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The functional characterisation of murine CLEC-2 and analysis of the expression of its ligand, podoplanin, on macrophages

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A thesis submitted for the degree of Doctor of Philosophy at the University of Cape Town, December 2009
This thesis is dedicated to my parents
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Declaration

I, Ann Kerrigan, hereby declare that the work on which this thesis is based, is my original work (except where acknowledgments indicate otherwise) and that neither the whole work nor any part thereof has been, is being or is to be submitted for another degree in this or any other university.

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Ann Kerrigan
December 2009
Abstract

Characterisation of CLEC-2 and its ligand podoplanin

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Submitted for the degree of Doctor of Philosophy, December 2009

CLEC-2 is a member of the 'Dectin-1 cluster' of C-type lectin-like receptors and was originally thought to be restricted to platelets. In this study it was demonstrated that murine CLEC-2 is also expressed by peripheral blood neutrophils, but only weakly by bone-marrow or elicited inflammatory neutrophils. It was also shown that on circulating neutrophils, CLEC-2 can mediate phagocytosis of antibody-coated beads and the production of proinflammatory cytokines, including TNFα, in response to the CLEC-2 ligand, rhodocytin. CLEC-2 possesses a tyrosine-based cytoplasmic motif similar to that of Dectin-1, and it was shown using chimeric analyses that the activities of this receptor are dependent on this tyrosine. These data therefore demonstrate that CLEC-2 expression is not restricted to platelets and that it functions as an activation receptor on neutrophils.

Like Dectin-1, CLEC-2 can recruit the signalling kinase Syk in myeloid cells; however, it was found that stimulation of this pathway does not induce the respiratory burst. This suggests that there is another component / pathway stimulated by Dectin-1, in addition to Syk, that is required for the induction of the respiratory burst. To explore the mechanism of Dectin-1 mediated induction of this response some preliminary experiments were performed and it was found that the tyrosine within the ITAM-like motif of Dectin-1 is crucial for this response, whereas the membrane distal tyrosine is not required. Furthermore, a number of chimeric receptors which will be used to further investigate the underlying mechanisms were generated.

Podoplanin is a transmembrane mucin-like sialoglycoprotein that is expressed on a variety of cell types. It was recently described as a CLEC-2 ligand and its expression on myeloid cells was demonstrated for the first time here. Inflammatory macrophages and neutrophils were found to display high levels of podoplanin expression. In contrast, resident alveolar and peritoneal macrophages, and bone marrow derived macrophages were found to have negligible podoplanin expression. Furthermore, it was shown that certain TLR agonists and cytokines cause upregulation of podoplanin on bone marrow derived macrophages.
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First and foremost, I would like to thank my supervisor Professor Gordon Brown, for his patience, teaching, support and encouragement throughout this project. His enthusiasm, energy and interest, helped and inspired me a great deal, especially when I faced various problems. I feel genuinely privileged to have had the opportunity to complete this work under his supervision.

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Abbreviations used in this thesis

Aa  Amino acid
Ab  Antibody
APC  Allophycocyanin
BSA  Bovine serum albumin
CFSE  Carboxyfluorescein diacetate succinimidyl ester
CTL  Cytotoxic T lymphocyte
CTLD  C-type lectin-like domain
CTLDcps  CTLD-containing proteins
Cy  Cyanine
DAG  Diacylglycerol
DC  Dendritic cell
DHR 123  Dihydrorhodamine 123
DMSO  Dimethylsulfoxide
DNA  Deoxyribonucleic acid
dATP  Deoxyadenosine Triphosphate
dCTP  Deoxycytidine Triphosphate
dGTP  Deoxyguanosine Triphosphate
dNTP  Deoxynucleotide Triphosphate
dTTP  Deoxythymidine Triphosphate
ECL  Enhanced chemiluminescence
EDTA  Ethylenediamine tetracetic acid
ELISA  Enzyme-linked immunosorbent assay
FACS  Fluorescence activated cell sorting
FAD  Flavin adenine dinucleotide
FCS  Foetal calf serum
FITC  Fluorescein isothiocyanate
fMLP  Formyl-methionyl-leucyl-phenylalanine
FSC  Forward scatter
GDP  Guanosine diphosphate
GPCR  G-protein coupled receptor
GTP  Guanosine triphosphate
HA  Hemagglutinin
His  Histidine
HIV  Human immunodeficiency virus
HRP  Horseradish peroxidase
IFN  Interferon
Ig  Immunoglobulin
IgSF  Immunoglobulin superfamily
ITAM  Immunoreceptor tyrosine-based activation motif
ITIM  Immunoreceptor tyrosine-based inhibitory motif
IL  Interleukin
IP3  Inositol-1,4,5-triphosphate
kDa  Kilodalton
LB  Luria Bertani
LPS  Lipopolysaccharide
MALDI-TOF  Matrix-assisted laser desorption/ionization – time of flight
M-CSF  Macrophage colony stimulating factor
MFI  Mean fluorescent intensity
MHC  Major histocompatibility complex
MS  Mass spectrometry
NADPH  Nicotinamide adenine dinucleotide phosphate
NK  Natural killer
NKC  Natural killer gene complex
NLR  Nod-like receptors
Nod  Nucleotide-oligomerisation domain
OxLDL  Oxidised low-density lipoprotein
Protein abbreviations / common names

For ease of reading, common protein names or abbreviations are usually used throughout the thesis. Full names are listed below.

ADAM  A Disintegrin And Metallopeptidase
Bcl-2  B cell CLL/lymphoma-2
Bcl-10  B-cell CLL/lymphoma 10
BDCA-2  Blood dendritic cell antigen 2
BDCA-3  Blood dendritic cell antigen 3
Btk  Bruton's tyrosine kinase
Card9  Caspase recruitment domain-containing protein 9
Card11  Caspase recruitment domain-containing protein 11
CCL3  C-C motif chemokine 3
Cdc42  Cell division control protein 42 homolog
CEACAM3  Carcinobryonic antigen-related cell adhesion molecule 3
c-IAP  Cellular Inhibitor of Apoptosis
CLL-1  C-type lectin-like molecule-1
CXCL2  Chemokine (C-X-C motif) ligand 2 (CXCL2)
DAP12  DNAX-activating protein of 12kDa
DCAL-2  Dendritic-cell associated C-type lectin 2
DCAR  Dendritic cell immunoactivating receptor
DCIR  Dendritic cell immunoreceptor
DC-SIGN  Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
Dectin-1  Dendritic cell-associated C-type lectin-1
<table>
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<td>DNGR-1</td>
<td>DC, NK lectin group receptor-1</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated mitogen activated protein kinase</td>
</tr>
<tr>
<td>FcRγ</td>
<td>Fc receptor gamma chain</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>ICAM-1</td>
<td>Inter-Cellular Adhesion Molecule 1</td>
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<td>HSP</td>
<td>Heat-shock protein</td>
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<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis protein</td>
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<tr>
<td>ikB</td>
<td>Inhibitor of kB</td>
</tr>
<tr>
<td>IKK</td>
<td>ikB kinase</td>
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<tr>
<td>KLRL-1</td>
<td>Killer cell C-type lectin like receptor L 1</td>
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<tr>
<td>LAT</td>
<td>Linker for activation of T cells</td>
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<tr>
<td>LOX-1</td>
<td>Lectin-like oxidised low-density lipoprotein receptor-1</td>
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<tr>
<td>MALT1</td>
<td>Mucosa-associated lymphoid tissue lymphoma translocation protein 1</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MARCKS</td>
<td>Myristoylated, alanine-rich C-kinase substrate</td>
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<td>MCL</td>
<td>Myeloid inhibitory C-type lectin-like receptor</td>
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<td>MT1-MMP</td>
<td>Membrane type 1 matrix metalloproteinase</td>
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<td>Platelet endothelial cell adhesion molecule</td>
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<td>Triggering receptor expressed on myeloid cells</td>
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<td>Vascular cell adhesion molecule-1</td>
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<td>Zap-70</td>
<td>Zeta-chain-associated protein kinase 70</td>
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1

Introduction

1.1 Homeostasis
The concept of homeostasis refers to the ability or tendency of a system to maintain internal equilibrium. In physiological terms, this is an exceptionally dynamic process during which internal change continuously compensates for external change to keep conditions relatively constant. The human body consists of several systems which together function to maintain homeostasis. Each system is composed of cells which are progressively organised into tissues and organs, and the concept of homeostasis can be applied at all levels. At the most basic level cells perform functions that are essential for their own survival, in addition to specific tasks that play a part in the maintenance of an organism's overall homeostasis. Membrane proteins such as those that form channels, serve as carrier molecules and act as enzymes or receptors; are all crucial for a cell's role in this system. Cells express a wide range of surface receptors which are involved in a variety of functions, including differentiation, growth and survival, adhesion, migration, phagocytosis, activation and cytotoxicity \[1, 2\]. Even though there has been a rapid development of powerful technologies which has resulted in an ever increasing list of membrane proteins, the entire repertoire of membrane expressed receptors remains to be determined. Nonetheless, there has been a vast amount of research devoted to their characterisation and their roles in homeostasis and host defence.

1.2 Receptor classification
Although protein families have long been grouped according to structure and function, the extraordinary explosion in protein sequence, structure and functional data in recent times, makes classification a significantly complex and dynamic process \[3\]. Structural classifications derive groups on the basis of molecular similarity in terms of primary or tertiary structure, whereas functional classifications derive groups on the basis of functional similarity. Family members grouped according to structural homology frequently have related functions, but may also be functionally divergent. Structurally homologous receptors are usually derived from a common ancestor and are likely to have been generated by genetic changes such as exon shuffling, gene duplication, retroposition and gene fusion \[4\]. An example of a structurally defined group is the immunoglobulin (Ig) superfamily (IgSF). This superfamily is one of the largest in vertebrates and its members can be diverse in terms of sequence and structure. However, all proteins of the IgSF possess a common Ig-fold which has a sandwich like structure formed by two sheets of antiparallel \(\beta\)-strands \[5\]. The members of the IgSF are involved in immune functions, recognition, binding, and adhesion processes of cells. The superfamily of G-protein coupled receptors, the Interleukin (IL)-1
receptor/Toll-like receptor (TLR) superfamily and the Tumor Necrosis Factor (TNF) superfamily are other examples of major groups defined by their structural domains.

1.3 Receptors linking homeostasis and immunity

In vertebrates, a complex immune system has evolved to defend against the potentially detrimental effects of microbial infection. This immune system consists of two branches; innate and adaptive. Innate immune recognition is based on the recognition of conserved, invariant structures that are unique to microorganisms. This recognition is mediated by the pattern recognition receptors (PRRs), a family of functionally related germ-line encoded proteins [6, 7]. According to Charles Janeway's original hypothesis, PRRs evolved to recognise microbial ligands and functioned to distinguish infectious non-self from non-infectious self with the resultant initiation of adaptive immunity [7, 8]. For a long time this theory seemed to hold true, and reports emerged that various PRRs such as TLRs, C-type lectin receptors, nucleotide-oligomerisation domain (Nod)-like receptors (NLRs) and retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs), did recognise pathogen-associated molecular patterns (PAMPs) and could induce immune responses.

TLRs were the first class of cellular PRRs described and are present at the cell surface or within endosomes. They recognise specific microbial components and play a crucial role in immunity that has been reviewed extensively elsewhere [9, 10]. C-type lectins comprise both soluble and transmembrane receptors and can recognise a wide range of mainly carbohydrate structures on pathogens. NLRs and RLRs are intracellular cytosolic receptors involved primarily in the detection of bacteria and viruses respectively [11-13]. As our understanding of these receptors has deepened, it has however become necessary to reconsider Janeway's original theory. For instance, it is now known that the bacterial ligands recognised by TLRs are not unique to pathogens but are also present on commensal microorganisms. For example, studies have revealed that TLR signalling induced by commensal bacteria has an essential role in maintaining epithelial homeostasis, and protection from direct epithelial injury [14-20]. Furthermore, growing evidence suggests that PRRs respond to endogenous ligands which are released from, or exposed on the surface of dying cells. For example the C-type lectins, Mincle and CLEC9A, were both shown to recognise ligands on necrotic cells with the resultant activation of adaptive immunity [21, 22]. It has also been demonstrated that TLRs can respond to endogenous ligands which are released during inflammatory responses and tissue damage. One example is the induction of signalling and dendritic cell (DC) activation via TLR2 and/or TLR4 recognition of hyaluronan [23-25]. Finally, it is becoming increasingly apparent that the recognition of endogenous ligands by certain PRRs contributes to immune homeostasis. This is particularly apparent in the case of C-type lectin receptors [26]. A select example is DC-SIGN, which in addition to recognising several pathogens, also binds endogenous proteins and in this way mediates adhesion between DCs and endothelial cells during DC migration, and between DCs and T cells during antigen presentation [27]. We have learned a tremendous amount about the immune system in the 20 years since Janeway proposed the idea of PRRs and it has become clear that his hypothesis does not encompass all scenarios. However, as Ruslan Medzhitov recently put it, 'no theory is ever
complete and (the) pattern recognition concept will continue to evolve and eventually integrate with other concepts into a more general theory’ [28]. Furthermore, our growing knowledge raises interesting questions about the evolution of such receptors and Siamon Gordon, amongst others, has speculated that the further identification of endogenous ligands and an increasing appreciation of homeostatic requirements will result in the realisation of the innate immune system as ‘primarily a self-regulating, physiologic system, with defence as a secondary, albeit vital, by-product’ [29].

1.4 C-type lectin-like domain superfamily

The term ‘C-type lectin’ was originally used to describe a group of Ca\(^{2+}\)-dependent carbohydrate binding proteins containing a conserved carbohydrate recognition domain [30, 31]. Other proteins containing the same domain were subsequently identified, but carbohydrate ligands have not been identified for many of these proteins. To clarify this ambiguity, the term C-type lectin-like domain (CTLD) was introduced to refer to such domains [32, 33]. The C-type lectin superfamily is now defined as a large group of proteins that are characterised by the presence of one or more CTLDs. It should be noted that the use of the word ‘lectin’ to describe this family is in fact misleading, as the definition of a lectin is a protein with carbohydrate binding capabilities. It was proposed by Zelensky in 2005 that the family instead be referred to as CTLD-containing proteins (CTLDcps) [34]. However, several reviews published since then have continued using the term ‘C-type lectin’ and as this is the more common usage in the current literature, it will be used here.

The CTLD is a structural motif which was originally identified as a protein fold in the carbohydrate recognition domain of mannose-binding lectin [35]. It consists of two antiparallel \(\beta\)-sheets and two \(\alpha\)-helices. A \(\beta\)-strand divides the structure into two loops, the upper and the lower. The lower loop is formed by the vertical \(\beta\)-sheet and both \(\alpha\)-helices. The upper loop consists of the long loop region and the second antiparallel \(\beta\)-sheet, and three distinct core hydrophobic regions are present. A WIGL amino acid (aa) sequence (aromatic aa, aliphatic aa, glycine, aliphatic aa) which is extremely well-conserved in CTLDs, is involved in formation of all three hydrophobic cores which stabilises the overall structure [34, 36]. Disulfide bridges at the bases of the loops are formed by four cysteines [34].

Initially, the superfamily was divided into seven groups based on their domain architecture (Groups I to VII) [37]. This grouping conveniently correlated with the results of phylogenetic analysis and incorporated functional similarity. The classification was modified some years later with the addition of seven new groups (Groups VIII to XIV) [38]. Subsequently three new groups were added to incorporate novel proteins identified in the interim (Groups XV to XVII) [34]. The latest classification is shown in Figure 1.1 [34]. Despite the presence of a highly conserved domain, C-type lectins are functionally diverse and have been implicated in various processes including, cell adhesion, tissue integration and remodelling, platelet activation, complement activation, pathogen recognition, endocytosis, and phagocytosis [39-43].
CTLDs recognise a range of ligand types, including carbohydrates as the superfamily name suggests, but also proteins, lipids and inorganic compounds [34]. As mentioned previously there are now many C-type lectins which have not been ascribed any carbohydrate binding function. Nevertheless, the recognition of carbohydrates by these receptors is relatively well studied and is worth considering. Many C-type lectins possess four Ca\(^{2+}\) binding sites, although Ca\(^{2+}\)-binding site 2 is the important site for carbohydrate binding [34]. Residues with carbonyl sidechains are involved in Ca\(^{2+}\) coordination at this site and together with the Ca\(^{2+}\) atom are directly involved in carbohydrate binding. These highly conserved motifs contributed by the long loop region of the CTLD are Glu-Pro-Asn (EPN) or Gln-Pro-Asp (QPD) and generally have specificity for mannose or galactose respectively [44]. A second highly conserved group of residues is a Trp-Asn-Asp (WND) motif and is contributed by a \(\beta\) strand in the upper \(\beta\) sheet [36]. These characteristic residues have been widely used in the literature as indicative of whether a C-type lectin with unknown binding function is likely to bind carbohydrates (EPN/QPD and WND present), and furthermore whether it would preferentially bind mannose or galactose type carbohydrates (EPN v QPD) [34]. However, this approach has some limitations as it is based on the assumption that Ca\(^{2+}\)-dependent binding is the only major mechanism of carbohydrate binding by CTLDs. This is known to be untrue, as demonstrated by Dectin-1, a C-type lectin which does not contain the motifs described above and binds a carbohydrate ligand in a Ca\(^{2+}\)-independent manner [45]. Furthermore, it also should be noted that the presence of these motifs does not guarantee carbohydrate binding functions, as there are several examples of CTLDs containing the conserved motifs that are not known to bind carbohydrates [34].

