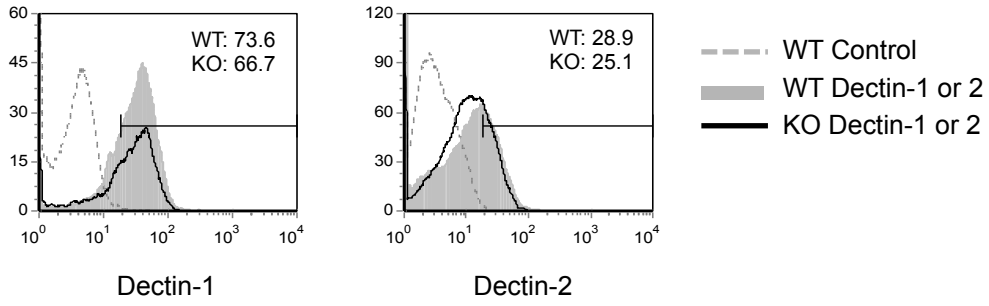

Figure 5.2. WT and CD45 KO BMDM express similar levels of the zymosan receptors Dectin-1 and Dectin-2. Day 7 BMDM were stained with Alexa-647-conjugated anti-Dectin-1 or anti-Dectin-2 followed by Alexa488-conjugated anti-rat, or corresponding isotype controls. FACS plots are representative of two independent experiments.

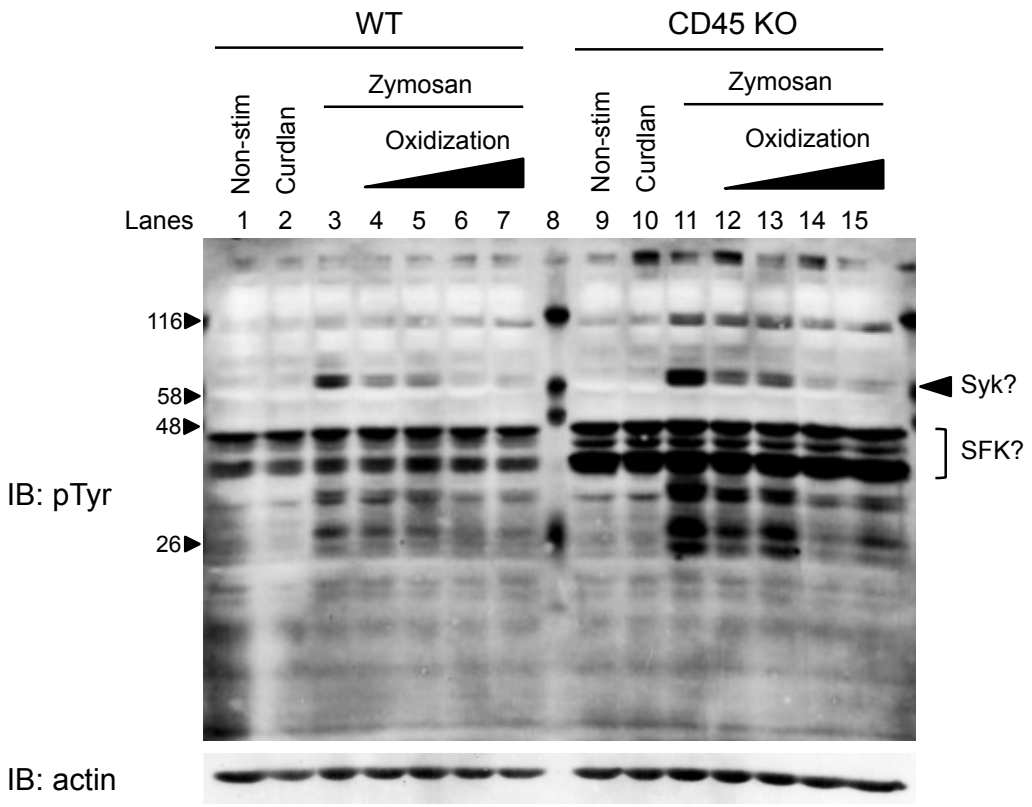

Figure 5.3. Tyrosine phosphorylation upon zymosan treatment is not triggered through beta-glucan recognition in WT and CD45 KO BMDM. BMDM were stimulated for 15 minutes with curdlan, zymosan or zymosan treated with increasing amounts of NaClO (0.01, 0.1, 0.5 and 1.0%) followed by lysis and Western blots with anti-phosphotyrosine and anti-actin as a loading control. Western blot is representative of two independent experiments.

stimulation of BMDM with zymosan triggered the tyrosine phosphorylation of multiple proteins (Figure 5.3, lanes 3 and 11). Many of these proteins showed increased tyrosine phosphorylation in CD45 KO BMDM in comparison to WT BMDM, including proteins in the 30-40 kDa, 50 kDa, 80 kDa, and 115 kDa range. It is important to note that the molecular weight standards used migrate slower than their predicted molecular weight.

In mouse BMDM, Dectin-1 is considered the main receptor for zymosan (Brown et al., 2002). Moreover, as Dectin-1 contains an ITAM motif in its cytoplasmic tail and induces tyrosine phosphorylation of protein kinases upon ligation, it was logical to expect that this receptor triggered tyrosine phosphorylation in BMDM (Herre et al., 2004; McCann et al., 2005; Rogers et al., 2005; Slack et al., 2007). To test this possibility, BMDM were stimulated with β-glucan stimuli: curdlan and oxidized zymosan. Curdlan is a pure preparation of β-1,3-glucans known to specifically bind to Dectin-1 and to induce downstream signalling in macrophages and DCs (Goodridge et al., 2009; Palma et al., 2006; Xu et al., 2009). In addition, zymosan was treated with increasing amounts of sodium hypochlorite (NaClO). This treatment of zymosan results in a product mainly composed of beta-glucans recognized by Dectin-1 (Hida et al., 2006). I found that stimulation of BMDM with curdlan failed to induce any significant tyrosine phosphorylation in either WT or CD45 KO BMDM (Figure 5.3, lanes 2 and 10). Moreover, although the native zymosan preparation induced tyrosine phosphorylation in both WT and CD45 KO BMDM, zymosan-induced tyrosine phosphorylation was inversely correlated with the beta-glucan content of zymosan

(Figure 5.3). Thus, in contrast to my inital hypothesis, beta-glucan recognition by Dectin-1 did not appear to contribute to the induction of tyrosine phosphorylation in BMDM.

5.2.4 The main receptor for zymosan in BMDM signals through Syk

It is well established that Syk is tyrosine phosphorylated downstream of Dectin-1 and Dectin-2/FcRgamma upon treatment with unopsonized zymosan (Robinson et al., 2009; Rogers et al., 2005). Its activation occurs through a multistep process initiated by its autophosphorylation at 10 major tyrosine sites, including Y352 and Y525/526 in humans (Y346 and Y519/520 in mice, respectively) (Furlong et al., 1997). CD45 has been shown to be upstream of Syk in NK cells (Huntington, Xu et al, 2005). In these cells, the absence of CD45 is associated with decreased phosphorylation of Syk upon stimulation through Ly49D.

Following our observation that treatment of BMDM with zymosan triggered the phosphorylation of a protein of ~ 70 kDa (Figure 5.3), I hypothesized that this protein could be Syk and that the absence of CD45 would lead to the disregulation of this kinase. To examine whether Syk phosphorylation was triggered by zymosan treatment in our assay and whether this was regulated by CD45, I stimulated WT or CD45 KO BMDM with various amounts of zymosan for 15 minutes followed by cell lysis and SDS-PAGE. Syk phosphorylation was assessed by Western blot by using antibodies specific to phosphorylated tyrosines 352 and 525 of Syk. Syk phosphorylation was induced

upon stimulation with as little as 50μg/ml of zymosan in both WT and CD45 KO cells (Figure 5.4). No significant differences were observed with respect to the degree of phosphorylation detected with these antibodies. A Western blot for total levels of Syk protein was also done and the bands co-migrate with the bands observed with the Syk phospho-specific antibodies. It is noteworthy to mention that Syk appears as a doublet on SDS-PAGE gels as it undergoes multiple conformational and phosphorylation states, making quantification difficult. However, I have observed that in CD45 KO BMDM lysates, Syk appears to have a more substantial migratory shift; the significance of which could not be determined with the available reagents. I cannot therefore eliminate the possibility that CD45 could regulate phosphorylation at one of the other sites on Syk. In conclusion, there are no detectable differences in Syk phosphorylation at Y352 and Y525/26 upon zymosan stimulation between CD45 KO and WT BMDM. Taken together, these results confirm that zymosan induces Syk activation in macrophages that is not dependent on CD45.

5.2.5 The absence of CD45 leads does not affect cytokine secretion upon stimulation with zymosan

Macrophages lacking CD45 have been shown to produce less TNF-α upon stimulation with WGP (Goodridge et al., 2011). I thus examined whether the absence of CD45 altered the inflammatory response to zymosan. More specifically, I investigated whether the absence of CD45 would alter the secretion of two cytokines known to be secreted upon zymosan stimulation, TNF-α and IL-

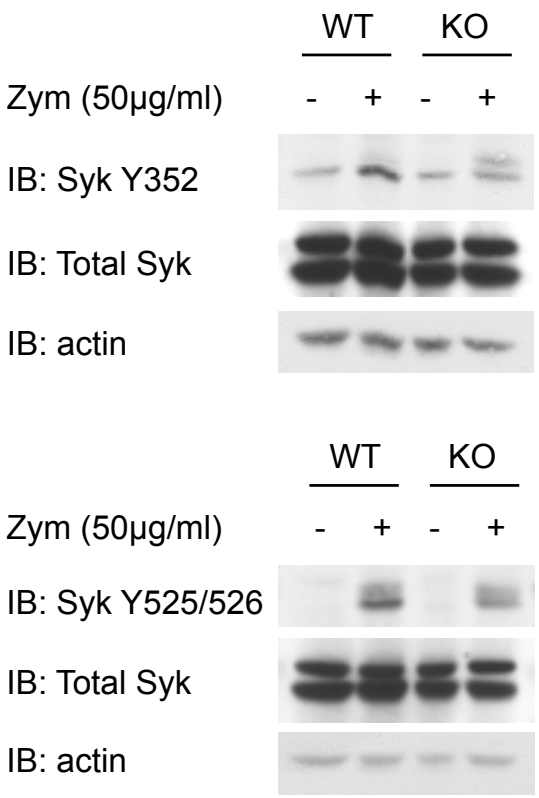

Figure 5.4. Syk is phosphorylated downstream of zymosan stimulation in both WT and CD45 KO BMDM. Day 7 BMDM from CD45 KO or WT mice were stimulated with with 50µg/ml of zymosan for 15 minutes, or left unstimulated. Cells were then lysed and samples were loaded onto SDS-PAGE gel. Syk phopshorylation at Tyrosines 352 and 525/526 was assessed by Western blot with site-specific phospho-Syk antibodies. Western blots are representative of two independent experiments.

10 (Du et al., 2006; Stein and Gordon, 1991). For this purpose, WT or CD45 KO BMDM were incubated with zymosan for various times. Supernatants were then collected and cytokine secretion was measured by ELISA. As shown in Figure 5.5a, TNF-α and IL-10 exhibited similar kinetics and levels of secretion by WT and CD45 KO BMDM stimulated with zymosan. No significant differences were also detected in the threshold of activation for TNF-α and IL-10 secretion, as measured by cytokine secretion in the presence of increasing amounts of zymosan (Figure 5.5b). Thus, although signalling events downstream of zymosan are regulated by CD45, this does not appear to affect secretion of TNF-α and IL-10.

5.2.6 The absence of CD45 leads to enhanced binding to zymosan particles and increased phagocytosis

Phagocytosis is a tightly coordinated process, which involves multiple cell-surface molecules, signalling components, and elements of the cytoskeleton. There is ample evidence for the participation of SFKs in the phagocytosis of zymosan (reviewed in Berton et al., 2005). This role is likely dependent on two aspects: 1) the ability of SFKs to phosphorylate ITAM motifs on zymosan receptors; and/or 2) on receptor-associated molecules and their regulatory function in cytoskeletal rearrangements. For example, IgG-mediated phagocytosis of erythrocytes was greatly reduced in BMDM isolated from $hck^{-/-}$ $fgr^{-/-}lyn^{-/-}$ mice as compared to WT mice (Fitzer-Attas et al., 2000).