1.5 Myeloid C-type lectins

A prominent role for C-type lectins in immunity was originally identified in Natural Killer (NK) cells, where they were implicated in controlling NK function in response to transformed and virally infected cells [46]. The functions of NK cells were found to be controlled by paired C-type lectins which delivered activation and inhibitory signals, the balance of which controlled NK cell function [47]. The identification of C-type lectins on myeloid cells raised the possibility that they may serve similar functions in controlling cellular activation in these cells. Of particular interest to our research group are myeloid expressed C-type lectins belonging to the Group II, V and VI subgroups, many of which have been implicated in innate immunity and homeostasis. In several instances their functions have been linked to the presence of signalling motifs in their cytoplasmic tails, or to the ability to couple with signalling chains. For example, Mincle (discussed below) induces cellular activation by associating with Fc receptor \(\gamma\) chain (Fc\(\gamma\)), an immunoreceptor tyrosine-based activation motif (ITAM) containing adaptor. An ITAM consists of a short amino acid sequence containing a duplicate of the sequence YxxL/I with six to twelve intervening residues \((YxxL/IX_{(6-12)}YxxL/I, \text{where } Y \text{ is tyrosine, } L \text{ is leucine, I is isoleuine and } x \text{ is any residue}) [48-50]. The conserved tyrosines and leucine/isoleucine residues, and the spacing between them are critical for the biological function of an ITAM [51]. Immunoreceptors which activate intracellular signalling through ITAMs include T cell receptors, B cell receptors and Fc receptors. Signalling via this motif is instigated by ligand binding which induces receptor clustering and phosphorylation of the ITAM.
Figure 1.1: Domain architecture of vertebrate C-type lectin domains (CTLDs) from different groups. Group numbers are indicated next to the domain charts. I - lecticans, II - the ASGR group, III - collectins, IV - selectins, V - NK receptors, VI - the macrophage mannose receptor group, VII - REG proteins, VIII - the chondrolectin group, IX - the tetranectin group, X - polycystin 1, XI - attractin, XII - EMBP, XIII - DGCR2, XIV - the thrombomodulin group, XV - Bimloc, XVI - SEEC, XVII CBCP. From Zelensky and Gready, FEBS Journal, 2005 [34].
tyrosines by Src family kinases. This phosphorylation promotes recruitment of Syk family kinases which initiates a series of downstream signalling events [49, 50].

ITAM signalling often results in the activation of cellular responses, including cell migration and adhesion [52], proliferation and differentiation [53, 54], and phagocytosis and degranulation [55]. This activation may be kept in check by immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptors [49, 50]. An ITIM is a cytoplasmic sequence consisting of the amino acid residues INIUSxYxxI/LN, where I is isoleucine, L is leucine, S is serine, Y is tyrosine, and x is any residue [56, 57]. Generally, ITIM containing receptors are tyrosine phosphorylated by Src kinases in response to immunoreceptor engagement. This results in recruitment and activation of protein tyrosine phosphatases such as SHP-1, which inhibit cellular activation by dephosphorylation of various immunoreceptor-regulated substrates [56, 57].

The concept of paired receptors, one activatory and the other inhibitory, working in collaboration to ensure controlled cellular responses was prevalent for some time and seems a plausible model of regulation [58]. This model certainly appears to hold true when one considers the tight regulation of NK cell function [47]. However, in recent years a number of examples have emerged which have questioned this paradigm [49, 50, 59]. For instance, it has been shown that ITAM signalling under certain conditions can induce negative responses. One example is TREM2 which associates with the ITAM containing adaptor DAP12. TREM-2/DAP12 signalling was shown to trigger nitric oxide production by macrophages [60], cytokine production, antigen uptake and maturation in DCs [61, 62], and differentiation of osteoclasts [63]. However it was also shown that in certain contexts, TREM-2/DAP12 signalling can negatively regulate TLR and FcRy signalling in macrophages and inhibit inflammatory cytokine production [64, 65]. There are also examples of ITIM-containing receptors that function to activate cellular responses. For instance, TLT-1 was shown to enhance FcεRI-mediated calcium signalling, an outcome which was dependent on its association with SHP-2 [66]. To add further complexity to ITAM/ITIM signalling models, ITAM-like motifs which vary from the traditional consensus sequence have now been identified in a number of receptors. Dectin-1 exemplifies an ITAM-like motif containing receptor and its mechanisms of signalling will be discussed later. Such examples of non-traditional ITAM-signalling are becoming increasingly common and demonstrate a flexibility of the mechanisms of signalling [49].

1.5.1 Dectin-2 cluster: an example of myeloid expressed C-type lectins

An example of myeloid expressed C-type lectins is the Dectin-2 cluster of receptors which are members of Group II of the superfamily [67]. The group consists of Dectin-2, DCIR, DCAR, BDCA-2, Mincle and Clecsf8. These receptors are encoded by a cluster of genes within a single large region known as the ‘natural killer gene complex’ (NKC) that occurs on syntenic regions of mouse chromosome 6 and human chromosome 12 [68, 69]. Mincle, Dectin-2 and DCAR have all been shown to associate with the ITAM-containing FcRy, (and BDCA-2 with FcεRI), which triggers intracellular signalling via Syk [21, 70-73]. Mincle provides a further example of a receptor involved in both homeostasis and pathogen recognition. As mentioned, it has been demonstrated that
Minde acts as an activating receptor that senses damaged cells and in this way has a homeostatic function [21]. On the other hand, it has also been shown that Minde mediates recognition of *Candida albicans* and *Malassezia* species and is involved in the immune response to these fungi [74, 75]. In a similar vein, Dectin-2 can recognise hyphal forms of various fungi and is capable of inducing innate immune responses via coupling to FcRy [71]. Another member of the group, DCIR, is an ITIM-containing protein and although neither endogenous nor exogenous ligands have been identified, it has been shown to act as an inhibitory receptor [76]. It is possible that under physiological conditions, DCIR may function in concert with Minde, and/or other activating members of the group to modulate signalling.

### 1.5.2 Dectin-1 cluster

The Group V receptors, identified only in higher vertebrates, are almost exclusively encoded by genes within the NKC [68, 69, 77, 78]. They are type II transmembrane receptors meaning they have a single membrane spanning region, with the N-terminal domain extending into the cytoplasm and an extracellular C-terminal domain. Within the NKC, another cluster of genes encode a subfamily of related Group V receptors known as the ‘Dectin-1 cluster’, that are expressed primarily, but not exclusively, on myeloid cells (Fig. 1.2) [77, 79, 80]. These type II receptors contain a single extracellular CTLD which lacks the typical residues involved in Ca\(^{2+}\) coordination required for carbohydrate binding by other C-type lectins. Although this group of receptors show considerable similarity in structure, they nevertheless recognise a diverse range of structurally unrelated ligands and mediate an assortment of functions that contribute to both immunity and homeostasis [79, 81]. Table 1 lists the members of the ‘Dectin-1 cluster’ and includes the approved gene symbol and approved gene name as designated by the HUGO Gene Nomenclature Committee\(^1\) (European Bioinformatics Institute, Cambridge, UK). Considering the structural and evolutionary relationship to CLEC-2, each of these receptors will be considered in turn here. I will summarise the current literature for each receptor under the headings: identification, structure and expression; ligands, and function. However, in the case of Dectin-1, I will enter into more detail because the sequence homology of the cytoplasmic tails of Dectin-1 and CLEC-2 was what instigated this project, and knowledge of Dectin-1 function directed the initial stages of the investigation. The order in which the receptors are discussed is based on the type of signalling motif present in the cytoplasmic tails. CLEC-1 and LOX-1 do not contain any characterised signalling motifs and are the first two receptors discussed. Next, I consider MICL and CLEC12B which contain ITIMs in their cytoplasmic tails. Finally, I discuss the ITAM-like containing members of the cluster, Dectin-1, CLEC9A and CLEC-2. Cartoon representations of the typical structures of receptors in the Dectin-1 cluster are shown in Figure 1.3.

\(^1\) [http://www.genenames.org/](http://www.genenames.org/)
Figure 1.2: Schematic view of part of human chromosome 12 and mouse chromosome 6 highlighting the presence of the Dectin-1 cluster within the context of the natural killer gene complex (NKC). Coloured boxes indicate families of genes that are present in both species. Figure is not drawn to scale. AICL, activation-induced C-type lectin; A2M, α-2 macroglobulin; CLEC, C-type lectin-like receptor; Clr, C-lectin related; DECTIN, dendritic cell-associated C-type lectin; KLR, killer-cell lectin-like receptor; LLT, lectin-like transcript; LOX, oxidized low-density lipoprotein; MAFA, mast cell function-associated receptor; NKG2, natural killer group 2; NKRP, natural killer cell receptor protein; Ocil, osteoclast inhibitory lectin; PRH, proline-rich Haelili; Prp, proline-rich protein. Adapted from Yokoyama & Plougastel, Nature Reviews Immunology, 2003.
Table 1 Members of the Dectin-1 cluster of C-type lectin proteins

<table>
<thead>
<tr>
<th>Common name</th>
<th>Aliases</th>
<th>Approved gene name</th>
<th>Approved gene symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLEC-1</td>
<td></td>
<td>C-type lectin domain family 1, member A</td>
<td>CLEC1A</td>
</tr>
<tr>
<td>LOX-1</td>
<td>CLEC6A</td>
<td>Oxidized low density lipoprotein (lectin-like) receptor 1</td>
<td>OLR1</td>
</tr>
<tr>
<td>MICAL</td>
<td>CLL-1, KLR-1, DCAL-1</td>
<td>C-type lectin domain family 12, member A</td>
<td>CLEC12A</td>
</tr>
<tr>
<td>CLEC12B</td>
<td>Macrophage antigen H</td>
<td>C-type lectin domain family 12, member B</td>
<td>CLEC12B</td>
</tr>
<tr>
<td>Dectin-1</td>
<td>β-glucan receptor (5GR)</td>
<td>C-type lectin domain family 7, member A</td>
<td>CLEC7A</td>
</tr>
<tr>
<td>CLEC9A</td>
<td>HEEE, DNGR-1</td>
<td>C-type lectin domain family 9, member A</td>
<td>CLEC9A</td>
</tr>
<tr>
<td>CLEC-2</td>
<td></td>
<td>C-type lectin domain family 1, member B</td>
<td>CLEC1B</td>
</tr>
</tbody>
</table>

Figure 1.3: Cartoon representation of the typical structure of receptors in the Dectin-1 cluster. Dectin-1 and MICAL are shown as representatives of activation and inhibitory receptors. Proximal signalling components are also included. Lollipop structures indicate sites of N-linked glycosylation. Adapted from Huysamen & Brown, FEMS Microbiology Letters, 2009[76].
1.6 CLEC-1 (CLEC1A)

1.6.1 Identification, structure and expression
CLEC-1 was originally identified through a computational screen of myeloid cells for C-type lectin receptors homologous to those expressed by NK cells [82]. CLEC-1 displays the typical structure of Group V C-type lectins and it contains a cytoplasmic tyrosine within the sequence YSST which may represent a novel signalling motif [82]. In addition to six cysteine residues in the extracellular region that are typical of C-type lectins, it also contains two more cysteines in this region which may mediate receptor dimerisation. RNA blot analysis of various human tissues revealed expression of CLEC-1 in placenta, lung, thymus, heart, pancreas, kidney, bladder, prostate, testis, ovary, small intestine and colon. RT-PCR analysis of cellular distribution revealed expression on unstimulated as well as TNF or CD40L-stimulated DCs, endothelial cells and weak expression in monocytes [77, 82]. The expression of rat CLEC-1 has also been examined and RT-PCR analysis revealed high expression in the lung, lymph nodes, spleen and aorta and lower expression in the heart and thymus [83]. In terms of cellular distribution, rat CLEC-1 was found at the protein level on the surface of bone marrow derived DCs and to a lower extent on endothelial cells. Furthermore, it was demonstrated that rat CLEC-1 expression was down-regulated by inflammatory stimuli such as LPS, and increased by immunoregulatory mediators such as IL-10. It was also found that CLEC-1 expression was up-regulated on myeloid cells and endothelial cells in various models of allograft tolerance. This study also suggested that the action of regulatory T cells (Treg) enhanced CLEC-1 expression in tolerated allografts which in turn moderated allogenic T helper 17 (Th17) responses. These findings led to the suggestion that CLEC-1 may be a potential therapeutic agent for modulation of immune responses in transplantation, autoimmunity or cancer settings [83].

1.6.2 Ligands and function
CLEC-1 is the least characterised of the Dectin-1 cluster of receptors. No ligands have been identified and limited functional studies have been carried out. As referred to above, it has been demonstrated that CLEC-1 moderated Th17 differentiation in an experimental transplant model [83]. As for the other ‘orphan’ receptors of this cluster, the identification of ligands will potentially provide direction towards clarification of the physiological function of CLEC-1.

1.7 LOX-1 (OLR1)

1.7.1 Identification, structure and expression
Lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1) was originally identified by screening a vascular endothelial cDNA expression library for receptors for oxidised low-density lipoprotein (OxLDL) [84]. LOX-1 is a type II transmembrane protein with a single CTLD connected by a stalk to a transmembrane region and a cytoplasmic tail which does not contain any known signalling motifs. The receptor contains the characteristic six cysteines of CTLDs which form intrachain disulfide bonds [85]. Human LOX-1 (hLOX-1) also contains a cysteine residue in the stalk region that mediates homo-dimerisation which is important for effective recognition of certain ligands [86]. Furthermore, evidence suggests that dimers of hLOX-1 may form noncovalently associated oligomers, although neither the mechanism nor the functional significance of these oligomers has
been determined [86, 87]. In contrast to hLOX-1, the rat and murine forms do not have a cysteine residue in their stalk regions. Interestingly, both rat and murine LOX-1 have longer stalk regions than the human form, consisting of triple repeat sequences [88, 89]. Sequence similarities between these long-stalked LOX-1 forms and the myosin tail, which is also highly repetitive, have been noted [89]. The spacing of hydrophobic and charged residues in myosin allows dimerisation to occur by two α-helices wrapping around each other to form an α-helical coiled coil [90]. Rat and murine LOX-1 may form dimers in a similar way, even though they lack the cysteine in the stalk region that mediates dimerisation of human LOX-1 [86].

In humans, alternative splicing generates an isoform which lacks exon 5 and exhibits functional differences to the full length form. This variant is called LOXIN and contains a stop codon in the open reading frame which leads to premature termination and generates a protein that lacks a large portion of the extracellular domain [91]. LOX-1 is glycosylated, although precise glycosylation patterns and chain length vary between species and cell type [85]. It can also be proteolytically cleaved at the cell surface and released in a soluble form [92]. IL-18 can stimulate this shedding of LOX-1, and ADAM10 is involved in the cleavage process [93]. The function of soluble LOX-1 is unknown but it was found to be elevated in obese patients and in the serum of patients with acute coronary disease and diabetes [94-96].

LOX-1 is widely expressed with highest levels in placenta, lung, marrow and spinal cord [97]. In terms of cellular distribution, LOX-1 is present at low levels on endothelial cells, monocytes, activated platelets, smooth muscle cells, fibroblasts, macrophages and immature DCs [84, 98-102]. However, expression can be enhanced by several pathological conditions including hypertension, diabetes and hyperlipidemia [98, 103-105]. LOX-1 can also be rapidly induced by pro-inflammatory, pro-oxidant and mechanical factors such as OxLDL, angiotensinII, TNFα, shear stress and free radicals [101, 106, 107].

1.7.2 Ligands
As mentioned, LOX-1 recognises OxLDL [84]. In addition to this ‘modified self ligand, LOX-1 recognises several other structurally unrelated macromolecules including advanced glycation endproducts, aged and apoptotic cells, heat shock protein-70, Gram-positive and -negative bacteria and C-reactive protein [99, 108-111]. The diversity of LOX-1 ligands highlights this receptor as yet another example of one which functions in both pathogen recognition and homeostasis.

1.7.3 Function
Even though LOX-1 does not contain any classical signalling motifs in its cytoplasmic tail, it can still mediate a range of cellular responses. For example, LOX-1 can mediate endocytosis of OxLDL by a dynamin-2 dependent, clathrin independent mechanism which requires a cytoplasmic acidic motif, DDL [84, 112]. The receptor can also mediate phagocytosis of aged/apoptotic cells, possibly via an interaction with phosphatidylerine [109, 112]. Furthermore, LOX-1 can induce apoptosis via
a mechanism that includes the downregulation of antiapoptotic proteins c-IAP and Bcl-2, the release of cytochrome c and Smac, and activation of caspase-9 and caspase-3 [113, 114]. LOX-1 can also mediate activation of NF-κB and the production of reactive oxygen species via a pathway that involves MT1-MMP, and Rac-1 mediated NADPH oxidase activation [115-118]. Binding and endocytosis of HSP-70 by LOX-1 can induce DC mediated antigen cross-presentation [99]. Although the pathways leading to these cellular responses are not understood in their entirety, other various downstream components have been implicated in LOX-1 signalling including PKC, p38 MAPK and PTK [119, 120]. The fact that LOX-1 can bind bacteria and induce superoxide generation suggest that it plays a role in immunity. However, a precise function in terms of protective responses has not been shown directly and further investigations are needed to clarify its role in the immune response.

1.7.3.1 Function in vascular disease

Much research on LOX-1 has focused on its involvement in vascular disease states. OxLDL is a key contributing factor to atherosclerosis [121, 122], and it mediates several of its adverse effects via LOX-1. Several other lines of evidence support a role for LOX-1 in vascular pathology. For example, LOX-1 is highly upregulated in human and animal atherosclerotic lesions [103, 123, 124], and many of the previously mentioned factors which upregulate its expression are associated with atherosclerosis and other vascular disease. OxLDL ligation of LOX-1 results in upregulation of adhesion molecules E-selectin, P-selectin, VCAM-1 and ICAM-1, which have been shown to have a role in atherosclerosis by facilitating entry of leukocytes into the artery wall [122, 125]. Additionally it was shown that OxLDL triggers the CD40/CD40L signalling pathway through LOX-1 [119]. This signalling system is known to play a central role in the development and progression of atherosclerosis [126]. Furthermore, LOX-1 mediated uptake of OxLDL in macrophages results in the formation of lipid laden cells that are similar to foam cells which are present in atherosclerotic lesions [127]. Other evidence which indicates a role for this receptor in cardiovascular disease is the observation that the deletion of LOX-1 in mice limits atherogenesis despite a high cholesterol diet, at least in part by preserving endothelial function and integrity [128]. Finally, LOX-1 allelic polymorphisms can confer an increased risk of heart attacks [129, 130]. Interestingly, subjects homozygous for the ‘non-risk’ haplotype showed a small increase in LOXIN expression [91]. Further in vitro studies demonstrated that hetero-oligomerization of LOX-1/LOXIN isoforms impaired OxLDL binding and uptake [131]. This suggests that hetero-oligomerisation between naturally occurring isoforms of LOX-1 may be a mechanism of LOX-1 regulation [131]. This would constitute an elegant control system and it is tempting to speculate that the existence of receptor isoforms that mediate self regulation may be a feature of the Dectin-1 cluster of receptors. However, this is purely conjecture and further studies involving the various receptor isoforms are required to investigate the idea.