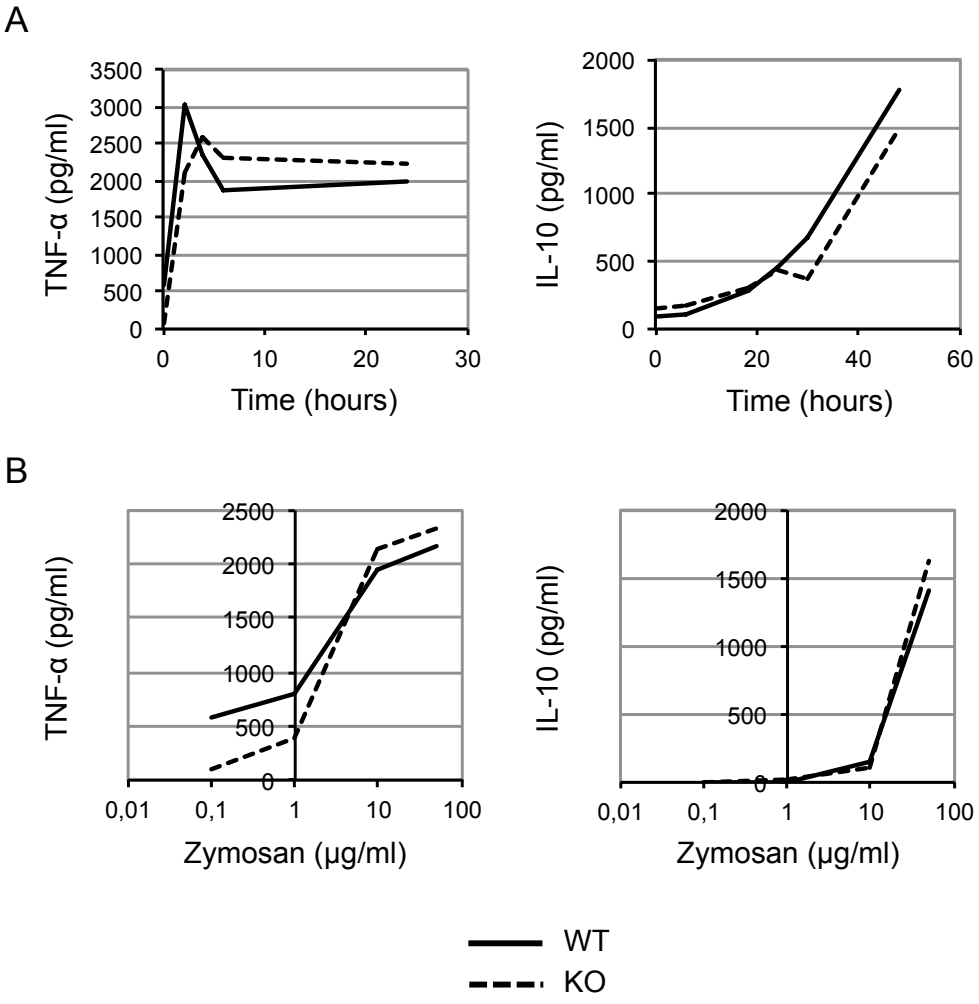

Figure 5.5. The absence of CD45 does not affect TNF-α and IL-10 secretion upon stimulation with zymosan. (A) Day 7 BMDM were stimulated with 50μg/ml of zymosan for various times. Supernatants were analyzed for TNF-α and IL-10 secretion by ELISA. (B) Day 7 BMDM were stimulated with various amounts of zymosan for 24 hours. Supernatants were analyzed for TNF-α and IL-10 secretion by ELISA. Data presented is representative of three independent experiments.

BMDM from these triple knockout mice exhibited reduced phosphorylation of the ITAM-containing FcγR, as well as delayed actin cup formation. SFKs have also been shown to participate in the phagocytosis of zymosan particles. For example, in human granulocytes, neutrophils and macrophages, Hck and Lyn have been shown to localize at the phagosome upon zymosan internalization (Astarie-Dequeker et al., 2002; Mohn et al., 1995; Welch and Maridonneau-Parini, 1997a, b). Also, PP2 has been shown to decrease Dectin-1-mediated phagocytosis of zymosan in Dectin-1-transfected NIH 3T3 cells (Herre et al., 2004).

Phagocytosis also triggers the phosphorylation of paxillin (Greenberg et al., 1994), a cytoskeletal-associated protein, which I have shown to be regulated by CD45 (Chapter 4). In macrophages, FcR-mediated phagocytosis triggered the phosphorylation of paxillin, and paxillin colocalized with actin at the phagocytic cup (Greenberg et al., 1994). A similar finding was also found in human monocyte-derived macrophages, where FcγR-mediated phagocytosis triggered the phosphorylation of paxillin, Pyk2 and Hck while showing localization of paxillin around the phagosome (Kedzierska et al., 2001). Because CD45 regulates SFK signalling and cytoskeletal rearrangements, I sought to determine whether the absence of CD45 would lead to impaired phagocytosis of zymosan particles by BMDM. To test this hypothesis, FITC-conjugated zymosan particles were incubated with BMDM at a 5:1 ratio for various times and phagocytosis was assessed by flow cytometry. Two negative controls were used to determine the amount of bound, but non-internalized, zymosan particles. The first control consisted of BMDM incubated with fluorescent zymosan particles at 4°C for 30

minutes while a second control consisted of BMDM incubated with zymosan particles for 30 minutes at 37°C in presence of lidocaine, an inhibitor of phagocytosis (Das and Misra, 1994). Phagocytosis of zymosan was followed over 30 minutes at 37°C. CD45 KO BMDM showed increased binding to zymosan particles compared to WT compared to the two negative controls (Figure 5.6, left panels). More specifically, I found that between 57 and 66% of BMDM from CD45 KO mice bound to zymosan particles in absence of phagocytosis as compared to approximately 45% in the case of BMDM from WT mice. Differences in phagocytosis upon incubation of macrophages with zymosan at 37°C were also observed: BMDM from CD45 KO mice reached a maximal level of phagocytosis after 5 min of incubation, whereas it took 30 min for BMDM fromWT mice to reach such maximal a level (Figure 5.6, right panels). Taken together, these results show that the presence of CD45 on the cell surface of macrophages alters their binding to zymosan particles and that CD45 influences the intake of zymosan by these cells.

5.2.7 Discussion

In this chapter, I examined the role of CD45 in the response of macrophage to fungal stimuli, more specifically the downstream tyrosine phosphorylation, phagocytosis and cytokine secretion triggered upon stimulation with zymosan. I found that CD45 deficiency did not drastically alter the capacity of macrophages to activate to PAMPs. Moreover, although the absence of CD45 led to the enhanced tyrosine phosphorylation of many proteins downstream of

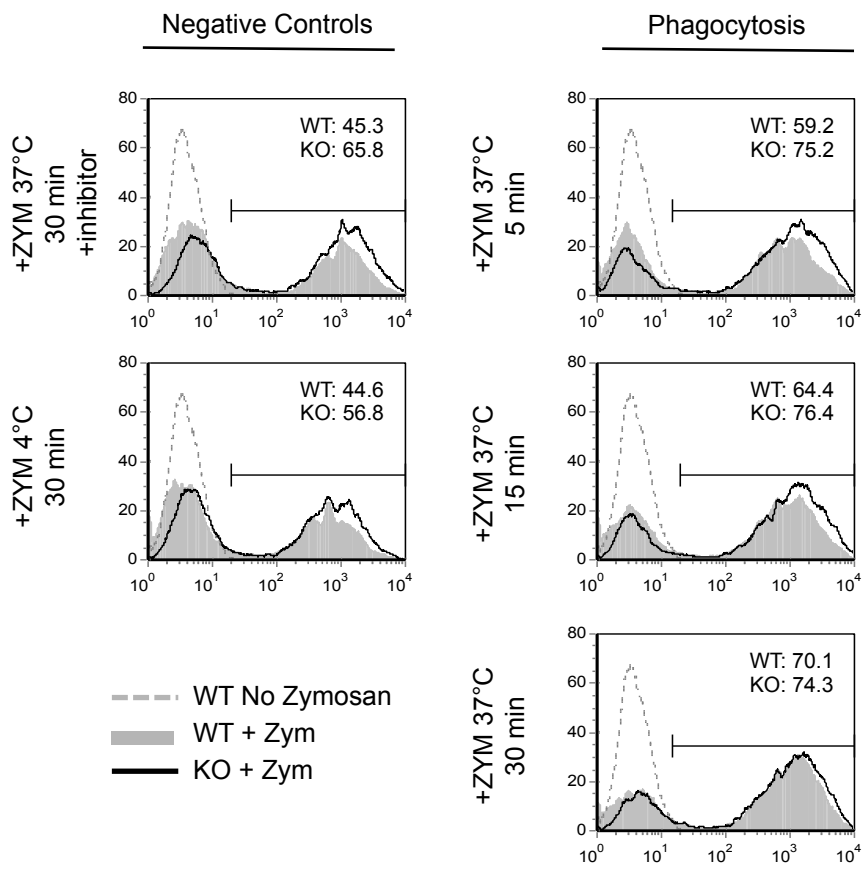

Figure 5.6. The absence of CD45 leads to enhanced binding to zymosan particles and increased rate of phagocytosis. FITC-conjugated zymosan particles were incubated with BMDM at a ratio of 5:1 for the indicated times at 37°C. Phagocytosis was halted at various times with an inhibitor of phagocytosis, lidocaine hydrochloride. As negative controls, zymosan particles were incubated with BMDM at 4°C or in the presence of lidocaine hydrochloride for 30 minutes. FACS plots are representative of two independent experiments.

zymosan treatment, no significant changes in Syk phosphorylation or secretion of TNF-α and IL-10 were observed. The absence of CD45 did, however, lead to increased binding of zymosan particles and rate of phagocytosis of these particles by macrophages. Therefore, I have shown that CD45 regulates only certain aspects of the macrophage response to zymosan.

The difference I observed in the tyrosine phosphorylation pattern between BMDM from WT and CD45 KO mice is interesting. Several of these proteins had a molecular mass in the 40 kDa to 120 kDa range. A study by Liles et al. (1995) showed that TNF-α induced tyrosine phosphorylation of several proteins within the same molecular range following cross-linking with anti-CD45 Abs. Many signalling proteins downstream of zymosan receptors are found in this range. The identification of these proteins will be necessary in order to dissect the signalling pathways that are regulated by CD45. Nonetheless, I have found that Syk is phosphorylated downstream of zymosan stimulation, however, I found no quantifiable difference in the phosphorylation status of this kinase at Y352 and Y525/26 between CD45 KO and WT cells. CD45 does not appear to regulate the ability of these sites to be phosphorylated since they are indeed phosphorylated upon zymosan stimulation in the absence of CD45. I cannot eliminate a contribution to the regulation of phosphorylation at other sites by CD45, however they could not be examined with the reagents currently available. Considering these results, it is possible that the tyrosine phosphorylated protein observed in the 70 kDa MW range in Figure 5.3 corresponds to Syk. Western blots for total Syk did co-migrate with the bands upon stripping and reprobing of the same blots.

Zymosan-induced tyrosine phosphorylation does not seem to be triggered by the recognition of beta-glucan contained in the zymosan preparation, as stimulation of BMDM with curdlan or with oxidized zymosan did not induce tyrosine phosphorylation of these proteins. Therefore, although Dectin-1 and CR3 (CD11b) are expressed at high levels on BMDM, they do not appear to be upstream of these phosphorylated proteins in response to zymosan. The PRR responsible for tyrosine phosphorylation downstream of zymosan in BMDM is likely to bind to mannans or chitins contained in the zymosan preparation. This is consistent with earlier reports that demonstrated that in human monocyte-derived macrophages, zymosan uptake is mediated via mannans (Speert and Silverstein, 1985).

Although I have not identified the PRR responsible for triggering tyrosine phosphorylation downstream of zymosan, the fact that it binds to mananns or chitin and induces Syk phosphorylation considerably narrows down the list of putative receptors involved. Dectin-2 is a likely candidate as it binds to mannans and its downstream signalling is SFK-dependent (Sato et al., 2006). It has also been shown to signal through Syk upon recognition of *Candida albicans* (Robinson et al., 2009). Other signalling molecules known to be downstream of Dectin-2 are phospholipase C gamma 2 (PLC γ 2) and Erk (Gorjestani et al., 2011; Suram et al., 2010). CD45 has been shown to regulate the phosphorylation of both of these molecules (Bijian et al., 2007; Fernandis et al., 2003; Hesslein et al., 2006; Kanner et al., 1992; Kiener and Mittler, 1989; Koretzky et al., 1992; Ledbetter et al., 1991; Ledbetter et al., 1988; Marvel et al., 1991; Ogimoto et al.,

2001; Shiroo et al., 1992; Volarevic et al., 1992). Additional studies will be needed to identify the phosphorylated proteins and the upstream PRR in order to determine whether this pathway is regulated by CD45.

The contribution of CD45 to the regulation of zymosan-induced cytokine secretion in macrophages had not been previously reported. Although CD45 regulates TNF-α production upon stimulation of Dectin-1 with WGP, I did not observe any differences in the secretion of TNF-α or IL-10 between BMDM from WT or CD45-deficient mice upon stimulation with zymosan (Goodridge et al., 2011). These results are consistent those reported by (Zhu et al., 2008) which showed that TNF-α secretion was unchanged by the absence of CD45 in BMDM stimulated through the FcR. These results would be consistent with Dectin-2/FcR γ being the primary receptor for zymosan in this system.

Although I have demonstrated that CD45 deficiency in macrophages leads to defects in cytoskeletal-associated functions such as adhesion and motility, it did not affect the amount of phagocytosed zymosan particles at 30 minutes. This is consistent with a recent study from Goodridge et al. (2011) that showed that BMDM from CD45-deficient mice did not exhibit defects in zymosan internalization as compared to normal BMDM. This result might reflect a redundancy in the function of phosphatases. For example, the CD148 cell surface phosphatase has been shown to compensate for the lack of CD45 and KO of both is necessary to significantly alter zymosan phagocytosis (Goodridge et al., 2011). Alternatively, absence of effect in zymosan internalization might also reflect a redundancy in the phagocytic receptors, as deficiency in any one of these

receptors does not significantly affect internalization of zymosan (Underhill and Ozinsky, 2002). The effect of CD45 on phagocytosis might therefore be subtler than what is represented at 30 minutes of incubation at 37°C and might require and more quantifiable approach than a FACS-based phagocytosis assay. Nonetheless, this approach did demonstrate that BMDM from CD45 KO mice bound more zymosan particles compared to normal BMDM and such differences have not been reported to date.