1.8 Myeloid inhibitory C-type lectin-like receptor (MICL) (CLEC12A)

1.8.1 Identification, structure and expression
MICL was originally identified through data base searches for molecules homologous to Dectin-1 [80]. Subsequent studies by a number of groups described the same receptor, but used the alternative names C-type lectin-like molecule-1 (CLL-1), Killer cell C-type lectin like receptor L 1 (KLRL1) and Dendritic-cell associated C-type lectin 2 (DCAL-2) [132-134]. Alternative splicing results in at least three isoforms of human MICL (hMICL) which have been designated MICL-α, β and γ [80]. hMICL-α displays the type II transmembrane structure characteristic of Group V C-type lectins and contains an ITIM signaling motif in its cytoplasmic tail. The transmembrane region is not present in hMICL-β, suggesting that it may be either secreted or withheld in the cytosol as has been described for other equivalent C-type lectin isoforms [135]. The hMICL-γ isoform is unspliced at the second intron, resulting in the introduction of a stop codon after 18 codons. Although hMICL contains cysteine residues in its stalk region, which in other cases mediate dimerisation by forming disulfide bridges, this receptor appears to be expressed as a monomer [80]. hMICL is also highly N-glycosylated, although the precise level of glycosylation varies between cell types [136]. In contrast, no isoforms have been identified for murine MICL (mMICL) and its N-glycosylation is not as extensive as that of the human form. Furthermore, mMICL is expressed as a dimer [137].

Both murine and human MICL are predominantly expressed by myeloid cells (granulocytes, monocytes, macrophages and DCs) [136-138]. hMICL is also present at low levels on CD4⁺ T cells [137]. mMICL is expressed on B cells and at low levels on CD8⁺ T cells making it more broadly expressed than its human counterpart [137]. One study described expression of MICL on NK cells, but other studies have definitively concluded that MICL is not present on these cells [134, 136-138]. hMICL was also identified as a marker for acute myeloid leukemia [132]. Expression of MICL is downregulated following inflammatory conditions, including those triggered by microbial components [80, 136, 137].

1.8.2 Ligands
There have been no ligands identified for MICL to date. However, a cellular reporter system based on BWZ.36 cells and related to the one used in this thesis (see Chapter 5) shows that MICL recognises an endogenous ligand that is broadly expressed in many mouse tissues [137].

1.8.3 Function
MICL can inhibit cellular activation, a process which most likely occurs by signalling though the ITIM motif located in its cytoplasmic tail. Accordingly, it has been shown that MICL can associate with the signalling phosphatases SHP-1 and SHP-2, but not SHIP [80, 137]. This is consistent with other ITIM containing C-type lectins [139]. It has also been reported that MICL inhibits NK cell cytotoxicity, although this was shown indirectly and is unlikely to be a true function as NK cells do not express MICL [80, 134, 137]. Furthermore, antibody cross-linking of hMICL in DCs has been reported to result in antibody internalisation and to influence cytokine production and receptor expression. Studies also suggest that MICL can selectively interact with TLR signalling pathways or the T-cell signal CD40L, to alter cytokine production by DCs and thereby regulate downstream T-cell activation [133]. Although the physiological role of MICL has not yet been identified, the observation that it has a broadly expressed endogenous ligand suggests that it may play a role in
homeostasis [137]. Its ability to inhibit cellular activation suggests a regulatory role and furthermore, the various isoforms may also function cooperatively in order to maintain some type of regulation.

A recent investigation examined whether targeting of MICL could induce immune responses. This study concluded that antigen targeting of MICL in the presence of minimal amounts of adjuvant resulted in the mounting of a robust humoral response, but only moderate cytotoxic T-lymphocyte (CTL) responses [138]. Lahoud et al postulate that the poor induction of CTLs may be a consequence of inhibitory signalling through the MICL ITIM motif or alternatively that antigen delivered via MICL may be inefficiently shuttled into the cross-presentation pathway. The authors also suggest that there may be scenarios in which the induction of a humoral response without the activation of CTLs is desirable, in which case immunisation via MICL may be a useful strategy [138].

1.9 CLEC12B

1.9.1 Identification, structure and expression
CLEC12B was identified during a data base search for proteins with a high homology to the extracellular domain of the activating NK cell receptor NKG2D [140]. It is a type II transmembrane receptor with a CTLD, a transmembrane region and a cytoplasmic tail containing an ITIM sequence. RT-PCR analysis in human tissues showed that CLEC12B is widely expressed at low levels. Alternative splicing generates a variant which lacks exon 4. This transcript is present in mammary gland and ovary and is predicted to yield a non-functional protein. In transfected cells, CLEC12B formed disulfide-linked homodimers. Expression of CLEC12B was detected on differentiated human macrophages and on a stimulated human pro-myelocytic cell line, but there was no expression detected on freshly isolated monocytes or any other leukocyte population [140].

1.9.2 Ligands
To date there have been no ligands identified for CLEC12B.

1.9.3 Function
Like MICL, CLEC12B can recruit the inhibitory phosphatases SHP-1 and SHP-2 and it was shown to be capable of inhibiting certain cellular functions via its ITIM in an experimental in vitro system [140]. An obvious speculation is that such ITIM containing receptors function to downregulate and control cellular activation. In this context it is possible that CLEC12B and indeed MICL function as the inhibitory counterparts of ITAM containing receptors in this cluster. The identification of ligands and further functional investigations will give more insight into the role of this receptor.

1.10 Dectin-1 (CLEC7A)

1.10.1 Identification, structure and expression
Dectin-1 was initially identified as a DC specific receptor that modulated T cell function through recognition of an unidentified ligand on T cells [141, 142]. It was re-identified as a receptor for β-
glucans following a screen of a murine macrophage cDNA expression library with zymosan, a β-glucan rich extract of Saccharomyces cerevisiae [143]. β-glucans are carbohydrate polymers with well-characterised immunostimulatory activity that are found primarily in the cell walls of fungi, but also in plants and some bacteria [143, 144].

Dectin-1 is a glycosylated type II transmembrane receptor with a single extracellular CTLD connected by a stalk to a transmembrane region and a cytoplasmic tail containing an ITAM-like motif (described later). Like the other members of the Dectin-1 cluster, it lacks the residues for calcium ligation. The murine form of the receptor is expressed by many cell types, including macrophages, DCs, monocytes, neutrophils and a subset of splenic T cells [145]. There are at least two isoforms of murine Dectin-1, encoding the full length receptor and a stalkless version and they exhibit differences in their ability to recognise zymosan and to induce cellular responses [146]. Both murine forms are N-glycosylated at two sites in the extracellular region [141]. The expression of human Dectin-1 differs slightly in that it is also expressed on B cells, eosinophils and mast cells [147, 148]. Human Dectin-1 mRNA is also alternatively spliced generating eight isoforms (isoforms A-E), of which only two are functional for β-glucan binding (isoforms A and B). hDectin-1 isoform E lacks exon 3, which results in a variant that does not contain the transmembrane and stalk regions [147]. It has been shown that this isoform is retained in the cytoplasm and is capable of associating with a Ran-binding protein, RanBPM, although the functional relevance of this interaction is undetermined [135]. Of the two β-glucan binding isoforms, the most predominant lacks the stalk region and has no glycosylation sites. The other form contains one N-glycosylation site in its stalk region [147, 149]. It has been shown that glycosylation contributes to Dectin-1 surface expression [150], and the two major human isoforms are expressed differently in various cell types, although the significance of this remains unclear [147]. The highest levels of Dectin-1 expression are on inflammatory cells and at sites of pathogen entry such as the lung [145, 151]. It has been shown that the level of Dectin-1 expression in murine macrophages can be influenced by various cytokines and microbial factors [152-154]. For example IL-4, IL-13 and GM-CSF cause Dectin-1 expression to be highly upregulated. In contrast IL-10, LPS and dexamethasone cause down-regulation of Dectin-1 expression [153].

1.10.2 Ligands
Oligosaccharide microarray technology has shown that Dectin-1 specifically binds β-1,3-linked glucose oligomers [155]. The Ca\(^{2+}\) independent mechanism for recognition of carbohydrates by Dectin-1 is still unclear, however it is known that at least two residues flanking a shallow groove on the protein surface, Trp221 and His223, are crucial for β-glucan binding [156]. By way of its β-glucan specificity, Dectin-1 can recognise a number of fungal species [157-165]. Recent studies have also shown that Dectin-1 interacts with various mycobacterium species; however the specific ligand(s) recognised by Dectin-1 on these microbes remain unidentified [166-169]. Although β-glucans have been detected in bacteria such as Brucella, Agrobacterium and Rhizobium [170, 171], their presence has not been described in mycobacteria. As briefly mentioned earlier, Dectin-1 also recognises an unidentified endogenous ligand on T-cells resulting in cellular activation and
proliferation [141, 142, 147]. It is more likely that this T-cell ligand is a protein rather than a carbohydrate because it is sensitive to protease but not glycosidase treatment [141]. Based on the similarity of Dectin-1 to other NK-cell-receptor-like C-type lectins, it has been suggested that the ligand may be an MHC class I molecule or related protein, or another C-type lectin [45]. Studies using cell lines also showed that Dectin-1 recognises a ligand on apoptotic cells [172].

1.10.3 Function

Signalling from Dectin-1 following ligand binding can induce many cellular responses including the production of various cytokines and chemokines, the respiratory burst, production of arachidonic metabolites, ligand uptake through phagocytosis and endocytosis, and DC maturation [45, 173]. In recent years, several studies have sought to decipher the Dectin-1 induced signalling pathways that underlie different cellular responses. Before discussing the findings below, it is worth noting that as evidence of cross-talk and branching in various systems is emerging, the traditional visualisation of a linear signalling pathway is giving way to the realisation that these pathways are in fact much more intricate [174]. This holds true for Dectin-1 induced signalling which in some cases can trigger a response directly but in other cases requires cooperative signalling with MyD88-coupled TLRs. This is complicated further by recent evidence which shows variability in Dectin-1 signalling in different myeloid cell types [175, 176].

1.10.3.1 Dectin-1 signalling through the ITAM-like motif

Dectin-1 signalling is mediated through a cytoplasmic ITAM-like motif. It contains two tyrosines in its cytoplasmic tail that are positioned in an arrangement that is similar to an ITAM but does not conform exactly to the consensus sequence. The two tyrosines are appropriately spaced, but the N-terminal tyrosine resides in a YxxXL sequence (or YxxxL for mouse), as opposed to the traditional YxxL ITAM sequence. The membrane proximal tyrosine resides within a YxxL (YxxxL for mouse) sequence conforming to the traditional ITAM motif [45, 49]. Studies revealed that the membrane distal tyrosine was dispensable for signalling [157, 177-180] and this suggested that a single tyrosine based motif was sufficient for mediating signal transduction. Subsequently, it emerged that CLEC-2 and CLEC9A (discussed below) also signal via similar single tyrosine based motifs. The convention now is to use the term ‘ITAM-like motif’ to describe this type of sequence, and in the case of Dectin-1 the designation pertains to the tyrosine containing YxxL sequence and excludes the C-terminal tyrosine. This motif has also been referred to as a ‘hemiITAM’ motif [42], although the term ‘ITAM-like’ will be used here.

Following ligand binding, Dectin-1 becomes tyrosine phosphorylated by Src kinases, thereby providing a docking site for Syk which initiates downstream signalling [180]. This is reminiscent of traditional ITAM signalling where ligand binding similarly results in receptor phosphorylation by Src kinases and thus promotes the recruitment of Syk kinases as described above. However in contrast to ITAM receptors where dually phosphorylated tyrosines are necessary for Syk recruitment, phosphorylation of only the membrane proximal tyrosine is sufficient for Syk association with Dectin-1, even though both SH2 domains of Syk are still required [177, 181].
Destin-1 was the first receptor shown to signal via this pathway which although is quite similar to ITAM signalling, is unique in its dependence on only a single tyrosine present within the ITAM-like motif. As mentioned, other receptors have now been identified which signal in such a manner and these could perhaps be the first of many Syk-coupled ITAM-like receptors that have yet to be identified.

The precise nature of the interaction between Syk and these receptors is not yet fully understood; however, a model proposing that Syk is recruited by bridging two monophosphorylated molecules has become widely accepted [45, 177, 181, 182] (Fig 1.4). The presence of a highly flexible inter-SH2 domain linker region in Syk confers a conformational flexibility on the molecule that allows it to accommodate a number of relative orientations and to recognise a variety of dually phosphorylated ITAMs which vary significantly both in sequence and in the length of the region between the two tyrosines [183-186]. This attribute may also allow Syk to act in the bridging capacity proposed by this model. On the other hand, the related Zap-70 tyrosine kinase is highly specific for dually phosphorylated ITAMs of the T-cell receptor on T-cells and NK cells, and it is not capable of binding to singly phosphorylated ITAMs [187-190]. Structural differences account for the stringent binding of Zap-70 versus the flexibility of Syk [185, 191]. One wonders whether the plasticity that Syk displays in terms of binding could go so far as to allow its recruitment to ITIMs in myeloid cells, and whether this could account for instances of cellular activation brought about by signalling via ITIMs.

![Figure 1.4: Immureceptor tyrosine-based motif (ITAM)-like signalling. On ligand binding, receptors become tyrosine phosphorylated by Src kinases, thereby providing a docking site for Syk which initiates downstream signalling. It is proposed that Syk bridges two receptor molecules.](image-url)
1.10.3.2 Syk-dependent activation of NF-κB

Signalling initiated via Dectin-1 results in activation of NF-κB through both the canonical and non-canonical pathways (Fig 1.5) [192, 193]. Before discussing the details below, it is first useful to give a brief overview of the two established pathways of NF-κB activation (for review see [194]). In mammals, there are five members of the NF-κB family: p65, RelB, c-Rel, NF-κB1 (p50/p105) and NF-κB2 (p52/p100). NF-κB1 and NF-κB2 are both synthesised as large precursors (p105 and p100 respectively), which are post-translationally modified to become p50 and p52 respectively. The NF-κB family members form numerous homo- or hetero-dimers that are associated with differential regulation of gene transcription. Usually in unstimulated cells, NF-κB is retained in the cytoplasm by IkBs (inhibitors of κB). NF-κB1/p105 and NF-κB2/p100 precursors contain the inhibitory function of the IkB proteins in their C-terminal halves and also function to retain their partners in the cytoplasm. Two major signalling pathways result in translocation of NF-κB dimers from the cytoplasm to the nucleus. The canonical (or classical) pathway is mediated by activation of an IKK (inhibitor of κB kinase) complex which catalyses the phosphorylation of IkBs resulting in their ubiquitylation and subsequent proteasomal degradation. The released NF-κB dimers translocate to the nucleus and activate gene transcription. In the non canonical pathway (or alternative) pathway, NF-κB2/p100 (which is most commonly associated with RelB), is targeted through the successive activation of NF-κB-inducing kinase (NIK) and IKKa, which catalyses the phosphorylation of p100 and its ubiquitylation. This results in the proteasomal degradation of only the inhibitory C-terminal half of p100 and the remaining p52 protein is released resulting in translocation of the dimers (usually p52-RelB) to the nucleus [194].

In terms of Dectin-1 mediated activation of NF-κB, studies in DCs have demonstrated that recruitment of Syk to Dectin-1 results in activation of PLCγ2, leading to the engagement of the caspase recruitment domain (CARD) containing protein, CARD9, which together with Bcl10 and Malt1 signal for activation of the transcription factor NF-κB (Fig 1.5) [193, 195, 196]. The signalling events that link PLCγ2 activation to CARD9 recruitment are still not known. However in lymphocytes, signalling through ITAM containing proteins leads to recruitment of Syk tyrosine kinases that activate PLCγ, which in turn catalyses the hydrolysis of membrane phospholipids generating diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) [197]. DAG activates PKC isoforms which then activate the CARD9 related protein, CARD11, allowing the formation of a signalling complex consisting of CARD11, Bcl10 and Malt10 [197]. It is likely that a pathway analogous to this one culminating in CARD11 activation in lymphocytes, results in CARD9 activation in myeloid cells [195]. How signals are transmitted from the CARD9/Bcl10/Malt1 complex to NF-κB is also not yet described. However if one looks again to defined pathways in other cells, it seems reasonable to consider that the direct interaction of Malt10 with the adaptors Traf2 and Traf6 may lead to activation of IKK, which in lymphocytes results in translocation of NF-κB to the nucleus where it influences gene transcription [197, 198]. Dectin-1 is also the first PRR shown to induce Syk dependent activation of NF-κB in DCs via the non-canonical pathway. Previously, this activation of NF-κB had been reported for only a few members of the TNF receptor superfamily which do so by a Syk-independent mechanism [199, 200].
Figure 1.5: Ligation of Dectin-1 results in the recruitment of Syk leading to the activation of PLCγ2 and the formation of a complex composed of CARD9, Bcl-10 and MALT1. Unknown signals transmitted from this complex result in activation of an IKK complex which catalyses the phosphorylation of IκBs resulting in their ubiquitylation and proteasomal degradation. This results in the release of NFκB dimers and their translocation to the nucleus. Dectin-1 mediated recruitment of Syk also triggers the non-canonical pathway of NFκB activation through the successive activation of NIK and IκB which catalyses the phosphorylation of p100 and its subsequent degradation. This results in the nuclear translocation of p52-RELB dimers. Ligation of Dectin-1 also induces a Syk independent, Raf1 signalling pathway which integrates with the Syk pathway at the point of NFκB activation, as well as activation of NFAT via an unknown signalling pathway. Adapted from Geijtenbeek & Gringhuis, Nature Reviews Immunology, 2003 [196].
The intricacies of the pathway which relays signals from Syk to the activation of RelB-p52 dimers remain undefined at present. Ligation of Dectin-1 also induces a Syk independent, Raf1 signalling pathway which integrates with the Syk pathway at the point of NF-κB activation (Fig 1.5) [192].

It has recently come to light that Dectin-1 signalling via Card9 is differentially regulated in myeloid cells [175, 176]. For example, bone marrow derived DCs, resident peritoneal cells and alveolar macrophages activate NF-κB and drive TNFα production as described above, whereas bone marrow derived macrophages, thioglycollate elicited macrophages and Flt3L-derived DCs fail to do so. This failure to drive proinflammatory cytokine production is in spite of Dectin-1 induced activation of the Card9/Bcl10/Malt1 signalling complex [175]. This variability in Dectin-1 signalling in different cell populations is potentially a novel mechanism by which myeloid cells can be tweaked to regulate immune responses and may have implications for therapeutic uses [175, 176].