The reason for increased binding of CD45-deficient macrophages to zymosan particles may lie in the sheer size of CD45 on the cell surface. CD45 is an abundant cell-surface protein that possesses a large and heavily glycosylated extracellular domain (Alexander, 2000; Thomas, 1989). Interestingly, upon phagocytosis of fluorescently-labelled zymosan, CD45 is excluded from the contact site with zymosan in the phagosome (Goodridge et al., 2011). This is reminiscent of TCR signalling, where CD45 is excluded from the TCR complex upon antigen presentation by APCs (Leupin et al., 2000). This is thought to allow SFK activation at the TCR complex (Thomas and Brown, 1999). Steric constraint has been proposed as a reason for the exclusion of CD45 from the T-cell-APC contact site (Springer, 1990). Thus, the absence of CD45 from the cell surface of macrophages would favour increased binding of receptors to PAMPs, as observed in the phagocytosis experiment.

The rate of phagocytosis of zymosan particles by CD45-deficient macrophages was also increased compared to WT. The amount of zymosan ingested by CD45-deficient BMDM plateaued at 5 minutes of incubation at 37°C

whereas WT BMDM took 30 minutes to reach similar levels. Although the initial increased binding would contribute to this, it is also possible that this is a CD45-regulated occurrence. This is supported by the fact that although the SFKs Hck, Fgr and Lyn are not required for phagocytosis, their absence significantly reduces the rate of Fc γ R phagocytosis (Fitzer-Attas et al., 2000; Majeed et al., 2001). In a scenario where SFKs are hyperactive, as is the case in CD45-deficient macrophages, this may contribute to an increase in the rate of phagocytosis of zymosan. Additionally, because SFKs are key regulators of the cytoskeleton, they might positively regulate elements of the cytoskeleton that are involved in phagocytosis. All things considered, although the net effect of CD45 deficiency on phagocytosis of zymosan is not significant at later time-points, CD45 might still actively participate in the regulation and coordination of signalling pathways necessary for microbial internalization.

In summary, the data showed that CD45 is involved in the regulation of the tyrosine phosphorylation of many proteins downstream of zymosan recognition. Moreover, in contrast to the paradigm stating that Dectin-1 is the main receptor for zymosan recognition in macrophages, tyrosine phosphorylation was beta-glucan independent. I also showed that absence of CD45 led to an increase in binding to zymosan particles and kinetics of phagotycosis. The absence of CD45, however, did not affect the production of TNF- α and IL-10 upon stimulation of macrophages with zymosan. Further characterization of the specific PRRs and their respective downstream pathways involved in these

responses will provide additional information into which of these are regulated by CD45.

CHAPTER SIX: GENERAL DISCUSSION

6.1 Summary of results

The ability of macrophages to regulate their adhesion and motility is narrowly linked to their capacity to respond to infection. In this study, I investigated the mechanisms that regulate Pyk2 and paxillin in macrophages during adhesion, as well as the role of the protein tyrosine phosphatase CD45 in macrophage adhesion, motility and responses to a fungal stimulus.

In the first aim of this study, I examined the requirements for Pyk2 and paxillin interaction and localization in macrophages through the use of two polyclonal antibodies, which recognize the C-terminal and the N-terminal of Pyk2 (F245 and F298, respectively). I demonstrated that Pyk2 recovered with the F245 antiserum corresponded to a population of Pyk2 that displayed a lower MW on SDS-PAGE gel and Western blot and associated with paxillin at the MTOC. The F298-immunoreactive fraction of Pyk2, on the other hand, migrated slower on SDS-PAGE gel, localized below the membrane and did not associate with paxillin. Moreover, Pyk2 recovered with the F298 antiserum displayed higher serine/threonine phosphorylation levels than the F245-recovered Pyk2 population. I also found that the differences in MW displayed by each fraction of Pyk2 were not due to phosphorylation or to expression of Pyk2 isoforms. Instead, I showed that the slower migrating form of Pyk2 was immunoprecipitated with an anti-SUMO-1 antibody, suggesting that this form of Pyk2 is either directly SUMOylated or associates with a SUMOylated protein. In all, these results show

that Pyk2 is found as distinct molecular species that are defined by their serine/threonine phosphorylation, their association with paxillin as well as their intracellular localization in macrophages.

The function of CD45 has been extensively studied in lymphocytes, however, not much is known of its role in macrophages. Previous studies had shown that CD45 regulates macrophage adhesion although the molecular mechanisms involved had not been defined (Roach et al., 1997). In chapter 5, I presented data that revealed that the lack of CD45 expression in macrophages leads to a reduced ability to maintain attachment and defects in cell spreading and motility. I established that these adhesion defects are likely due to the enhanced calpain-mediated cleavage of paxillin in CD45 KO macrophages. Moreover, tyrosine phosphorylation is likely involved in calpain-mediated cleavage of paxillin as not only are several proteins hyperphosphorylated in macrophages in the absence of CD45, but inhibition of the kinase activity of Pyk2/FAK in these cells restores paxillin protein levels. Together, these results provide insight in the mechanisms by which CD45 is capable of regulating macrophage adhesion.

Finally, I demonstrated that CD45 possesses a minimal role in the regulation of macrophage responses to zymosan, despite the involvement of many ITAM-containing molecules in mediating signalling downstream of this fungal PAMP. Indeed, the absence of CD45 did not significantly alter the ability of macrophages to upregulate activation markers and to secrete TNF-α and IL-10 upon zymosan stimulation. The absence of CD45 did, however, alter the ability of macrophages to bind to zymosan particles and hence, enhanced rate of

phagocytosis of these particles. Through these experiments, I also revealed that although Dectin-1 is considered the main receptor for zymosan in macrophages (Brown et al., 2002), it did not possess a major role in triggering downstream tyrosine phosphorylation in murine BMDM stimulated with zymosan particles. Instead, another receptor, which remains to be identified, induced tyrosine phosphorylation of several proteins, including Syk. In all, these results show that CD45 does not possess a major role in the regulation of many of the macrophage responses to zymosan and that Dectin-1 does not contribute significantly to tyrosine phosphorylation upon stimulation of BMDM.