1.10.3.3 Syk-dependent activation of NFAT

Apart from activation of NF-κB in DCs, ligation of Dectin-1 also triggers activation of the transcription factor NFAT in macrophages and DCs (Fig 1.5) [196, 201]. The role of NFAT and the sequence of events leading to its activation are most well defined in T cells. The signalling pathway induced by ITAM containing T-cell receptors in lymphocytes (discussed earlier) branches after the generation of DAG and IP₃. As described, DAG initiates a cascade of events resulting in the activation of NF-κB. The action of IP₃ initiates another signalling pathway by triggering the release of Ca²⁺ from intracellular stores which in turn triggers the opening of Ca²⁺ channels in the plasma membrane resulting in the maintenance of the increased levels of intracellular Ca²⁺. This results in the activation of the phosphatase calcineurin which dephosphorylates NFAT transcription factors, leading to their nuclear translocation and regulation of gene expression [202, 203]. Although the signalling events linking Dectin-1 signalling with NFAT activation have yet to be elucidated, it is possible that NFAT activation in myeloid cells occurs via a pathway analogous to the one described for T cells. This is all the more likely since zymosan has been reported to trigger calcium flux, and Dectin-1 is known to signal via Syk in macrophages and DCs [196, 204, 205]. Dectin-1 mediated activation of NFAT regulates cyclooxygenase-2 and prostaglandin production in macrophages, and the induction of IL-2, IL-10 and IL-23 in DCs [201].

1.10.3.4 Syk-dependent, CARD9 independent signalling

It has also been shown that certain cellular responses controlled by Dectin-1/Syk signalling are not dependent on CARD9. For example, in DCs there is Syk-dependent activation of ERK, a mitogen activated protein (MAP)-kinase, through a CARD9-independent pathway [206]. Another example of this type of signalling is the induction of phagocytosis via Dectin-1 in DCs. Although this pathway is not fully understood, it is known that it is Syk dependent but does not require CARD9 [177, 193]. In macrophages however, Dectin-1 mediated phagocytosis is induced in a Syk independent manner (see below). An artificial system which demonstrated the phagocytic capacity of Dectin-1 by expressing the receptor in normally non-phagocytic fibroblasts, showed that Dectin-1 signalling caused activation of Cdc42 and Rac-1 triggering actin polymerisation and pseudopod extension.
around the particle [179]. It also showed that PKC was required. The precise role of PKC in phagocytosis is unclear, however one of its substrates, MARCKS (myristoylated, alanine-rich C-kinase substrate), possesses actin cross-linking activity that is involved in zymosan phagocytosis [207].

1.10.3.5 Syk-independent signalling
Dectin-1 is also capable of inducing signalling via Syk-independent pathways. In macrophages, ligation of Dectin-1 results in phagocytosis. This response requires the ITAM-like motif and a triacidic DED sequence, but does not require Syk and is possibly propagated by a novel kinase [45, 177, 179]. This is in contrast to the Syk requirement for Dectin-1 mediated phagocytosis by DCs discussed above. Recently, it was found that Dectin-1 induced a signalling pathway through the kinase Raf-1 that was independent of the Syk pathway but integrated with it at the level of NF-κB activation for regulation of cytokine production [192].

1.10.3.6 Interactions between Dectin-1 and other proteins
Continuing research in the field of pathogen recognition and the receptors involved in mediating immune responses has highlighted that receptor collaboration, as opposed to receptors functioning in isolation is an important facet of controlling infection [208, 209]. This holds true for Dectin-1 which collaborates with various TLRs to promote anti-fungal and anti-mycobacterial responses. In addition to β-glucans, there are a number of PAMPs in the cell walls of fungi that are recognised by several PRRs whose cooperation is required for efficient anti-fungal immune responses [210]. Dectin-1 has been shown to collaborate with TLRs for optimal cytokine production and enhancement of the respiratory burst. This collaboration depends on cell-type. For example, in macrophages, collaborative signalling by TLRs and Dectin-1 is required for TNFα production [157, 178]. On the other hand, in DCs, Dectin-1 signalling alone can trigger TNFα and IL-10 production, although collaborative signalling with TLRs enhances these responses [208, 211]. In macrophages, it was shown by a recent study in which I participated, that the Syk kinase pathway triggered by Dectin-1 is required for collaborative signalling with the TLRs which induced sustained IκB degradation resulting in enhanced nuclear translocation of NF-κB [208]. In fact, Dectin-1 can interact with several MyD88-coupled TLRs (TLR2, TLR4, TLR5, TLR7, TLR9) to induce production of multiple cytokines [173, 208, 212]. In addition to Syk, integration of TLR and Dectin-1 signalling requires the adaptor Raf-1 [192]. Functional cooperation between Dectin-1 and TLR2 has also been established during phagocytosis, ERK1/2 MAPK activation and the secretion of pro-inflammatory cytokines by macrophages and dendritic cells [165, 168, 210, 212, 213]. Indeed, a study which examined bone marrow derived macrophages challenged with Mycobacterium abscessus or zymosan, showed that Dectin-1 physically interacts and colocalises with TLR2 [167].

Apart from the TLRs, Dectin-1 also cooperates with a number of other plasma membrane proteins. In human DCs, costimulation of Dectin-1 and DC-SIGN triggers the arachidonic acid cascade [214]. In macrophages, Dectin-1 also cooperates with SIGNR1, the murine homologue of DC-SIGN, during nonopsonic recognition of yeast [215]. In addition to PRRs, Dectin-1 can interact with
tetraspanins, a family of proteins that have been shown to modulate signal transduction by interacting with many other transmembrane proteins to form ‘tetraspanin microdomains’ in the plasma membrane [216]. Dectin-1 associates with the ubiquitous tetraspanin CD63. Furthermore, it has been shown that phagocytosis of yeast particles by DCs was accompanied by a decrease in CD63 expression, which was inhibitable by a soluble β-glucan, laminarin [217]. Although the functional significance of a Dectin-1-CD63 interaction has not yet been elucidated, it may represent part of a signalling complex that could influence phagocytosis. Dectin-1 also interacts with the immune cell specific tetraspanin CD37 resulting in stabilisation of Dectin-1 in the cell membrane and inhibition of Dectin-1 mediated IL-6 production in response to zymosan [218, 219]. Whether the tetraspanin-Dectin-1 interactions are involved in Dectin-1 collaboration with TLRs is not known, although there has been speculation that tetraspanins may directly link the Dectin-1 and TLR signalling pathways [218].

1.10.3.7 Dectin-1 and innate immunity
β-glucans can constitute up to 50% of fungal cell walls and as mentioned, Dectin-1 recognises a number of fungal species by way of its β-glucan specificity including Candida, Pneumocystis, Saccharomyces, Aspergillus, Coccidioides and Penicillium [157-165]. Many studies have shown that detection of various fungal species by Dectin-1 results in the initiation of protective immune responses such as fungal uptake and killing by toxic reactive oxygen intermediates, and the production of protective inflammatory cytokines and chemokines, including TNFα, CXCL2, IL-1β, IL-1α, CCL3, GM-CSF, G-CSF and IL-6 [45, 220]. Dectin-1 also induces the production of IL-2 and IL-10, cytokines whose role in fungal infection is less clear. IL-10 in particular has been shown to have inhibitory effects on anti-fungal immune responses [221]. These cytokines also contribute to the development of Tregs, the implications of which will be discussed later.

In vivo studies, although not wholly consistent, have supported other evidence that Dectin-1 plays a role in antifungal immunity. Loss of Dectin-1 in mice with a 129Sv background resulted in increased susceptibility to systemic infection with C. albicans and invasive pulmonary infection with A. fumigatus as a consequence of inflammatory defects and decreased fungal killing [162, 222]. Interestingly, Dectin-1 deficient mice with a C57BL/6 background showed no increased susceptibility to C. albicans, but did exhibit increased susceptibility to Pneumocystis carinii due to defects in the respiratory burst [159]. This disparity is most likely because of the genetic backgrounds of the deficient mice, but could also be due to differences in the fungal strains and/or routes of infection used. Further evidence to support a role for Dectin-1 in fungal infections is the recent identification of a polymorphism in human Dectin-1 that is associated with an increased oral and gastrointestinal colonisation with Candida species [223].

1.10.3.8 Dectin-1 and adaptive immunity
In addition to triggering innate immune responses, Dectin-1 signalling can also induce adaptive immunity and was the first non-TLR PRR shown to do so. Dectin-1 activated DCs can ‘instruct’ the differentiation of Th17 and Th1 CD4+ T cells independently of TLR signalling in response to
C. albicans and Mycobacterium tuberculosis [211, 224]. It has been shown that Raf-1 signalling by Dectin-1 is a crucial determinant in the induction and regulation of the relevant polarising cytokines [192, 211]. While it is generally accepted that the generation of a Th1 response is required for protection against fungal infection in healthy hosts [225], the role of Th17 cells in anti-fungal immunity is rather more controversial. A number of studies have shown a detrimental role for IL-17 in anti-fungal immunity [226, 227]. On the other hand, mice that are unable to mount Th17 responses are very susceptible to oropharyngeal candidiasis [228, 229]. Furthermore Th17 deficiency in humans is also associated with increased susceptibility to C. albicans infection [230, 231]. Conditions such as route of infection and inocula used may account for the observed differences; however, IL-17 like other cytokines can have opposing roles in disease [232]. For example, in periodontal disease IL-17 may be either host-protective or destructive depending on how chronic and severe the disease is [233, 234]. It has also been shown that vaccination induced IL-17 producing T-cells are critical for the rapid recruitment of protective CD4+ T cells upon challenge with M. tuberculosis [235]. This suggests that the Th17 responses induced by targeting Dectin-1 may be a means of enhancing the efficacy of future vaccines to this pathogen.

Interestingly, Dectin-2 was recently identified as another Syk/CARD9-coupled C-type lectin that mediates DC activation and induction of Th17 immunity in response to C. albicans [236]. Dectin-2 has a positively charged residue in its transmembrane domain which mediates association with the ITAM containing FcRy chain and allows coupling to the Syk pathway [236]. Despite this distinct difference in Syk recruitment, Dectin-2 and Dectin-1 may be representative of a novel class of myeloid expressed receptors which signal via Syk and CARD9 to initiate immunity, as the authors pointed out [236].

Activation of DCs via Dectin-1 can also convert selected populations of Treg cells into IL-17 producing T cells that cannot be classified as either Treg or Th17 cells [237]. Furthermore, costimulation of Dectin-1 and TLR2 contributes to Treg expansion and function [238, 239]. Tregs suppress T effector cells and have protective effects in certain infections by means of their anti-inflammatory function [240]. They have also been shown to be essential components of the memory protective immunity to certain pathogens [241, 242]. On the other hand, Tregs were demonstrated as immunosuppressive during disseminated candidiasis [243]. These adaptive immune responses mediated via Dectin-1 remain somewhat unclear in terms of their contribution to host protection or immunopathology; however, it may turn out that Dectin-1 has a central role in balancing the pro-inflammatory and anti-inflammatory responses during fungal infection [173].

In addition to driving CD4+ T cell differentiation, Dectin-1 stimulation can also induce CD8+ T cell responses. DCs which were stimulated with curdlan, a selective β-glucan agonist, were found to promote the expansion and differentiation of CTL precursors in vitro [244]. The precise role of CTLs in direct antifungal responses is unclear, however it has been shown that CD8+ T cells are activated during fungal infections and can play a protective role in some cases [245-248]. Curdlan was also found to act as a potent adjuvant for CTL crosspriming in vivo, eliciting a cytotoxic
response that was sufficient to protect against tumor challenge [244]. This may explain the antitumor activity of β-glucans which are used in cancer therapy in some countries [249], and this study suggests that Dectin-1 agonists may be beneficial as immunotherapeutic agents. Antibody-mediated targeting of Dectin-1 also resulted in the induction of CTL responses as well as CD4⁺ T cell and Ab responses [250]. This study demonstrated that targeting to different DC subsets using receptors can induce qualitatively different immune responses, with anti-Dectin-1 preferentially triggering a CD4⁺ T cell response but anti-DEC-205 favouring induction of a CD8⁺ T cell response [250].

1.10.3.9 Dectin-1 and autoimmunity
Dectin-1 mediated responses have also been implicated in driving autoimmunity. SKG mice, which are genetically susceptible to develop autoimmune arthritis, did not develop arthritic symptoms in a pathogen free environment. However, treatment with zymosan or purified β-glucans induced severe chronic arthritis in these animals which could be inhibited by blocking Dectin-1 [251]. This may be as a result of dysregulated generation of IL-17, a cytokine which has been shown to be involved in the pathogenesis of various autoimmune diseases in mice [252]. Furthermore, blockage of Dectin-1 could prevent experimental autoimmune uveoretinitis, a Th1/Th17 disease and it has been suggested that Dectin-1 will be implicated in various other autoimmune diseases in future [173].

1.10.4 Dectin-1 – summary
The expression of Dectin-1 on myeloid cells allows for the recognition of fungal and mycobacterial species and the subsequent induction of all branches of immunity. Dectin-1 was the first Syk-coupled non-TLR PRR shown to drive such responses and it may be the prototype of a new class of receptor. Furthermore, Dectin-1 was the first example of a PRR which induces intracellular signalling via a single-tyrosine based ITAM-like motif. Further research into the role of Th17 and Treg responses in fungal and mycobacterial infection is required before we will fully understand the role of Dectin-1 in these immune responses, but studies suggest that Dectin-1 is potentially an immunotherapeutic target. The potential role of Dectin-1 in autoimmunity is also an area that requires further research and the identification of the endogenous Dectin-1 ligand(s) on T-cells will assist in the elucidation of the role of Dectin-1 in homeostasis.

1.11 CLEC9A
1.11.1 Identification, structure and expression
CLEC9A is a type II transmembrane protein with a single CTLD, a transmembrane region and a cytoplasmic tail containing an ITAM-like motif similar to that of Dectin-1 [253, 254]. It has also been described as DNGR-1 (DC, NK lectin group receptor-1) [254]. In mice, alternative splicing generates at least five isoforms of CLEC9A. Similar to related C-type lectins one of these isoforms contains six exons, and this form is expressed as a non-glycosylated monomer at the cell surface [253]. Conversely, a seven exon version which contains an additional exon in the CTLD is expressed as a dimer at the cell surface [254, 255]. The various murine isoforms may display different physiological functions and cellular distribution, although this has not yet been examined.
Investigation of cellular distribution has only been carried out for the seven exon version of murine CLEC9A. It was found to be expressed at low levels on plasmacytoid DCs and on CD8α+ DCs in the spleen, lymph nodes and thymus [254, 255]. The CD8α+ DCs are specialised for, and are by far the most efficient at, presenting exogenous peptides on MHC class I molecules, a process known as cross-presentation [250, 256-258].

In humans, CLEC9A (hCLEC9A) is present as a glycosylated dimer at the cell surface and RT-PCR analysis showed that it is broadly expressed as a single transcript in most organs, with highest expression in brain, thymus and spleen [253]. In peripheral blood, hCLEC9A expression is restricted to BDCA3+ DCs, a small subset of CD14+CD16- monocytes, an unidentified population of CD14-CD64+ cells and B-cells [253, 254, 259]. Little is understood about the BDCA3+ population, although they are known to express multiple TLRs and are thought to be immature precursors of interstitial DCs [260-262]. These cells also express high levels of CD62L and it has been suggested that this may mediate the migration of BDCA3+ DCs from the blood into lymphoid tissues [262]. Functionally, little is known about the BDCA3+ DCs but based on the expression of CLEC9A and several other surface markers, it is believed that they may represent a DC lineage equivalent to the murine CD8α+ DC subset [253, 254, 259, 263, 264]. CD14+CD16- monocytes are considered 'classical monocytes' and are believed to migrate to sites of inflammation where DC differentiation occurs [265, 266].

1.11.2 Ligands
Specific ligands for CLEC9A have yet to be identified. However, it is known that CLEC9A acts as a receptor for necrotic cells and it is thought that this is mediated by recognition of a ubiquitous preformed acid-labile ligand(s) that is normally sequestered in healthy cells but becomes exposed following disruption of membrane integrity during necrosis [22].

1.11.3 Function
As mentioned, CLEC9A contains an ITAM-like motif in its cytoplasmic tail, YxxL, and it induces intracellular signalling via recruitment of Syk [253, 254]. It is possible that Syk associates with CLEC9A in a similar way to that which has been proposed for Dectin-1; via a bridging interaction between two CLEC9A molecules (Fig.1.4) [177, 181, 253]. As mentioned, it was recently demonstrated that CLEC9A acts as a receptor for necrotic cells [22]. CLEC9A also regulates the cross-presentation of dead-cell associated antigens in a Syk-dependent manner [22]. This investigation found that although CLEC9A is not required for uptake of necrotic cell material, it is necessary for efficient cross-presentation of dead-cell associated antigens by CD8α+ DCs [22].

As mentioned earlier, another Syk-coupled C-type lectin in addition to CLEC9A, Mincle, has also been shown to recognise necrotic cells [21]. It may be that CLEC9A and Mincle are part of a family of Syk-coupled proteins that are involved in sensing and mediating the response to dead cells [22]. Mincle has also been described as a fungal PRR as mentioned previously [74, 75]. In light of recent discoveries regarding CLEC9A, Dectin-1, Dectin-2 and Mincle, it appears that a paradigm is
emerging of Syk-coupled myeloid expressed C-type lectins that mediate recognition and initiate cellular responses to pathogens and necrotic cells.

Because CLEC9A is an endocytic receptor that is largely restricted to a specific DC subset that importantly is common to both human and mice, it holds some promise as a candidate for delivery of antigens to DCs. This potential was explored recently by two independent groups. Sancho and colleagues demonstrated that antigen epitopes covalently coupled to an anti-CLEC9A monoclonal antibody were selectively cross-presented to CD8⁺ T cells in vivo and induced potent CTL responses in the presence of adjuvant [254]. They also determined that targeting of antigen to DCs via CLEC9A did not result in presentation by plasmacytoid DCs, a result which they attribute to the fact that CLEC9A is expressed at very low levels on these cells and that plasmacytoid DCs are nevertheless understood to be inefficient at cross-presentation. They then proceeded to explore whether CLEC9A targeting could be used for tumour immunotherapy. In a cancer model, they tested the efficacy of antigen targeting via CLEC9A to either prevent or treat tumors. In their model, tumor derived peptides were covalently coupled to anti-CLEC9A and administered to mice in the presence or absence of adjuvants either before or after tumor challenge. They found that the CTL responses mounted resulted in both tumour eradication and tumor prevention [254]. Caminschi and colleagues also showed that targeted delivery of antigens to DCs using anti-CLEC9A monoclonal antibodies induced a striking enhancement of humoral immunity, and enhanced CD4⁺ and CD8⁺ T cell proliferative responses [255]. Interestingly all of the responses in this investigation were observed in the absence of additional adjuvants.