6.2 Spatial organization of Pyk2 populations in macrophages

A role for Pyk2 in macrophage migration and adhesion has been previously demonstrated, however not much is known on the regulation of this kinase in macrophages (Allen et al., 1997; De Nichilo and Yamada, 1996; Hirano and Kanno, 1999; Okigaki et al., 2003). The data presented in chapters 3 and 4 provide insight into the localization and regulation of Pyk2 during macrophage adhesion. I have demonstrated that Pyk2 of distinct apparent MW possess distinct staining patterns by confocal microscopy and overall serine/threonine phosphorylation levels. Indeed, the slower migrating form of Pyk2 was localized below the membrane, possibly with the cytoskeleton, and the faster migrating Pyk2 localized predominantly at the MTOC. Concordant with our localization data and Pyk2 migration patterns on SDS-PAGE gel presented in Chapter 3, Kacena et al (2012) have reported that a higher MW form of Pyk2 is pulled down

with the cytoskeletal fraction during a cell fractionation assay of osteoblast lysates whereas a doublet of lower MW of Pyk2 is found in the cytoplasmic and nuclear fractions of these cells. It is noteworthy to mention that these cells are capable of forming similar focal complexes that contain Pyk2 and paxillin, similar to macrophages (Guignandon et al., 2006; Saldana and Vilaboa, 2010; Silber et al., 2012). The authors attribute these MW differences to differential expression of Pyk2 isoforms however their evidence for this is merely correlative. The data presented in chapter 3 does not support this as these MW differences are observed when Pyk2-H is transfected alone in NIH 3T3 cells. Phosphorylation was also ruled out as a possible explanation of the shifts in MW observed by SDS-PAGE and Western blot. Rather, other post-translational modifications of Pyk2, such as SUMOylation, might explain this increase in apparent MW. Indeed, Pyk2 pulled down with anti-SUMO-1 migrates similarly to the fraction of Pyk2 recovered with the F298 antiserum. Alternatively, it is possible that the F245 population of Pyk2 might be subjected to cleavage, which would result in its faster migration on SDS-PAGE gel. Nonetheless, I can conclude from our data and from the study by Kacena et al. (2012) that modifications that cause a Pyk2 migratory shift on SDS-PAGE gel may dictate its intracellular localization in macrophages, as well as in osteoblasts.

6.3 Serine/threonine phosphorylation as a means to regulate Pyk2

Pyk2 recovered with the F245 or the F298 antisera displayed differences in serine/threonine phosphorylation levels; F298-recovered Pyk2 displayed

increased serine/threonine phosphorylation. I have shown that although phosphorylation did not cause the apparent MW differences between the Pyk2 populations, it did interfere with the recognition of F298-recovered Pyk2 with the F245 antiserum by Western blot. It is thus possible that many of the serine or threonine residues phosphorylated in the F298 population occur within the region of the F245 epitope that overlaps a part of the proline-rich region and part of the FAT domain.

The biochemical difference between the Pyk2 populations characterized may reflect differences in their functional state or dictate binding partners. For example, Pyk2 is thought to regulate its activity by autoinhibition, similar to FAK (Lietha et al., 2007). More precisely, it is thought that the N-terminal FERM region folds over the kinase region, a conformation that does not allow for tyrosine phosphorylation by SFKs. Autophosphorylation of Pyk2 would allow for an "open" conformation of the molecule, allowing for docking of SFKs, subsequent phosphorylation of tyrosines in the kinase and FAT domains and activation of Pyk2. The "open" conformation of Pyk2 would also allow for phosphorylation of many serine/threonine residues, many of them found in the F245 epitope region. Phosphorylation of these residues may lead to stabilization of the enzymatic activity of Pyk2 by further preventing autoinhibition. Therefore, the F298 population may reflect a population of Pyk2 that has been fully activated and stabilized.

Another possible reason for serine/threonine phosphorylation of the F245 epitope region could be to regulate Pyk2 association to various binding partners.

Two identified Pyk2 binding partners are known to bind near this region: paxillin and Grb2. Paxillin association with Pyk2 in regards to serine/threonine phosphorylation is discussed below. Grb2 association with FAK and Pyk2 is dependent on tyrosine phosphorylation at Y925 or Y881, respectively (Li et al., 1996; Schlaepfer and Hunter, 1996). Serine/threonine phosphorylation could interfere with Grb2 association by inducing a conformation of Pyk2 incompatible with Grb2 association, despite tyrosine phosphorylation of Y881. In opposition, stabilization of the "open" conformation of Pyk2 by serine/threonine phosphorylation could promote Grb2 binding of Pyk2. Finally, Grb2 binding could be regulated by competition of the Pyk2 FAT domain with paxillin. Therefore, mechanisms regulating paxillin association, like serine/threonine phosphorylation, could indirectly regulate Grb2 binding to Pyk2. Examination of Grb2 binding to the F245 and F298 populations of Pyk2 is therefore needed in order to determine if, and how, serine/threonine phosphorylation may influence this association.

6.4 Determinants of Pyk2 and paxillin association

Direct interaction between Pyk2 and paxillin has been confirmed by resolution of the crystal structure of the FAT domain of Pyk2 (Lulo et al., 2009). Interaction of these proteins in macrophages, however, does not seem constitutive as Pyk2 colocalizes with paxillin at podosomes, but not focal complexes (Duong and Rodan, 2000; Pixley et al., 2001). This suggests that certain parameters dictate their interaction and spatial organization within macrophages.

Until recently, it was thought that phosphorylation at Tyrosine 881 in Pyk2 caused its dissociation from paxillin as phosphorylation at this site was thought to induce a conformational state of the FAT domain incompatible with paxillin association. However, Lulo et al. (2009) showed that this site did not affect Pyk2 binding to paxillin. My data revealed that paxillin-associated Pyk2 population displayed reduced overall levels of serine/threonine phosphorylation. Phosphorylation of serine/threonine residues near or in the FAT domain could interfere with paxillin binding by disrupting the conformation of the FAT domain compatible with paxillin association. Supporting this, Lulo et al. (2009) found that the T870 of Pyk2 appeared to link the Pyk2 FAT domain to paxillin through its interaction with D267 found in the LD4 region of paxillin. Other yet undescribed serine or threonine interactions may similarly be important for Pyk2 and paxillin association.

Another mechanism implicated in paxillin association with Pyk2 may be related to the apparent MW shift observed in the F298 population as this population was not found in association with paxillin. Identification of the possible post-translational modification that causes this MW shift, and in which regions it occurs, may provide further insight in this scenario. Alternatively, paxillin and Pyk2 dissociation would allow for Pyk2 association with various serine/threonine kinases or other post-translational modifications.

6.5 CD45 regulation of paxillin

In chapter 4, I have shown that the absence of CD45 leads to increased calpain cleavage of paxillin in macrophages. During cellular adhesion and deadhesion in WT macrophages, paxillin protein levels are not overtly affected by calpain cleavage. This is possibly due to the fact that the fraction of paxillin cleaved by calpains is replaced by *de novo* synthesis. In the absence of CD45, however, cleavage of paxillin is enhanced and *de novo* synthesis may not occur rapidly enough to compensate for this loss in paxillin proteins levels, leading to its depletion in CD45 KO BMDM.