It will be interesting to see whether CLEC9A turns out to function in pathogen recognition as has been described for Dectin-1, Dectin-2 and Mincle. The studies described above which use CLEC9A to target antigens to the CD8α⁺ DC subset indicate that CLEC9A is a promising target for therapeutic applications and further research in mouse models could possibly translate to an immunotherapeutic strategy for cancer in clinical settings.

1.12 CLEC·2 (CLEC1B)

1.12.1 Identification, structure and expression

CLEC-2 is a type II transmembrane receptor displaying an extracellular CTLD, a single transmembrane region and a short cytoplasmic tail that contains an ITAM-like signalling motif. CLEC-2 was originally identified through a computational screen of myeloid cells for C-type lectin-like receptors homologous to those expressed by NK cells [82]. This study identified human CLEC-2 (hCLEC-2) and its murine homologue (mCLEC-2) which showed approximately 60% identity with the human form. RT-PCR analysis of full length hCLEC-2 showed transcripts in PBMC, bone marrow cells, monocytes, DCs, granulocytes and in a few NK cell clones [82]. RNA blot analysis of CLEC-2 on different human tissues also showed selective expression in the liver [82]. In mice, two further splice variants in addition to full length mCLEC-2, have been identified. One, named mCLEC-2B, lacks exon 2 which encodes the transmembrane region. The other, named mCLEC-2C, lacks exons 2 and 4, which causes a frameshift and generates an open reading frame of only
The various isoforms of mCLEC-2 showed different expression profiles and subcellular localisation. All three transcripts were expressed in PBMC and monocytes. However, only mCLEC-2B and mCLEC-2C were detected in a T-cell line, a B-cell line and a macrophage cell line, and furthermore, only mCLEC-2C in peritoneal macrophages and a fibroblast cell line [267]. In transfected fibroblasts, full length mCLEC-2 was expressed on the cell surface, while mCLEC-2B and mCLEC-2C were retained in the cytoplasm. This is consistent with the cytoplasmic retention of other type II transmembrane receptors which have been alternatively spliced to generate variants lacking the transmembrane region, for example human Dectin-1 isoform E [135]. Surface expression of CLEC-2 has been shown in platelets where it has been shown to undergo differential glycosylation [268]. During the course of this study I also described surface expression of mCLEC-2 on peripheral blood neutrophils [269].

mCLEC-2 can be cleaved by serine proteases releasing a soluble form of the receptor. Soluble CLEC-2 has been shown to exist as disulfide-linked homodimers which led to the suggestion that full-length mCLEC-2 also exists as homodimers, although this has not been directly shown [267]. In fact, the structure of the extracellular domain of hCLEC-2 has been solved to 1.6-

The crystal structure of the extracellular region of hCLEC-2 revealed that its basic core consists of key features of the CTLD, with two antiparallel β-sheets flanked by two α-helices. A semi-helical long loop region dominates the upper surface of CLEC-2 and there is a 3-10 α-like helix contained in this region. Mutational binding analysis identified the long loop as important for ligand binding [270]. The residues which typically coordinate a calcium ion in related carbohydrate binding proteins are not present in CLEC-2.

1.12.2 Ligands

Both endogenous and exogenous ligands of CLEC-2 have been identified. The first exogenous ligand described was rhodocytin [268], a toxin purified from the venom of the Malayan pit viper Calloselasma rhodostoma. It was long known that rhodocytin elicited powerful activation of platelets [271] but the exact mechanism was unclear until an investigation by Suzuki-Inoue and colleagues. This investigation used rhodocytin-affinity chromatography combined with mass spectrometry analysis to reveal that CLEC-2 was the receptor underlying platelet activation by rhodocytin [268]. It has been speculated that as rhodocytin is multimeric, ligand binding may initiate surface clustering of CLEC-2 on platelets causing localisation of cytoplasmic signalling domains and augmentation of signal transduction [270]. CLEC-2 is likely to recognise a proteinaceous ligand on rhodocytin, as the venom protein is not glycosylated which rules out the possibility of a CLEC-2-carbohydrate interaction [270].

Podoplanin, a transmembrane sialoglycoprotein involved in tumor cell-induced platelet aggregation, tumor metastasis, and lymphatic vessel formation, was recently identified as an endogenous ligand for CLEC-2.
for CLEC-2 and it has been suggested that their interaction may be involved in tumor growth and/or metastasis [272-274]. Using recombinant glycopeptides to study the interaction of hCLEC-2 with podoplanin, Kato et al concluded that the interaction was carbohydrate-mediated, and that a disialylated core O-glycan sequence on Thr52 of podoplanin was essential for recognition by CLEC-2 [273]. This is in agreement with a structural study which indicated that an endogenous ligand is likely to be a protein with a predominantly negatively charged binding surface [270]. However, it remains unclear whether the oligosaccharide sequence alone is sufficient for CLEC-2 binding, or whether any of the flanking peptide region is also involved.

CLEC-2 was also identified as a HIV-1 attachment factor that may capture and transfer infectious HIV-1 in cooperation with DC-SIGN in platelets [275]. The structures recognised by CLEC-2 on HIV-1 particles are so far unidentified. However, binding of HIV-1 to CLEC-2 occurred in the absence of the envelope protein which led to the suggestion that CLEC-2 may recognise a cellular factor which is incorporated into the viral envelope upon budding from infected cells [275]. It is well known that a vast array of host proteins can be incorporated by HIV-1 during viral budding [276, 277]. It is therefore a plausible idea that CLEC-2 may recognise an HIV-1 incorporated host factor. In fact, there is evidence that HIV-1 can infect renal cells where podoplanin is expressed and it has been suggested that podoplanin may be incorporated into the budding virion leading to recognition by CLEC-2 [272, 278, 279].

1.12.3 Function
A number of studies have explored the downstream signalling pathways initiated by ligation of CLEC-2. CLEC-2 signalling is critically dependent on both SH2 domains of Syk, similar to Dectin-1/Syk signalling [181]. Furthermore, it was also proposed that a single Syk molecule forms a bridge between two CLEC-2 receptors, as has been suggested for both Dectin-1 and CLEC9A (Fig. 1.4) [181, 253]. It was also shown that in addition to Syk phosphorylation, stimulation of CLEC-2 results in tyrosine phosphorylation of PLCy2, Vav1/3, LAT, SLP-76 and Btk [181, 268, 280]. Indeed, platelets deficient in Syk, and CLEC-2 transfected T-cells deficient in Syk, Btk or PLCy2 failed to respond to rhodocytin, demonstrating a critical role for these proteins in mediating CLEC-2 signalling in platelets [181, 268]. On the other hand, a significant but diminished response of CLEC-2 transfected T-cells to rhodocytin was observed in the absence of BLNK or SLP-76, demonstrating that CLEC-2 is only partially dependent on the SLP-76/BLNK family of adapter proteins for signalling [181, 280]. Platelets lacking Rac1 display severely impaired CLEC-2 dependent activation demonstrating a role for this Rac GTPase in CLEC-2 signalling [281].

Over-expression of CLEC-2 in a cell line generated constitutive signalling via Src and Syk kinases that led to NFAT activation [282]. Co-expression of the ITIM-containing platelet protein, G6b-B, resulted in inhibition of both constitutive and agonist-induced CLEC-2 signalling. The authors of this study speculated that this may represent an important role of G6b-B and other ITIM containing platelet receptors in inhibition of platelet activation which is an essential component of physiological blood flow [282]. A further study examined the effect of another ITIM-containing platelet protein,
PECAM-1, on CLEC-2 signalling using both PECAM-1 deficient mice and antibody cross-linking of PECAM-1. This work demonstrated that PECAM-1 also had an inhibitory effect on CLEC-2 signalling, although the results suggested that this inhibitory effect was mild and unlikely to be physiologically relevant [283].

Dectin-1 and CLEC-2 have an identical sequence consisting of four amino acid residues DEDG, preceding their YxxL motifs. Analysis in transfected B cells revealed that mutation of the glycine residue of CLEC-2 resulted in a significant reduction in signalling in response to rhodocytin demonstrating that the glycine residue is important for CLEC-2 signalling [181]. In a similar vein, mutation of this highly charged cluster in Dectin-1 was shown to abolish particle uptake [180]. Given the significance of the glycine residue in CLEC-2 signalling, the requirement of the DED cluster for Dectin-1 mediated phagocytosis, and the demonstration that CLEC9A which lacks such a sequence does not mediate phagocytosis [253], it seems highly likely that the ability of CLEC-2 to mediate phagocytosis involves this highly charged cluster.

Although many of the CLEC-2 signalling studies were carried out in platelets, the importance of CLEC-2 for platelet activation during hemostasis and in the course of thrombotic events was not defined until very recently. May et al demonstrated that antibody-targeting of CLEC-2 resulted in complete and highly specific loss of the receptor in circulating platelets for several days [284]. This study demonstrated that CLEC-2 is not required for adhesion of platelets to collagen during thrombus formation, but is necessary for subsequent stable aggregate formation. Furthermore, antibody-induced CLEC-2 deficiency resulted in increased bleeding times of mice indicating that CLEC-2 plays a significant role for normal hemostasis [284]. These results suggest that CLEC-2 may represent a novel antithrombotic target.

I have demonstrated that CLEC-2 is a phagocytic receptor expressed on neutrophils which can mediate the production of proinflammatory cytokines [269]. The expression of this receptor on neutrophils and the recognition of exogenous ligands imply a role in innate immunity. Although, a direct role in immunity has not been demonstrated, it is likely that future investigation of CLEC-2 will reveal yet another example of a C-type lectin which functions in both homeostasis and pathogen response.

### 1.13 Aims of this thesis

CLEC-2 is the focus of this thesis. I originally embarked on this project in 2006 with the primary aim to carry out a functional characterisation of this receptor. At the outset of this work, CLEC-2 had merely been identified with some minimal analysis of expression [82]. However, quite a number of investigations of CLEC-2 have been published in the interim, including further analysis of expression and structure; and identification of ligands and signalling pathways. The emergence of new discoveries and technologies, and the development of general concepts, especially within a field, often influence the path of one's research and/or the interpretation of results.
My initial investigation into CLEC-2 was prompted by the observation that a short, but potentially relevant sequence, was present in the cytoplasmic tails of Dectin-1 and CLEC-2: DEDGYxxL. As previously outlined, the YxxL motif of Dectin-1 is required for its functionality and given the presence of this motif in CLEC-2 and the upstream homology between the two proteins, I sought to determine whether CLEC-2 was functionally related to Dectin-1. As I will describe in the forthcoming chapters, I found that in some respects CLEC-2 was functionally related to Dectin-1. However, I also established that unlike Dectin-1, CLEC-2 could not induce production of reactive oxygen species despite signalling via Syk. This observation initiated an investigation into the mechanisms that underlie ITAM-mediated activation of the respiratory burst and the role of Syk in this process. In addition, the identification of podoplanin as a ligand for CLEC-2 resulted in a facet of this project which aimed to characterise expression of this CLEC-2 ligand on macrophages.

In summary the aims of this thesis were to extend the functional characterisation of CLEC-2, explore the mechanisms that underlie Syk-coupled ITAM-mediated induction of the respiratory burst and investigate the expression of podoplanin on various macrophage populations.
2 Materials and Methods

2.1 General reagents
General chemicals of analytical grade were purchased from Gibco/Invitrogen, Sigma Aldrich and Merck. Distilled water was produced using a milliQ water purification system.

2.2 Solutions
- **Cell lysis buffer for extraction of genomic DNA**: dH₂O, 10mM Tris pH 7.8, 100mM NaCl, 10mM EDTA pH 8.0, 0.5% SDS (w/v) and 50µg/ml Proteinase K were added immediately prior to use.
- **Cell lysis buffer for immunoprecipitations**: dH₂O, 25mM Tris pH 8.0, 140mM NaCl, 4mM EDTA, 1.1% (v/v) NP40, 10mM NaF, 1mM Na₃VO₄
- **Coomassie staining solution**: dH₂O, 0.125% (w/v) Coomassie brilliant blue, 45.5% (v/v) Methanol, 9.2% (v/v) acetic acid
- **Coomassie destaining solution**: dH₂O, 7.5% (v/v) glacial acetic acid, 5% (v/v) methanol
- **Coupling buffer**: dH₂O, 0.1M NaHCO₃, 0.5M NaCl, pH 8.5
- **ELISA stop solution**: 2M H₂SO₄
- **FACS block**: PBS, 5% (v/v) heat inactivated serum, 0.5% (w/v) BSA, 2mM NaN₃
- **FACS wash**: PBS, 0.5% (w/v) BSA, 2mM NaN₃
- **Formaldehyde fix**: PBS, 2% (w/v) formaldehyde
- **Gey’s solution**: 20% A, 5% B, 5% C, 70% tissue culture grade dH₂O. Filter sterilised.
  - **Gey’s solution A**: 3.5% (w/v) NH₄Cl, 0.185% (w/v) KCl, 0.15% (w/v) Na₂HPO₄.12H₂O, 0.5% (w/v) glucose
  - **Gey’s solution B**: 0.42% (w/v) MgCl₂.6H₂O, 0.34% (w/v) CaCl₂, 0.14% (w/v) MgSO₄.7H₂O
  - **Gey’s solution C**: 2.25% (w/v) NaHCO₃
- **Lidocaine/EDTA**: PBS, 4mg/ml Lidocaine hydrochloride, 10mM EDTA pH8.0, filter sterilised
- **Paraformaldehyde solution**: dH₂O, 4% (v/v) paraformaldehyde, 250 mM HEPES
- **Pervanadate solution**: PBS, 20mM Na₃VO₄, 0.05M H₂O₂, prepared fresh for each use
- **PBS-Tween wash buffer**: PBS, 0.1% Tween-20
- **Ponceau stain**: dH₂O, 0.2% (w/v) ponceau S, 5% (v/v) acetic acid
- **SDS-PAGE running buffer**: dH₂O, 25mM Tris, 90mM glycine, 0.1% (w/v) SDS
2.3 General nucleic acid manipulation techniques

2.3.1 Polymerase Chain Reaction (PCR)

For general PCR reactions, 2X PCR Master Mix (Fermentas) containing 0.05u/µl Taq DNA polymerase, reaction buffer, 4mM MgCl2 and 0.4mM of each dNTP (dATP, dCTP, cGTP, dTTP) was used. PCR grade H2O (Sigma), 0.2µM of appropriate forward and reverse primers and approximately 100ng of template DNA per 20-50µl reaction were added and reactions were incubated using a Gradient PTC-200 Peltier thermal cycler (from MH Research). Cycling parameters were dependent on primers and template size. However, standard parameters involved a denaturing step at 94°C for 2 minutes, followed by 30-35 cycles of denaturation, annealing and extension. Annealing temperatures were set at 5°C below the lowest primer melting temperature. If TOPO cloning was to be performed, a final incubation at 72°C for a further 10 minutes was carried out to ensure that all PCR products were full length and 3’adenylated. When increased sensitivity was required, PCR reactions were carried out using the Advantage® 2 PCR Kit (Clontech) according to manufacturer’s instructions. All PCR reactions were analysed by agarose gel electrophoresis.

2.3.2 Agarose gel electrophoresis

1% (w/v) agarose gels were prepared by dissolving electrophoresis grade agarose (BioAmerica) in 0.5X TBE (Roche) under high temperatures. Ethidium bromide (Sigma) was added to the dissolved agarose to a final concentration of 0.2µg/ml. DNA loading dye (Fermentas) was added to the PCR samples before they were loaded onto the gel. DNA molecular weight markers (Fermentas) were loaded in parallel to samples for identification of fragments of specific size. Gels were run at a constant voltage of 90-100V, to give a good separation of fragments. Gels were analyzed using a long wavelength (366nm) UV transilluminator (from UV Wirsam Scientific).
2.3.3 Purification of DNA from agarose gels
Gel slices containing the DNA band of interest were excised and DNA was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to manufacturer’s instructions.

2.3.4 Topo cloning
TOPO TA Cloning® (Invitrogen) provides a highly efficient, one-step cloning strategy for the direct insertion of Taq polymerase-amplified PCR products into a plasmid vector. Topo cloning of PCR products into the pCR2.1-TOPO vector was carried out according to manufacturer’s instructions. TOPO reactions were then used to transform chemically competent Escherichia coli.

2.3.5 Digestion of DNA using restriction enzymes
Where required, DNA was digested using appropriate restriction enzymes according to manufacturer’s guidelines. In general, 25-50ng of DNA was digested for 2 hours at 37°C. When indicated by the manufacturer, enzymes were then heat inactivated at appropriate temperatures. The success of each digest was confirmed by agarose gel electrophoresis and thereafter the digested DNA was excised and gel purified as described earlier.

2.3.6 Ligation of digested vectors and insert DNA
Ligation reactions were prepared according to Table 2.1 and incubated overnight at 16°C. Control reactions containing linearised vector DNA but no digested insert DNA were routinely included to analyse background from self-ligation of the vector. 2μl of ligation reactions were used to transform chemically competent E.coli.

Table 2.1 Ligation reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearised vector DNA</td>
<td>20-100ng</td>
</tr>
<tr>
<td>Digested insert DNA</td>
<td>1:1 to 5:1 molar ratio over vector</td>
</tr>
<tr>
<td>10X T4 DNA Ligase buffer (Fermentas)</td>
<td>2μl</td>
</tr>
<tr>
<td>T4 DNA Ligase (Fermentas)</td>
<td>0.2μl (1u)</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>to 20μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>20μl</td>
</tr>
</tbody>
</table>

2.3.7 Transformation of competent cells
One Shot® TOP10 Chemically Competent E.coli (Invitrogen) were thawed on ice from storage at -80°C. 2μl of DNA was added to 15-35μl of competent cells, mixed gently and incubated on ice for 5 to 30 minutes. Cells were heat shocked at 42°C for 30 seconds without shaking and immediately transferred to ice. 250μl of room temperature SOC medium was added and the reaction was incubated at 37°C on a horizontal shaking incubator for 1 hour. 200μl from each transformation was spread on prewarmed LB agar plates containing 100μg/ml ampicillin (Sigma) and incubated overnight at 37°C. All vectors used in this study contained an ampicillin resistance cassette.
2.3.8 Analysis of transformants by PCR
Transformants were screened by PCR to confirm that the insert was present in the desired orientation. Single colonies were sampled from selection plates using a pipette tip, and transferred to a replica plate containing the appropriate antibiotic which was incubated overnight at 37°C. Thereafter the colonies were transferred directly to a tube containing a PCR reaction mixture with the appropriate primers. PCR and agarose gel analysis were performed as previously described.

2.3.9 Inoculation of cultures and isolation of plasmid DNA
Single, well isolated colonies corresponding to transformants that showed the presence of the desired insert, were selected from the replica plate and used to inoculate 10-15ml of LB medium containing 100μg/ml ampicillin. This culture was incubated for 12-16 hours at 37°C in a shaking incubator. The Wizard® Plus SV Minipreps DNA Purification System (Promega) was used according to manufacturer's instructions to isolate plasmid DNA from these cultures. DNA samples were checked for concentration and quality using a NanoDrop® ND-1000 Spectrophotometer prior to sequencing analysis.

2.3.10 Genomic DNA isolation
To confirm that newly generated cell lines were expressing the full correct nucleotide sequence introduced by retroviral transduction (described later), genomic DNA was isolated from these cells and analysed by sequencing. Cells were detached with lidocaine/EDTA, pelleted at 1000rpm for 5 minutes at 4°C and lysed in cell lysis buffer which had been supplemented with 50μg/ml Proteinase K (Roche) immediately prior to use. Cell lysates were incubated overnight on a shaking platform at 55°C. To extract the genomic DNA, an equal volume of phenol, pH 8.0 was added, followed by vigorous shaking and then centrifugation at 13,000rpm for 2 minutes at 4°C. Supernatants were carefully transferred to new eppendorf tubes and an equal volume of Chloroform(24):Isoamylalcohol(1) was added. Tubes were mixed by inversion and centrifugation repeated. The aqueous supernatant was transferred to a new eppendorf tube and 50μl of 3M sodium acetate, pH 5.2 was added, followed by 500μl of isopropanol. Tubes were inverted several times before centrifugation at 13,000rpm for 15 minutes to precipitate DNA. Pellet was washed with 70% ethanol, air dried and resuspended in TE buffer. DNA samples were checked for concentration and quality using a NanoDrop® ND-1000 Spectrophotometer prior to sequencing analysis.

2.3.11 Sequencing
To confirm the fidelity of all constructs, purified plasmid DNA was analysed by sequencing at the Central Analytical Facility at the University of Stellenbosch, Cape Town, South Africa. Sequences were analysed using DNAMAN software.
2.4 Nucleic acid manipulations specific to this thesis

2.4.1 Generation of mCLEC-2HA and Dectin-1HA

Full length murine CLEC-2 (mCLEC-2) with a C-terminal hemagglutinin (HA) tag was generated in collaboration with Dr. Kevin Dennehy as follows: The complete CLEC-2 open reading frame was amplified from BALB/c mouse spleen cDNA by PCR using primers A and B (see Table 2.2). The PCR product was purified and inserted into TOPO vector as described (section 2.3.4) and the fidelity of the sequence was confirmed. Using primers C and D (see Table 2.2); recognition sites for Sal1 and Xho1 were introduced by PCR. The purified PCR product was digested with Sal1 and Xho1 restriction enzymes (Fermentas) and ligated with annealed primers E and F (see Table 2.2). This ligation resulted in the introduction of the influenza hemagglutinin (HA)-tag at the C-terminus of the receptor. This short sequence (YPYDVPDYA) encodes a simple and convenient method to detect the expression of genes by flow cytometry, Western blot or immunoprecipitation. The mCLEC-2HA construct was then digested with Sal1 and Not1 restriction enzymes (Fermentas) and ligated with pFBneo or pMXs-IP vectors which had also been digested with Sal1 and Not1 as described (section 2.3). Full-length HA-tagged Dectin-1 (Dectin-1 HA) was available in the laboratory and was generated as previously described [157].

2.4.2 Generation of mCLEC-2/Dectin-1HA chimeric constructs

A C-terminally HA-tagged mCLEC-2/Dectin-1 chimera in pFBneo was generated in collaboration with Dr. Kevin Dennehy using overlap extension PCR. The chimeric sequence translated as CLEC-21-27/Dectin-144-244, generating a chimeric receptor consisting of the cytoplasmic tail of CLEC-2 and the transmembrane, stalk and CTLD of Dectin-1. A C-terminally HA-tagged chimeric ITAM mutant in pFBneo (mCLEC2/Dectin-1HA Y7F, referred to from now as ‘Y7F’) in which the tyrosine in the DGYxxL motif was mutated to phenylalanine, was generated by PCR using primer G (see Table 2.2).

2.4.3 Generation of a soluble FcCLEC-2 fusion protein

A soluble fusion protein containing the extracellular CTLD and stalk region of mCLEC-2, fused to the Fc portion of human IgG1 was generated in collaboration with Dr. Kevin Dennehy as follows: The soluble Fc-mCLEC-2 chimera (FcCLEC-2) was generated by cloning the CTLD and stalk region of mCLEC-2 into the pSecTag2 expression vector containing the mutated Fc portion of human IgG1, described previously [285, 286]. The mCLEC-2 CTLD was amplified by PCR using primers H and I (see Table 2.2). To generate soluble protein, the construct was transfected into HEK293T cells. Transfected cells were selected with 200µg/ml zeocin (Invivogen). Conditioned supernatants were harvested and FcCLEC-2 was purified by chromatography on protein A sepharose (GE Healthcare).

2.4.4 Generation of chimeric constructs for respiratory burst assays

C-terminally HA-tagged chimeric constructs (ROS chimera 1, chimera 2, chimera 3) were generated by PCR from the mCLEC-2/Dectin1 HA construct using forward primers J, K and L and reverse primer FB neo. The PCR products were purified and inserted into TOPO vector as
described and the fidelity of the sequences were confirmed. The constructs were then digested with Sal1 and Not1 restriction enzymes (Fermentas) and ligated with similarly digested pFBneo vector.

2.4.5 Generation of CLEC-2 reporter constructs

Reporter chimeric constructs consisting of the extracellular and transmembrane portions of mCLEC-2 or Dectin-1 fused with the cytoplasmic portion of the CD3ζ chain were generated essentially as described previously [137]. Appropriate sequences were amplified by PCR and cloned into the pMXs-IP retroviral vector. The fidelity of all constructs was verified by sequencing.

Generation of reporter constructs was carried out by Dr. Elwira Pyż, University of Cape Town.

Table 2.2 Sequences of primers used

| A | 5'ATGCAGGATGAAGATGGGTA3' |
| B | 5'GTCCATCCTTCTGGATTA 3' |
| C | 5'AAAGACGACCCACCATTGCGATGAAGAT3' |
| D | 5'GTACTGAGCAGTTGGGTAC3' |
| E | 5'TCGAGTACCCCATACGATGTCCAGTTAAGCGCGCGGCTTGT3' |
| F | 5'AAAGCAGCGGGCTTAACGGTGATCTGGGATACGTATGGGTAC3' |
| G | 5'TCGAGTACCCCATACGATGTCCAGTTAAGCGCGCGGCTTGT3' |
| H | 5'GGGGGTACCTGGGGAGATCATGTCTGG3' |
| I | 5'CTCGTAATTCGAAGCAGTGTTGATGGACTTC3' |
| J | 5'ACGTCGACCACCATTGGAATATCATCTTTATAGAGATCGATGGGTATATC3' |
| K | 5'ACGTCGACCACCATTGGAATATCATCTTTATAGAGATCGATGGGTATATC3' |
| L | 5'ACGTCGACCACCATTGGAATATCATCTTTATAGAGATCGATGGGTATATC3' |

2.5 Animals

C57BL/6, Balb/c, 129/Sv mice and Wistar rats were obtained from the specific pathogen free animal unit at the University of Cape Town (UCT). Animals were kept and handled in accordance
with institutional guidelines. All procedures were reviewed and approved by the animal ethics committee of the University. All mice were used at 6-12 weeks of age.

2.6 Cells

2.6.1 Cell lines

All cell lines in this study were obtained from the IIDMM cell bank at UCT and are listed in Table 2.3.

Table 2.3 Summary of cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Media used</th>
<th>Subculture</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH3T3</td>
<td>Adherent murine embryonic fibroblast</td>
<td>DMEM or X-VIVO</td>
<td>as per ATCC guidelines</td>
<td></td>
</tr>
<tr>
<td>RAW264.7</td>
<td>Adherent murine macrophage</td>
<td>RPMI</td>
<td>as per ATCC guidelines except cells were detached by the addition of lidocaine/EDTA for 5 minutes</td>
<td></td>
</tr>
<tr>
<td>EL4</td>
<td>Non-adherent murine T lymphocyte</td>
<td>RPMI</td>
<td>as per ATCC guidelines</td>
<td></td>
</tr>
<tr>
<td>C35</td>
<td>Non-adherent Syk-deficient murine B cell line established from IIA1.6, a FcyR-defective variant of A20 B lymphoma cells</td>
<td>RPMI</td>
<td>as per ATCC guidelines for A20 cells</td>
<td>[287]</td>
</tr>
<tr>
<td>WT8</td>
<td>Non-adherent Syk-reconstituted murine B cell line derived from C35 cells</td>
<td>RPMI</td>
<td>as per ATCC guidelines for A20 cells</td>
<td>[287]</td>
</tr>
<tr>
<td>Plat E</td>
<td>Adherent ecotropic retroviral packaging cell line (a gift from Professor Kitamura, University of Tokyo)</td>
<td>RPMI or DMEM containing blasticidin (10μg/ml) and puromycin (1μg/ml)</td>
<td>as per ATCC guidelines for 293 cells</td>
<td>[288]</td>
</tr>
<tr>
<td>293T</td>
<td>Adherent derivative of the human embryonic kidney 293 cell line</td>
<td>RPMI</td>
<td>as per ATCC guidelines for 293 cells</td>
<td></td>
</tr>
<tr>
<td>BWZ.36</td>
<td>Non-adherent T-cell line established by transfecting BW5147 cells with an NFAT-lacZ construct (a gift from Wayne Yokoyama, Washington University)</td>
<td>RPMI</td>
<td>as per ATCC guidelines for BW5147.3 cells</td>
<td>[289]</td>
</tr>
</tbody>
</table>

2.6.2 General cell culture techniques

All cell culture was undertaken in a laminar flow hood using aseptic technique and following standard cell culture protocols to ensure sterility. For long term storage of cell lines, 1 to 2x10^6 cells/ml were resuspended in cryovials in foetal calf serum (FCS) containing 10% DMSO (v/v). Cells were frozen in a freezing container containing isopropanol at a rate of -1°C per minute. The following day, cryovials were transferred to a liquid nitrogen storage facility. Each cell line was...
maintained in the appropriate media as indicated and unless otherwise stated this media was supplemented with 10% FCS (Gibco), 2mM L-glutamine, 100U/ml penicillin and 0.1mg/ml streptomycin (all from Cambrex). FCS was heat inactivated at 56°C for 30 minutes before use. Cells were subcultured according to ATCC guidelines and maintained on tissue culture plastic in a 5% CO₂ humidified incubator at 37°C. Dulbecco's phosphate buffered saline (PBS) without calcium, magnesium and phenol red (Gibco) was routinely used for cell culture applications. To determine total cell counts and viable cell number, cells were diluted with Trypan Blue Stain (Sigma), a dye that is taken up only by non-viable cells. In the case of primary macrophages, total cell counts and viable cell number were determined by diluting cells with Turks Stain which is specifically taken up only by macrophages.

2.6.3 Primary cells
2.6.3.1 Isolation of bone marrow cells and generation of bone marrow derived macrophages
Mice were sacrificed by CO₂ asphyxiation. Femurs and tibias were dissected from sacrificed mice and fresh bone marrow was flushed from bone cavities with sterile ice-cold PBS using a 26G needle. Cells were strained through a 70μm nylon cell strainer before centrifugation at 850rpm for 5 minutes. Cell pellets were resuspended prior to analysis by flow cytometry. If cells were to be used for generation of bone marrow derived macrophages, cell pellets were resuspended in RPMI containing 20% L929 conditioned medium (containing M-CSF, laboratory stocks available) and transferred to Petri dishes. Fresh media was added on day 3 after isolation. On day 7, cells were detached and plated according to experimental requirements.

2.6.3.2 Isolation of peripheral blood leukocytes
Mice were sacrificed by CO₂ asphyxiation, and peripheral blood was collected by cardiac puncture using a 21G needle, into 0.1vol 100mM EDTA to prevent coagulation. Cells were harvested by centrifugation and resuspended in 50vol ice-cold Gey’s solution for 5 minutes to lyse erythrocytes. Peripheral blood leukocytes were then recovered by centrifugation through 100% FCS at 300g. Cells were counted and cell viability was established by the exclusion of trypan blue.

2.6.3.3 Purification of peripheral blood neutrophils
To isolate neutrophils from blood, mice were sacrificed by CO₂ asphyxiation and peripheral blood was collected by cardiac puncture as before. Samples were pooled and overlaid on a 3-layer Percoll™Plus (AEC Amersham) gradient 78%, 66% and 54% Percoll, respectively, diluted in PBS (100% Percoll= 9parts Percoll and 1 part 10X PBS) and centrifuged at 3141rpm for 30 minutes at 4°C (with acceleration and deceleration at lowest settings to avoid disruption of gradient). Neutrophils were harvested from the 77%/66% interface. After washing with PBS containing 1% BSA, remaining erythrocytes in the neutrophil fraction were eliminated by lysis in ice-cold Gey’s solution for 5 minutes. Cells were centrifuged for 10 minutes at 4°C and 1517rpm. Pellets were resuspended in FACS block or media depending on the following procedure. Cells were counted and viability was ≥95% as determined by the exclusion of trypan blue.
2.6.3.4 Isolation of alveolar macrophages
Mice were sacrificed by cervical dislocation. Bronchoalveolar lavage was performed by insertion of a 23G lavage tube into the trachea and repeated washes with 1ml 10mM EDTA in PBS. Resident alveolar macrophages, the major leukocyte population in the lungs, were identified by size and autofluorescence using flow cytometry.

2.6.3.5 Isolation of spleen and lymph node cells
Spleens and lymph nodes were dissected from sacrificed mice. Organs were disaggregated by maceration and cells were strained through a 70μm nylon cell strainer before centrifugation at 1500rpm for 5 minutes. Cell pellets were resuspended and cells were analysed by flow cytometry as described below. In BALB/c mice, systemic LPS challenge was achieved by a single intravenous injection of LPS from Salmonella typhimurium (1μg LPS diluted in PBS). Splenocytes and lymph node cells were harvested 20 hours after treatment as described above.

2.6.3.6 Induction of sterile peritonitis and recovery of peritoneal cells
To induce sterile peritonitis, mice were injected intraperitoneally with 1ml of 4% thioglycollate broth. 18 hours later (for inflammatory neutrophils) or 4 days later (for inflammatory macrophages), mice were sacrificed by CO₂ asphyxiation and cervical dislocation. Inflammatory cells were collected by peritoneal lavage with ice-cold 5mM EDTA in PBS. Lavage fluid was then centrifuged at 1000rpm for 5 minutes and cell pellets were resuspended in RPMI. Viability was determined by the exclusion of trypan blue. Inflammatory macrophages were identified by their expression of F4/80 and inflammatory neutrophils were identified by their expression of Gr-1 and CD11b. Resident peritoneal cells were harvested by lavage in the same way.

2.6.4 Cytokine and TLR agonist stimulation of cells
To test the effect of TLR agonists on CLEC-2 surface expression, PBLs were plated in 24 well plates and stimulated for 6 hours with Pam3CSK4 (TLR2/1, 100ng/ml), LPS (TLR4, 100ng/ml), flagellin (TLR5, 20ng/ml), and FSL-1 (TLR2/6, 20ng/ml). Cells were then analysed by flow cytometry.

To test the effect of LPS stimulation on podoplanin expression by RAW264.7 cells, 2x10⁵ cells/well were stimulated with various concentrations of LPS for 24 hours. Cells were then detached and analysed by flow cytometry as described below.

To test the effect of TLR agonists on podoplanin expression, bone marrow derived macrophages were stimulated for 20 hours with Pam3Csk4 (100ng/ml and 1μg/ml), LPS (100ng/ml and 1μg/ml), flagellin (10ng/ml and 100ng/ml), Pam2Csk4 (10ng/ml and 100ng/ml), FSL-1 (100ng/ml and 1μg/ml) or PolyIC (1μg/ml and 10μg/ml). (All TLR agonists were from Invivogen). To test the effect of various cytokines on podoplanin expression, cells were stimulated for 20 hours with IL-4, IL-10, GM-CSF, IFNγ or TNF at the indicated concentrations. (All cytokines were from R&D systems). Cells were then detached and analysed by flow cytometry as described below.
2.6.5 Generation of transduced cell lines

2.6.5.1 Retrovirus production

Retroviral vectors containing genes of interest were introduced into an ecotropic packaging cell line which enabled production of infectious retroviruses with the ability to infect target cells and stably transmit the genes of interest into chromosomes. In this study, pFB-neo and pMXs-IP were the retroviral vectors used and the Plat-E cell line was the packaging cell line used. Genes of interest were introduced into vectors using standard molecular biology techniques described earlier. To produce retrovirus particles, recently passaged Plat-E cells were plated in six well plates at 2x10^6 cells/well in a total volume of 2ml DMEM on Day 1. On Day 2, the medium was replaced without detaching cells, and cells were transfected with 1μg DNA using Fugene6 (Roche) according to the manufacturer’s instructions. On Day 3, plates were transferred to a 5% CO_2 filled airtight container and incubated overnight at 32°C. On Day 4, supernatants were harvested and 5μg/ml polybrene (Sigma) was added. Polybrene is a cationic polymer that acts to neutralise membrane charge and initiates virus aggregation resulting in an increased rate of sedimentation of virus onto target cells. Supernatants were filtered through a 0.45μm filter and were used immediately to transduce various cell lines.

2.6.5.2 Retroviral transduction of NIH3T3 and RAW264.7 cell lines

In parallel with retrovirus production, on Day 3, target cells were plated in six well plates (NIH3T3 cells were plated at 1x10^5 cells/well and RAW264.7 cells were plated at 1x10^6). On Day 4, RAW264.7 cells were treated for 7 hours with 0.2μg/ml tunicamycin (Sigma) which inhibits N-glycosylation and increases transduction inefficiency. Cells were then washed before 1.5ml of the viral supernatant was added. Plates were centrifuged at 2500rpm at 25°C for 90 minutes to bring viral particles into contact with cells. Plates were then returned to 3rC. On Day 5, media was replaced with fresh media and on Day 6, media was replaced with selection media containing 0.6mg/ml G418 (Invivogen). Cells were monitored daily and fresh selection media was added when required. Cells were detached and transferred to flasks only when colonies became clearly viable.