The reason for enhanced cleavage of paxillin in the absence of CD45 is not known, but could be due to several possibilities. In one scenario, the increase in activity of SFKs in CD45 KO cells could lead to hyperphosphorylation of Pyk2 and paxillin. As paxillin is a known substrate for Pyk2, and inhibition of Pyk2/FAK kinase activity in CD45-deficient macrophages prevents calpain-mediated degradation of paxillin, tyrosine phosphorylation of paxillin by Pyk2 may regulate its degradation. Phosphorylation of paxillin may induce conformational states within paxillin that promote availability of calpain cleavage sites to calpains. Interestingly, the major calpain cleavage site on paxillin (S95) is in close proximity to the two major sites of tyrosine phosphorylation in paxillin (Y31 and Y118). In another scenario, the absence of CD45 could lead to increased turnover of focal complexes, forcing their increased dismantlement through calpain cleavage. This could also be SFK- or Pyk2-dependent, as their increased activity may stimulate adhesion turnover and indirectly promote calpain

cleavage of paxillin. Finally, although I did not observe any differences in the levels of expression of calpains, it is possible that their activity levels are nonetheless increased in the absence of CD45.

Taken together, these results help outline a model in which the regulation of Pyk2 and paxillin association, as well as the CD45-dependent regulation of paxillin protein levels, contribute to the regulation of macrophage adhesion and occur at specific intracellular sites.

6.6 CD45 regulation of macrophage responses to zymosan

Macrophage responses to the fungal PAMP zymosan have been shown to require a variety of ITAM-containing molecules and SFKs (Ariizumi, Shen et al. 2000; Gantner, Simmons et al. 2003; Sato, Yang et al. 2006; Nimmerjahn and Ravetch 2007). I showed that stimulation of CD45 KO BMDM with zymosan led to the hyperphosphorylation of several proteins, when compared to WT BMDM. Given the fact that SFKs are hyperphosphorylated and hyperactivated in CD45-deficient cells, it is possible that these proteins are substrates of SFKs and hence are hyperphosphorylated downstream of these kinases. Given that the absence of CD45 also leads to increased binding of zymosan, it is also possible that increased binding leads to sustained signalling downstream of zymosan receptors, which could be dependent or not on SFKs. The effect of this tyrosine phosphorylation disregulation in the absence of CD45 did not translate to changes in cytokine secretion or activation marker expression in response to zymosan. It is therefore possible that although there may be disregulation of certain signalling

pathways, there exists redundant pathways that are not controlled by CD45 and that compensate for the absence of CD45. Indeed, as zymosan is bound by many receptors on the surface of macrophages, it may be that CD45 only regulates signalling downstream of a limited number of these. In all, these results demonstrate that not all aspects of macrophage biology that rely on SFKs are affected by the absence of CD45.

6.7 Model

In light of the data presented, I suggest a model that incorporates the regulation and localization data of chapters 3 and 4. In Figure 6, I propose that the MTOC serves as a depot for unactivated Pyk2 and when the MTOC translocates towards the contact point, with either a cell or the extracellular matrix, it may lead Pyk2 to the contact zone. Once at the contact point, Pyk2 would dissociate from paxillin, allowing paxillin to associate with various other cytoskeletal-associated proteins, proper to its role as a scaffolding protein at sites of adhesion. At these sites, paxillin would regulate rapid assembly and disassembly of adhesion structures that are required for the transient interactions between various hematopoietic cells of the immune system. Dismantlement of focal complexes would be, at least in part, accomplished by cleavage of paxillin by calpains, a process that is negatively regulated by CD45.

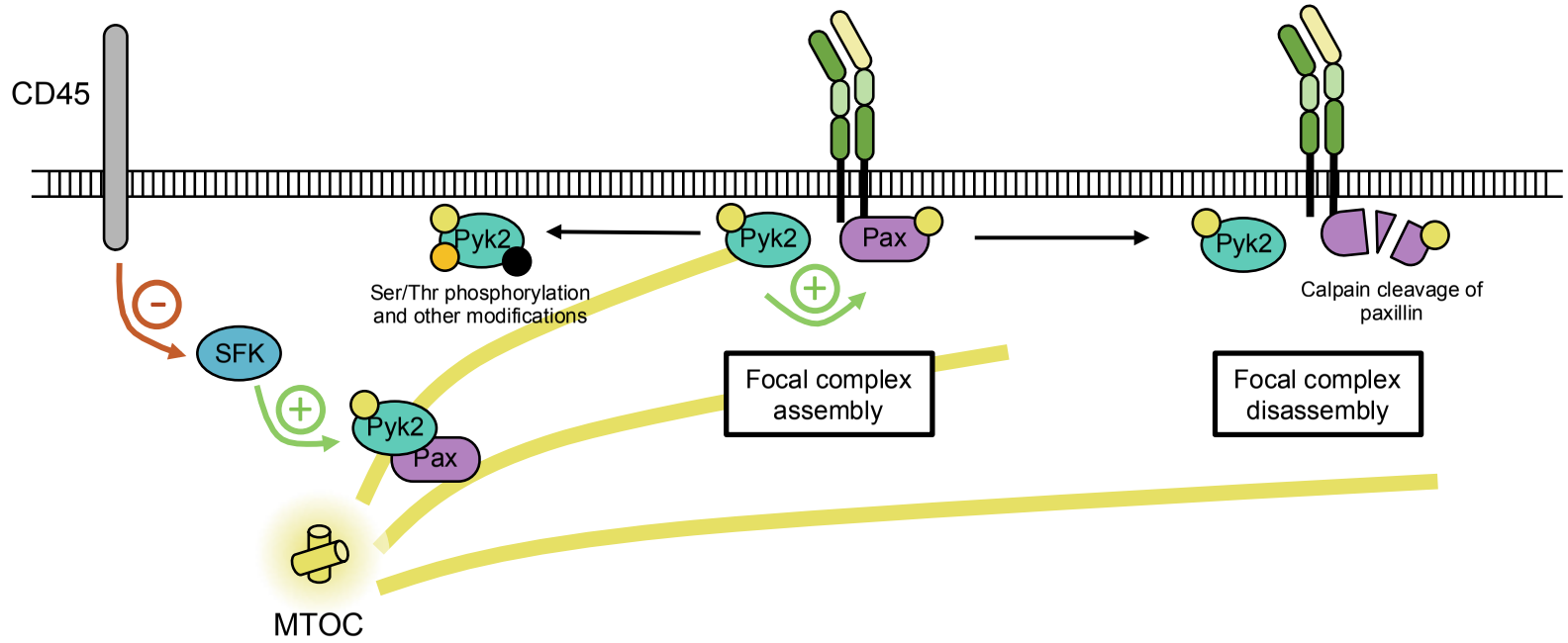

Figure 6. Model for CD45 and Pyk2 regulation of macrophage adhesion and migration. Presented is a model by which CD45 and Pyk2 participate in macrophage adhesion through the regulation of paxillin. SFKs positively regulate Pyk2 activity by the phosphorylation of two tyrosine residues in the kinase domain of Pyk2. Pyk2 activity, in turn, is necessary for the phosphorylation of paxillin. The presence of CD45 negatively regulates SFK activity, thereby also negatively regulating Pyk2 activity and paxillin phosphorylation. Pyk2 is in association with paxillin at the MTOC in macrophages. Once delivered near the membrane by migration along microtubules, Pyk2 and paxillin dissociate, allowing paxillin to associate with other cytoskeletal-associated proteins at focal complexes. Dissociated Pyk2 is then further modified by serine/threonine phosphorylation and possibly other post-translational modifications. At focal complexes, phosphorylation of paxillin by Pyk2 alters paxillin conformation, making it susceptible to calpain cleavage. Cleavage of paxillin, in turn, promotes focal complex disassembly. In all, focal complex assembly and disassembly regulate macrophage adhesion and migration.

6.8 Future directions

In light of the results presented, certain aspects of the regulation of Pyk2 and paxillin by CD45, as well as the role of CD45 in response to zymosan, remain to be elucidated.

The identification of the factor(s) contributing to the differences in MW between Pyk2 pulled down with the F298 or F245 antisera will provide further insight in the mechanisms that regulate Pyk2 function. Given the role of Pyk2 in many cellular functions, including adhesion, responses to PAMPs and cellular proliferation, it would not be surprising that this molecule is subjected to a variety of regulatory mechanisms in addition to phosphorylation. Moreover, whether the regulatory mechanisms that define Pyk2 populations within cells are in parallel with its functional differences or define its association with various binding partners will provide further information on the role of Pyk2 in macrophages as well as in other cell types. In line with these future investigations, whether Pyk2 is directly SUMOylated needs to be confirmed. SUMOylation of FAK occurs in the FERM domain and promotes its autophosphorylation, possibly by inducing an "open" conformation of the molecule (Kadare et al., 2003). If Pyk2 is indeed SUMOylated, whether its autophosphorylation is regulated by SUMO in a similar manner to FAK remains to be tested.

As Pyk2 is a substrate for SFKs, and CD45-dependent proteolytic cleavage of paxillin involves Pyk2 activity, the role of CD45 in the regulation of Pyk2 warrants further investigation. As I have been unable to detect basal levels

of Pyk2 phosphorylation in resting CD45 KO BMDM by Western blot, it may be necessary to treat CD45 KO macrophages with stimuli that induce phosphorylation of Pyk2 in order to fully appreciate the effect of the absence of CD45 on Pyk2 phosphorylation. Moreover, it will be of great interest to determine whether Pyk2 activity directly or indirectly promotes CD45-dependent degradation of paxillin by assessing whether paxillin is hyperphosphorylated prior to its cleavage by calpains or whether Pyk2 activity merely increases adhesion turnover, indirectly leading to paxillin degradation. If this is the case, other cytoskeletal-associated proteins are likely going to be affected as well and would require further examination. Moreover, this scenario would also warrant investigation into whether Pyk2 activity or paxillin degradation is the main mechanism by which CD45 regulates adhesion.

Paxillin has been shown to directly associate with the cytoplasmic tail of integrin α_4 (also known as CD49b), preferentially with the high affinity conformation of α_4, and this interaction is essential for signalling downstream of this integrin ((Hyduk et al., 2004; Liu et al., 1999). This alpha chain associates with the β_1 integrin chain to make up the $\alpha_4\beta_1$ integrin dimer, also known as Very Late Antigen 4 (VLA-4) (Stewart et al., 1995; Zhang and Wang, 2012). This integrin is expressed by leukocytes and is involved in cell spreading, adhesion, homing and transmigration, processes most important in mediating the immune response. As paxillin associates directly with this alpha chain, but not others, this poses the possibility that CD45, as a regulator of paxillin, preferentially regulates signalling downstream of certain integrins, such as α_4. As such, it would be of

great interest to assess whether CD45 exerts a greater influence downstream of specific integrins. Opposingly, CD45 could present itself as a master regulator of adhesion and spreading mediated by all integrins expressed by macrophages. This information could define CD45 as a potential target for the regulation of integrin adhesion downstream of certain, or all, integrins expressed by macrophages.

Furthermore, whether the CD45-mediates defects in adhesion translate to defects *in vivo* remains to be determined. As such, whether monocytes or macrophages are capable of normal adhesion or migration in CD45 KO mice warrants further examination. In addition, whether CD45-mediated regulation of macrophage adhesion is reproducible in human cells remains to be determined. Shenoi et al. (1999) have demonstrated a role for CD45 in regulating integrin-mediated adhesion in two human T-cell lines, however, no studies have investigated the role of CD45 in human primary macrophages or human macrophage cell lines. If these results are reproducible *in vivo* and in human macrophages, CD45 could be a potential target for controlling macrophage adhesion and migration.

As monocytes differentiate into macrophages, they go through changes in CD45 isoform expression at the same time as cytoskeletal rearrangements and changes in adhesion (Lehto, Hovi, Vartio et al, 1982; Trowbridge and Thomas 1994; DeFife, Jenney, Colton and Anderson, 1999). This could be coincidental, but this could also represent a way by which leukocytes regulate their adhesiveness and migratory capacities. Moreover, King et al. (1990) have shown

that T-cell adhesion to the monocytic cell line U-937 was inhibitable with anti-CD45 and anti-CD45RO antibodies, but not with an anti-CD45RA antibody, suggesting that certain isoforms may play a more important role in adhesion than others. As such, the examination of whether the expression of different CD45 isoforms has different effects on monocyte or macrophage adhesion could provide further insight in the mechanisms that regulate CD45 activity.

I have found that the effects of CD45 on macrophage responses to zymosan were limited to increased binding to zymosan particles and hyperphosphorylation of several proteins. Whether CD45 regulates macrophage responses other than the ones tested, such as reactive oxygen species production, remains to be assessed. Moreover, how CD45 participates in the regulation of zymosan binding and subsequent phagocytosis warrants further investigation. More precisely, whether steric hindrance from the extracellular domain, whether regulation of intracellular signalling pathways by the cytoplasmic phosphatase domain, or both, play a major role during phagocytosis of zymosan particles remains to be demonstrated. In addition, I have shown that Dectin-1 was not the major PRR responsible for tyrosine phosphorylation downstream of zymosan. The identification of the PRR responsible for triggering tyrosine phosphorylation, as well as Syk activation, remains to be determined.

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