2.6.5.3 Retroviral transduction of A20 cell line

In parallel with retrovirus production, on Day 3, A20 cells were treated overnight with 0.2μg/ml tunicamycin. On Day 4, A20 cells were washed, counted and plated at 7x10^5 cells/well in 24 well plates. The same day, 1.5ml of the viral supernatant was added. Plates were centrifuged at 2500rpm at 25°C for 90 minutes to bring viral particles into contact with cells. Plates were then returned to 37°C. On Day 5, media was replaced with fresh media and on Day 6, media was replaced with selection media containing 0.6mg/ml puromycin (Invivogen). Cells were transferred to six well plates and then flasks only when colonies became clearly viable.

2.6.5.4 Retroviral transduction of EL4, C35, WTB and BWZ.36 cell lines

EL4 cells, Syk deficient (C35) and Syk reconstituted (WT8) cells were retrovirally transduced with constructs by Dr. Kevin Dennehy in essentially the same manner as described for A20.
2.7 Generation and purification of antibodies against CLEC-2

2.7.1 Coupling of FcCLEC-2 to cyanogen bromide activated sepharose

Cyanogen bromide activated sepharose (1g, Sigma) was swollen with 100mls of cold HCL (1mM) for 30 minutes. The resin was then washed with a total of 200mls cold HCL (1mM) which was added in several aliquots. Supernatant was removed by gentle suction with a Büchner funnel between successive additions. Resin was then washed with 200mls of dH2O followed by 50mls of Coupling buffer. The resin was immediately transferred to a solution of FcCLEC-2 (0.8mg of protein in 5mls coupling buffer) and rotated overnight at 4°C. Unreacted ligand was removed by extensive washing with Coupling buffer. Unreacted groups were blocked with glycine, pH 8.0 (0.2M) for two hours at room temperature. To remove the blocking solution, resin was washed first with coupling buffer (pH 8.5) and then with acetate buffer containing NaCl, pH 4.0 (0.5M). This cycle of high and low pH washes was repeated five times. Resin was stored in NaCl (1M) containing sodium azide (10mM) as a preservative at 4°C.

2.7.2 Immunisation of rats

A purified polyclonal antibody, specific for CLEC-2, was affinity purified from the serum of Wistar rats after immunisation with FcCLEC-2. An emulsion of which 75% was FcCLEC-2 (20µg per rat in sterile PBS), and 25% was Titermax Gold (Sigma), was prepared according to manufacturer's instructions. This mixture was injected intraperitoneally. Booster injections were administered two and four weeks later. Four days prior to harvesting blood, FcCLEC-2 (30µg) was injected intravenously. Rats were anaesthetised and exsanguinated via the jugular vein. All injections and blood collection were performed according to standard protocols by Hiram Arendse, Animal Unit Manager at the University of Cape Town.

2.7.3 Purification of polyclonal antibody

Blood was immediately centrifuged for five minutes at room temperature at 3000rpm. The serum layer was harvested and rotated at 4°C overnight with an equal volume of human serum to precomplex IgG. This mixture was then centrifuged at 3000rpm for 15 minutes at 4°C before filtering through a serum filter. Filtered serum was run through the FcCLEC-2 coupled sepharose column. The column was then washed with PBS before fractions (12 x 1ml) were eluted into 500µl of Tris, pH 8.0 (1M), using glycine, pH 2.5 (50mM). The concentrations of the eluted fractions were measured using a NanoDrop® ND-1000 Spectrophotometer, and those containing the highest antibody concentrations were pooled and dialysed against PBS overnight using a Slide-A-Lyzer dialysis cassette according to manufacturer’s instructions. Quantification of antibody concentration was repeated and purified antibody was tested by flow cytometry as described later.
2.7.4 Generation of monoclonal antibody

A monoclonal antibody was generated and produced by Diego Mourão-Sá and Caetano Reis e Sousa (Cancer Research UK). Wistar rats were immunized 3-4 times with RBL-2H3 cells expressing mouse CLEC-2 fused to an HA epitope. Fusion of splenocytes with the rat myeloma cell line Y3 was carried out using standard procedures. Hybridoma screening was carried out as previously reported [254].

2.8 Flow cytometry

In all assays, cells were stained prior to fixation to preserve sensitive epitopes and to analyse only cell surface markers. FACS was performed according to conventional protocols at 4°C in the presence of NaN₃ (2mM) [290]. Cells were first blocked with FACS block buffer containing 5% heat-inactivated serum (serum was from goat, rat, rabbit or donkey depending on the secondary antibody used). Cells were stained in V-bottomed 96-well plates. Primary antibodies were generally used at 10μg/ml diluted in FACS block and secondary antibodies were used at 1/200 diluted in FACS block. Incubation times ranged from 30 to 60 minutes on ice. Cells were washed three times with ice-cold FACS wash after each staining step. All monoclonal antibodies used were compared with isotype-matched controls. Stained cells were fixed in 1% formaldehyde prior to flow cytometry. Data was acquired and analysed using a BD Biosciences FACSCalibur and CellQuest™ Pro.

The primary antibodies used are listed in Table 2.4.

Table 2.4 Primary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Target antigen</th>
<th>Source/supplier</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLEC-2</td>
<td>Rat IgG2b</td>
<td>Mouse CLEC-2</td>
<td>See section 2.7.4</td>
<td>[269]</td>
</tr>
<tr>
<td>CD61 (biotin)</td>
<td>Hamster IgG1,κ</td>
<td>Mouse CD61</td>
<td>BD Pharmlingen</td>
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</tr>
<tr>
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<td>HA</td>
<td>Covance</td>
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<td>Rat IgG2b</td>
<td>Mouse Dectin-1</td>
<td>Gordon Brown, (UCT)</td>
<td>[157]</td>
</tr>
<tr>
<td>5C6 (FITC)</td>
<td>Rat IgG2b</td>
<td>Mouse CR3</td>
<td>Siamon Gordon (Oxford)</td>
<td>[291]</td>
</tr>
<tr>
<td>Gr-1 (biotin)</td>
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<tr>
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<td>Mouse 7/4</td>
<td>Siamon Gordon (Oxford)</td>
<td>[292]</td>
</tr>
<tr>
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<td>Mouse F4/80</td>
<td>Siamon Gordon (Oxford)</td>
<td>[293]</td>
</tr>
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<td>Mouse CD3ε</td>
<td>Serotec</td>
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<td>Podoplanin</td>
<td>Rat IgG2a</td>
<td>Mouse podoplanin</td>
<td>MBL</td>
<td></td>
</tr>
</tbody>
</table>

Isotype-control antibodies were either obtained from the same suppliers or produced in house. Flow cytometric analysis with FcCLEC-2 and FcDectin-1 soluble proteins was performed using the Fc proteins at 10μg/ml. Secondary antibodies used were PE-conjugated donkey anti-rat IgG and PE-conjugated donkey anti-human IgG (Jackson). Biotinylated antibodies were detected using streptavidin-allophycocyanin (BD Pharmlingen). Fold changes in surface staining were determined.
using the following equation: receptor surface expression after treatment (mean fluorescence of receptor specific mAb – mean fluorescence of isotype control)/receptor expression of untreated cells (mean fluorescence of receptor specific mAb – mean fluorescence of isotype control)

2.9 Confocal microscopy
2.9.1 Preparation of acid-etched coverslips for confocal microscopy
13mm glass coverslips were soaked in 55% nitric acid for 4 hours followed by several washes with dH₂O. Coverslips were then stored in 70% ethanol until use.

2.9.2 Sample preparation and confocal analysis
The preparation of samples is outlined in later sections. Confocal laser scanning microscopy was performed by Dr. Dirk Lang at the UCT Imaging Centre. A Zeiss Axiovert 200M LSM 510 META confocal microscope was used. Images were processed using Adobe Photoshop version 6.0

2.10 Phagocytosis assays
2.10.1 Phagocytosis of zymosan particles by transduced NIH3T3 fibroblasts
Transduced cells were seeded at 2.5x10⁵ cells/well in six well plates the day before the assay. To inhibit phagocytosis, some cells were pretreated with Cytochalasin D (10μM) (Calbiochem) for 40 minutes before and throughout the assay. After washing, FITC-zymosan (1 to 5 particles/cell) (Invitrogen) was added and allowed to settle for one hour at 4°C. After washing three times with ice-cold media to remove unbound particles, cells were incubated at 37°C for one hour. Cells were then cooled to 4°C and detached using a cell lifter following 10 minutes incubation with Lidocaine-EDTA. Cells were then transferred to a 96 well plate and blocked with FACS block for 30 minutes prior to the addition of zymosan opsonising antibody (1:1000) (Invitrogen) for 60 minutes at 4°C. Cells were then washed three times with cold FACS wash. Secondary APC-conjugated goat anti-rabbit antibody (1:200) (Molecular Probes) was added for 45 minutes at 4°C before washing three times with cold FACS wash. Cells were resuspended in FACS wash and an equal volume of 2% formaldehyde was added to fix. Using flow cytometry, FITC⁺ cell populations which had bound or internalised zymosan particles were gated and the percentage of phagocytosis was determined by comparing the APC⁻ to the APC⁺ cell populations.

2.10.2 FITC labelling and antibody coupling of Sheep anti-rat IgG Dynabeads
Dynabeads Sheep anti-Rat (Invitrogen) are uniform, superparamagnetic polystyrene beads (4.5μm diameter) coated with polyclonal Sheep-anti-Rat IgG antibodies. 8x10⁸ of these beads were washed with bicarbonate buffer and then resuspended in 2mls of the same buffer. FITC solution (Sigma) was freshly prepared in DMSO at 1mg/ml. 90μl of FITC solution was added to beads in 5μl aliquots and rotated overnight at 4°C. The following day beads were washed twice with PBS and resuspended in PBS containing NaN₃ (10mM). Beads were then recounted and analysed via flow cytometry to confirm FITC labelling. Anti-CLEC-2 (monoclonal) or rlgG2b isotype control (0.5μg per 1x10⁸ beads) were rotated with beads for 45 minutes at 4°C. Beads were then washed twice with PBS before proceeding with phagocytosis assay described next.
2.10.3 Phagocytosis of anti-CLEC-2 coated beads by transduced NIH3T3 fibroblasts - analysis by flow cytometry

Transduced cells were seeded at 2.5x10^5 cells/well in six well plates the day before the assay. Cells were cultured in DMEM as usual, but plated out using X-VIVO media as this was found to reduce autofluorescence and allow easier interpretation of flow cytometry data. To inhibit phagocytosis, some cells were pretreated with Cytochalasin D (10μM) for 40 minutes before and throughout the assay. After washing with cold media, FITC-labelled anti-CLEC-2 coated Dynabeads (1 bead/cell), were added and allowed to settle for one hour at 4°C. After washing to remove unbound particles, cells were incubated at 37°C for two hours. Plates were then returned to ice and cells were washed three times with ice-cold media. Cells were then detached using a cell lifter following 10 minutes incubation with Lidocaine-EDTA. Cells were centrifuged for 5 minutes at 1000rpm and 4°C. For the detection of external beads, cells were incubated with a PE-conjugated anti-rat antibody (1:200) (Jackson) for 60 minutes at 4°C. Cells were then washed three times with cold FACS wash. Cells were resuspended in FACS wash and an equal volume of 2% formaldehde was added to fix. Using flow cytometry, FITC+ cell populations which had bound or internalised beads were gated and the percentage of phagocytosis was determined by comparing the PE to the PE+ cell populations.

2.10.4 Phagocytosis of anti-CLEC-2 coated beads by transduced NIH3T3 fibroblasts - analysis by confocal microscopy

Transduced cells were seeded at 2.5x10^5 cells/well on acid-etched glass coverslips in six well plates the day before the assay. Cells were washed with media and FITC-labelled anti-CLEC-2 coated Dynabeads (1 bead/cell) were added. Plates were incubated for two hours at 37°C. Cells were washed three times with cold media and then fixed using a 4% paraformaldehyde solution for 30 minutes. This solution was then removed and cells were incubated with a staining buffer for 30 minutes at room temperature. The staining buffer contained saponin which causes permeabilisation of the cell membrane. Actin was stained with TRITC phalloidin (1μM) (Sigma) for one hour, during which plates were covered with foil and shaken gently. Cells were then washed three times with staining buffer and finally with PBS. Coverslips were inverted onto drops of Vectashield (VECTOR laboratories) mounting medium containing Hoechst nuclear dye on microscope slides. Samples were then analysed by confocal microscopy.

2.10.5 Phagocytosis of zymosan particles by RAW2647 macrophages and Syk recruitment - analysis by confocal microscopy

Transduced cells were seeded at 2.5x10^5 cells/well on acid-etched glass coverslips in six well plates the day before the assay. After washing three times with cold media, FITC-zymosan (5 particles/cell) was added and cells were incubated at 37°C for one hour. Cells were washed three times with cold media and then fixed using a 4% paraformaldehyde solution for 30 minutes. This solution was then removed and cells were permeabilised by incubation with a staining buffer for 30
minutes at RT. Actin was stained with TRITC phalloidin (1μM) as before. Cells were then washed and coverslips were mounted as before.

Recruitment of Syk was investigated similarly, except after addition of FITC-zymosan, cells were incubated at 37°C for only two minutes. Cells were then fixed and permeabilised as before. Syk recruitment was detected with anti-phospho-Syk (1:100) (Cell Signalling) for one hour at 4°C, followed by cyanine (Cy)-3-conjugated anti-rabbit IgG (1:100) for one hour at 4°C. Coverslips were mounted as before.

2.10.6 Phagocytosis of anti-CLEC-2 coated beads by peripheral blood leukocytes

PBLs were obtained as described and 5x10^6 cells per sample were resuspended in RPMI. FITC-labelled Dynabeads, coated with anti-CLEC-2, 2A11 or isotype control Abs, were added at a ratio of two beads per cell and incubated with rotation for one hour at 4°C. To remove unbound beads, samples were layered over a two layer Percoll™Plus gradient- (40% and 70%) and centrifuged at 3140rpm for 30 minutes at 4°C (brake and acceleration at lowest setting). After washing, cells were resuspended in 100μl RPMI in the presence or absence of Cytochalasin D (5μM) and placed at 37°C for 45 minutes to allow phagocytosis to occur. Cells were washed and external beads were detected by incubation with a PE-conjugated anti-rat antibody (1:200) for 60 minutes at 4°C. Cells were then washed three times with cold FACS wash. Cells were resuspended in FACS wash and an equal volume of 2% formaldehyde was added to fix. Using flow cytometry, FITC+ cell populations which had bound or internalised beads were gated and the percentage of phagocytosis was determined by comparing the PE- to the PE+ cell populations.

2.11 Binding and cytokine production assays

2.11.1 Zymosan binding and TNFα production by transduced cells

RAW264.7 and NIH3T3 transfectants were plated at 2x10^5 cells/well and 5x10^4 cells/well respectively in 24-well plates the day before the assay. Cells were washed three times with ice-cold RPMI. To block Dectin-1, glucan phosphate (100μg/ml, a gift from David Williams, ETSU, TN) was added to appropriate wells and cells were incubated for 20 minutes on ice. Following the addition of FITC-zymosan (25 particles/cell), cells were incubated on ice for a further 60 minutes to allow binding, and then washed extensively to remove unbound particles. For the determination of TNFα production from transduced RAW264.7 macrophages, cells were incubated for a further three hours at 37°C in 5% CO₂. Supernatants were harvested and TNFα was measured by ELISA using the OptEIA™ murine TNFα ELISA kit (BD Biosciences) as described by the manufacturers. The amount of FITC-zymosan associated with the cells was quantified after lysis with 3% (v/v) Triton X-100, using a Thermo Fluoroskan Ascent FL. To account for any differences in cell density, control staining using Carboxyfluorescein diacetate succinimidyl ester (CFSE, Sigma) was performed. For this, untreated wells were rinsed with PBS. CFSE (10μM) was then added and cells were incubated at RT for 5 minutes, before washing three times with cold media and lysis as before. CFSE is a non-fluorescent dye that passively diffuses into the cytoplasm of cells where it is converted to anionic CFSE by intracellular esterases, becoming fluorescent. The succinimidyl ester group
covalently binds to amino groups on intracellular macromolecules, anchoring the dye. The CFSE fluorescence reading for each cell line was then used to normalise the zymosan fluorescence readings.

2.11.2 IL-2 production by C35 and WT8 cells
For the analysis of IL-2 production by Syk-sufficient (WT8) and Syk-deficient (C35) B-cells, 1x10^6 transduced cells were seeded in 24 well plates and stimulated with various concentrations of unlabelled zymosan (Sigma) for 16 hours at 37°C in 5% CO_2. IL-2 secreted into the supernatants was quantified by ELISA using the OptEIA™ murine IL-2 ELISA kit (BD Biosciences) as described by the manufacturers.

2.11.3 TNFα production by purified peripheral blood neutrophils
To examine cytokine production from primary cells, murine neutrophils were purified as described and plated at 3x10^5 cells/well, in 24 well plates. Cells were left unstimulated or stimulated with rhodocytin (15μg/ml) or LPS (1μg/ml) for six hours at 37°C. Rhodocytin was kindly provided by Johannes Eble, Frankfurt University Hospital, Germany [294]. Supernatants were harvested and TNFα was measured by ELISA using the OptEIA™ murine TNFα ELISA kit as described by the manufacturers.

2.12 Protein determination
2.12.1 SDS-PAGE
Protein separation on the basis of mass was carried out by SDS-PAGE. Protein mixtures were first mixed with sample buffer containing SDS and β-mercaptoethanol (Sigma) and boiled for 5 minutes. Using a Mini-PROTEAN® 3 System (BioRad), 1.5mm thick gels consisting of a 10% resolving polyacrylamide gel overlaid by a 10% stacking polyacrylamide gel were cast and submerged in SDS Running buffer. Samples were loaded into wells in the gel. A commercially available mixture of proteins having defined molecular weights was loaded in parallel. An electric current of 100V was applied across the gel, causing differential migration of proteins. Gels were subsequently stained with Coomassie staining solution for one hour, before destaining overnight, or were used for Western blots.

2.12.2 Western blotting
Western blotting was used for the detection of particular proteins. In order to make the proteins accessible to antibody detection, samples were transferred electrophoretically from acrylamide gels to Hybond C+ nitrocellulose membranes using the Mini-PROTEAN® 3 Cell System (BioRad). The uniformity and overall effectiveness of transfer of protein from the gel to the membrane was checked by soaking the membrane briefly in Ponceau S dye. The membrane was destained by several washes with water. After blocking overnight in PBS containing 0.5% BSA, membranes were probed with appropriate specific antibodies. After washing several times with PBS-Tween, membranes were incubated with an appropriate HRP-linked secondary antibody before washing with PBS-Tween again. Incubation times and antibody dilutions were dependent on the specific
antibody in use. Blots were developed with the ECL-plus kit (Amersham), used according to manufacturer's instructions.

2.12.3 Immunoprecipitations- RAW264.7 cells
To perform immunoprecipitations from RAW2647 macrophages, 1x10^7 cells were stimulated with pervanadate solution (5µl) for one minute at 37°C. Pervanadate is a protein tyrosine phosphatase inhibitor and it causes an increase in tyrosine phosphorylation and various cell signalling responses. Cells were lysed with ice-cold isotonic lysis buffer containing protease inhibitors (Roche). Lysates were centrifuged and supernatants were added to streptavidin beads (Sigma) precoupled with biotytinylated tyrosine phosphorylated or unphosphorylated signalling peptides (25µM). The CLEC-2 signalling peptides were generated by the Peptide SyntheSis laboratory at Cancer Research UK and corresponded to the following region of the CLEC-2 cytoplasmic tail: MQDEDGYITLNKPR. The Dectin-1 peptides corresponded to the following region of the Dectin-1 cytoplasmic tail: MKYHSHIENNEDGYTQDFSTQ and have been described previously [177]. Beads were rotated for two hours at 4°C and washed five times with lysis buffer before boiling for five minutes in SDS sample buffer. The immunoprecipitates were resolved by 10% SDS-PAGE and stained with coomassie staining solution or transferred electrophoretically to nitrocellulose membranes. After blocking membranes overnight in PBS containing 0.5% (w/v) BSA, proteins were detected with anti-phosphotyrosine (clone 4G10), and anti-Syk (Cell Signalling) followed by appropriate HRP-linked secondary antibodies.

2.12.4 Immunoprecipitations- A20 cells
For immunoprecipitations from A20 cells expressing a HA-tagged version of CLEC-2, 1x10^7 cells were precoated with anti-HA antibody and then stimulated with pervanadate and lysed as before. Cell lysates were added to Dynabeads Pan Mouse IgG (Invitrogen, dynabeads coated with a monoclonal human anti-mouse IgG antibody which recognises all mouse IgG subclasses and is Fc specific), and rotated for two hours at 4°C. Beads were washed and samples analysed by SDS-PAGE and Western blotting as described.

2.12.5 Mass spectrometry analysis
Stained gels were provided to Caroline Jefferies (Royal College of Surgeons in Ireland) who carried out MS/MS analysis using a MALDI-TOF system.

2.13 Respiratory burst assays
2.13.1 Respiratory burst assays in RAW 264.7 cells
For analysis of hydrogen peroxide (H_2O_2) generation, suspensions of RAW transductants in RPMI (1x10^6 cells/ml) were loaded with dihydorhodamine 123 (DHR 123, 2µM) (Sigma). Cells were incubated in a 37°C water bath for 30 minutes before stimulation with zymosan (Sigma), particulate β-glucan, or depleted zymosan (Invivogen) (all at 50µg/ml). For co-stimulation experiments cells were incubated as described before stimulation with particulate β-glucan (50µg/ml) in the presence or absence of LPS or Pam3CSK4 at various concentrations. Aliquots (1ml) were removed at
various time points and added to ice-cold PBS containing 1% BSA (3ml). Samples were centrifuged at 1000rpm, 4°C for 5 minutes and cell pellets were resuspended in PBS (200µl). Conversion of DHR 123 to rhodamine was analysed by flow cytometry. DHR 123 is a non-fluorescent reactive oxygen species (ROS) indicator that is oxidised by H₂O₂ to rhodamine which emits a bright fluorescent signal upon excitation by blue light. Cells loaded with DHR 123 but not treated with any stimulus were used to assess background levels of H₂O₂ production. Mean fluorescent intensity of untreated samples was subtracted from mean fluorescent intensity of treated samples.

2.13.2 Respiratory burst assay in primary cells
To examine H₂O₂ production from primary cells following stimulation with rhodocytin, murine neutrophils were purified as described and resuspended in RPMI in 24 well plates at 3x10⁵ cells/well in the presence of DHR 123 (2µM). Cells were unstimulated or stimulated with rhodocytin (15µg/ml) or phorbol 12-myristate 13-acetate (PMA, 100ng) for 1 hour at 37°C. Rhodocytin was kindly provided by Johannes Eble, Frankfurt University Hospital [294]. Aliquots (150µl) were removed at various time points and added to ice-cold PBS containing 1% BSA (350µl). Samples were centrifuged at 1000rpm, 4°C for 5 minutes and cell pellets were resuspended in PBS before immediate analysis by flow cytometry. Mean fluorescent intensity of untreated samples was subtracted from mean fluorescent intensity of treated samples.

2.14 Ligand screening
2.14.1 Screening for interactions of soluble CLEC-2 with polysaccharides
Carbohydrate microarray analysis was carried out in collaboration with Angelina S. Palma and Ten Feizi at The Glycosciences Laboratory, Imperial College London. Microarrays of a total of 326 lipid-linked oligosaccharide probes, neoglycolipids and glycolipids were robotically generated and microarray analyses with FcCLEC-2 and FcDectin-1 were performed essentially as previously described [155]. The lipid-linked oligosaccharide probes were printed in duplicate on nitrocellulose-coated glass slides at 2 and 7fmol/spot as previously described [295, 296], and encompassed diverse N-glycans, O-glycans, blood group-related sequences on linear or branched backbones and their sialylated and/or sulfated analogs, gangliosides, and oligosaccharide fragments of glycosaminoglycans and polysialic acid. Also included were homo-oligomers of glucose and of other monosaccharides. For a comprehensive list of probes see Schallus et al, Molecular Biology of the Cell, 2008 [297]. FcCLEC-2 and FcDectin-1 were tested at 20 and 5µg/ml, respectively and binding of these probes was detected after two hours incubation, using biotinylated anti-IgG, followed by streptavidin-conjugated horseradish peroxidise.

2.14.2 Ligand screening using reporter cells
For ligand screening, 1x10⁵ reporter cells were co-cultured with isolated mouse primary cells, or RAW264.7 cells (1x10⁵ cells), or PMA (40ng/ml) and ionomycin (1.5µg/ml) in 48 well plates for 20 hours. Following stimulation, IL-2 released into the supernatant was quantified by ELISA using the OptEIA™ murine IL-2 ELISA kit (BD Biosciences) as described by the manufacturers., and reporter
cells were tested for induction of β-galactosidase activity by X-gal staining using a commercially available kit [137]. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is a substrate for β-galactosidase and when cleaved by the enzyme it forms a visible blue precipitate. Following X-gal staining, plates were examined by microscopy and photographed. Photographs are shown in greyscale and β-galactosidase activity is indicated by the presence of black cells. Initial ligand screening with reporter cells was performed by Elwira Pyz, University of Cape Town.

2.15 Statistical analysis
As a measure of the variability amongst samples and sample populations, statistical analyses were performed using GraphPad Prism. The standard deviation (SD) was calculated where it was necessary to give an estimate of the spread around the sample mean. In some cases it was necessary to employ statistical methods to determine whether differences between data sets were significant. Where there were only two sets of data, unpaired t tests were performed. Where there were multiple data sets, one-way analyses of variance (ANOVA) was performed to ensure the most stringent comparison of the data. Two-tailed P values (probability of the difference seen being due to chance) are shown where appropriate (p < 0.05, *). P values above 0.05 were considered not significant (NS).
3 Generation of tools and subsequent functional characterisation of CLEC-2

3.1 Introduction
The focus of this chapter is the functional characterisation of C-type lectin-like receptor 2, CLEC-2 (also known as C-type lectin domain family 1, member B [CLEC1B]). I previously mentioned that minimal analysis of the expression and function of CLEC-2 had been carried out when I started this investigation in 2006. However, CLEC-2 was since described as a Syk-coupled activation receptor on platelets [268]. Here, I have explored the expression and regulation of CLEC-2 on other cell types. I also previously referred to the fact that CLEC-2 and Dectin-1 share a short, but potentially relevant, amino acid sequence in their cytoplasmic tails: DEDGYxxL. The YxxL portion of the sequence is called an ITAM-like motif and is important for the functionality of Dectin-1, as outlined in Chapter 1. The observation of a homologous DEDG sequence directly upstream of this motif in CLEC-2 and Dectin-1 raised the possibility that the receptors may be functionally related and initiated the work that is described here. As outlined in Chapter 1, Dectin-1 is a Syk-coupled activatory receptor that mediates phagocytosis and the induction of cytokines amongst other cellular responses. I therefore explored the possibility that CLEC-2 could also mediate phagocytosis and the induction of cytokines. I also investigated whether Syk is involved in CLEC-2 signalling in other cells types.

3.2 Results

3.2.1 The monoclonal and affinity-purified polyclonal antibodies specifically recognise mCLEC-2

Prior to this study, expression of murine CLEC-2 (mCLEC-2) had only been shown on platelets, although RT-PCR analysis did show transcripts in PBMC, bone marrow cells, monocytes, DCs, granulocytes and in a few NK cell clones [82, 268]. I wanted to generate anti-CLEC-2 antibodies to investigate the expression of CLEC-2 on other cell types. To produce polyclonal antibodies against mCLEC-2, a highly purified soluble FcCLEC-2 fusion protein comprising the CTLD of mCLEC-2 fused to the Fc portion of human IgG1 was generated. Wistar rats were immunised using FcCLEC-2 as the immunogen and anti-CLEC-2 polyclonal antibodies were then affinity-purified from the antisera of these immunised rats. In parallel, my collaborators at Cancer Research UK, generated an anti-CLEC-2 monoclonal antibody. This was achieved by immunising Wistar rats with cells expressing mCLEC-2 and generating hybridomas by standard protocols. An anti-CLEC-2 producing hybridoma was selected by screening.

To confirm the specificity of these antibodies, I used EL4 cells which were transduced to express a HA-tagged version of CLEC-2. Anti-HA staining and analysis by flow cytometry established that mCLEC-2 was expressed on the surface of transduced EL4 cells (Fig. 3.1A). Subsequent staining with the anti-CLEC-2 antibodies and further analysis by flow cytometry demonstrated that both the polyclonal and monoclonal antibodies specifically recognised CLEC-2 expressed on these cells (Fig. 3.1B&C).

3.2.2 mCLEC-2 is expressed on platelets and peripheral blood neutrophils

Using these antibodies, I then sought to confirm that CLEC-2 was expressed on the surface of platelets as had been previously described [268]. To do this, murine peripheral blood was collected and erythrocytes were removed by lysis. The remaining cells were stained with various antibodies and subjected to flow cytometric analysis. Analysis of CLEC-2 expression on CD61highSSClow platelets [298] confirmed that the receptor is expressed on the surface of these cells as expected [268] (Fig. 3.2A).

To determine whether CLEC-2 was also expressed on other cell types, I stained PBLs from BALB/c mice using a variety of markers to distinguish various cell populations. Expression of CLEC-2 on the surface of CD11bGr-1 neutrophils was detected (Fig. 3.2B). This neutrophil expression was not dependent on mouse strain, as similar levels of expression were also detected on these cells in C57BL/6 and 129/Sv mice (Fig. 3.2B). CLEC-2 was not detected on blood monocytes (CD11bF480SSCbw) or lymphocytes (CD3SSCbw) (Fig. 3.2C). Thus these data demonstrate that expression of CLEC-2 is not restricted to platelets, and that this receptor is also expressed by peripheral blood neutrophils.
Figure 3.1: A) Anti-HA staining of transduced EL4 cells, as determined by flow cytometry, showing expression of HA-tagged mCLEC-2 (filled histogram) at the cell surface. The vector-only control cells are indicated by the unfilled histogram. Primary antibody binding was detected using PE-donkey anti-mouse IgG. B) The monoclonal antibody raised against CLEC-2 specifically recognises EL4 cells transduced with HA-tagged mCLEC-2 (filled histogram), but not vector-only control cells (unfilled histogram), as determined by flow cytometry. Primary antibody binding was detected using PE-donkey anti-rat IgG. C) Similarly the affinity-purified polyclonal antibodies specifically recognise EL4 cells transduced with HA-tagged mCLEC-2 (filled histogram), but not vector-only control cells (unfilled histogram). Primary antibody binding was detected using PE-donkey anti-rat IgG.
Figure 3.2: Peripheral blood leukocytes were stained with various markers to identify different cell populations. A) mCLEC-2 is expressed on the surface of CD61$^{\text{high}}$SSC$^{\text{low}}$ platelets. B) mCLEC-2 is expressed on the surface of CD11b$^+$Gr-1$^+$ peripheral blood neutrophils from different mouse strains as indicated. C) mCLEC-2 is not expressed on CD11b$^+$F480$^+$SSC$^{\text{low}}$ blood monocytes or CD3$^+$SSC$^{\text{low}}$ blood lymphocytes. The polyclonal antibody was used for staining in A-C and is indicated by filled histograms. Primary antibody binding was detected using PE-donkey anti-rat IgG. Isotype control staining is indicated by the unfilled histograms.
3.2.3 Expression of CLEC-2 on bone marrow cells and inflammatory neutrophils is low

Under normal physiological conditions, the majority of neutrophils are located in the bone marrow, and only a small fraction of these cells are released into the blood, from where they can be recruited to sites of inflammation [299]. I wondered whether CLEC-2 was expressed on immature neutrophils located in the bone marrow and hence investigated its expression on CD11b^+Gr-1^ bone marrow cells from a number of mouse strains by flow cytometry. Anti-CLEC-2 staining of these cells revealed a much lower level of surface expression as compared with peripheral blood neutrophils (Fig. 3.3A). In addition, I examined 18 hour thioglycollate elicited CD11b^+Gr-1^ peritoneal neutrophils and found that these cells also had low expression of surface CLEC-2 (Fig. 3.3B). These results suggest that expression of CLEC-2 is upregulated upon neutrophil emigration from the bone marrow into the peripheral blood, but down regulated again following recruitment to sites of inflammation.

3.2.4 Regulation of CLEC-2 expression

As CLEC-2 expression was down regulated on recruited inflammatory neutrophils (Fig. 3.3B), I investigated whether stimulation of peripheral blood neutrophils with microbial agonists could induce regulation of this receptor, as had been described for other ‘Dectin-1 cluster’ receptors such as MICL [137]. PBLS were treated for six hours with a variety of TLR agonists and CLEC-2 expression on untreated and treated cells was then compared. I found that there was no significant regulation of surface expression of neutrophil-expressed CLEC-2 (Fig. 3.4A&B). However, stimulation with Pam3CSK4, a TLR2/TLR1 agonist, resulted in increased CLEC-2 expression on monocytes as defined by FSC and SSC profiles (Fig. 3.4A&B). Thus these results suggest CLEC-2 is not directly regulated on neutrophils following microbial stimulation, but that these conditions can induce upregulation of the receptor on other leukocytes.
Figure 3.3: Anti-CLEC-2 staining as determined by flow cytometry showing that mCLEC-2 is only weakly expressed on A) CD11b+Gr-1+ bone marrow cells from various mouse strains and on B) thioglycollate elicited inflammatory neutrophils (Balb/c). The polyclonal antibody was used for staining and is indicated by filled histograms. Primary antibody binding was detected using PE-donkey anti-rat IgG. Isotype control staining is indicated by the unfilled histograms.
Figure 3.4: A) Regulation of mCLEC-2 expression on peripheral blood neutrophils and monocytes following stimulation with various TLR agonists, as detected with monoclonal anti-CLEC-2 by flow cytometry. The histograms shown are gated on neutrophils defined by CD11b/Gr1 expression, and monocytes defined by forward and side scatter profiles. Anti-CLEC-2 staining is indicated by the black lined histograms and isotype control staining (rIgG2b) is indicated by the grey lined histograms. Primary antibody binding was detected with PE-donkey anti-rat IgG. B) To allow easier comparison of the data, isotype control staining is omitted and only anti-CLEC-2 staining for each treatment is shown.
3.2.5 CLEC-2 mediates phagocytosis

Having identified CLEC-2 on peripheral blood neutrophils, my next line of investigation sought to determine the function of the receptor on these cells. As CLEC-2 contains a tyrosine-based ITAM-like sequence which is similar to that used to mediate phagocytosis by Dectin-1, I explored the possibility that CLEC-2 might also mediate particle uptake. I initially examined the phagocytic potential of CLEC-2 using a chimeric receptor consisting of the extracellular and transmembrane regions of Dectin-1, fused to the cytoplasmic tail of CLEC-2 (Fig. 3.5). This chimeric receptor allowed triggering of CLEC-2 signalling using zymosan, a defined particulate ligand for the CTLD of Dectin-1 [143], and is a strategy that has been successfully used to characterise the phagocytic potential of other receptors in the Dectin-1 cluster [253]. To evaluate whether the cytoplasmic tyrosine of CLEC-2 contributes to its activity, a chimeric receptor construct in which the tyrosine within the cytoplasmic ITAM-like motif was mutated to a phenylalanine (Y7F) was generated (Fig. 3.5). These chimeric receptors also contained a C-terminal HA-tag which, as explained before, would allow me to determine whether the receptor was expressed on the surface of transduced cells. NIH3T3 fibroblasts were transduced with the chimeric constructs described and Dectin-1; and anti-HA staining followed by flow cytometric analysis confirmed that the various receptors were expressed on the cell surface (Fig. 3.6A).

Next, the ability of these normally non-phagocytic cells to bind and internalize zymosan was examined. To analyse zymosan binding, FITC-labelled zymosan particles in the presence or absence of glucan phosphate were added to the cells. The amount of zymosan associated with the cells was then quantified using a fluorometer. Glucan phosphate was included as it specifically inhibits Dectin-1 binding to zymosan [143, 147, 300]. As expected, expression of the chimeric receptors in the NIH3T3 cells conferred an ability to bind FITC-labelled zymosan, which could be inhibited by the inclusion of glucan phosphate (Fig. 3.6B). This inhibition demonstrated the specificity of the binding and the functionality of the receptors. The binding level of zymosan to NIH3T3 cells expressing the chimeric receptors was equivalent to cells expressing Dectin-1 which were included in the experiment as a positive control.

To analyse uptake, FITC-labelled zymosan particles were again added to transduced NIH3T3 cells and the cells were incubated at the appropriate temperature to allow phagocytosis. Samples were then stained with an anti-zymosan antibody, binding of which was detected using an APC-conjugated secondary antibody. All FITC positive cells were analysed (all cells which had bound zymosan, whether phagocytosis had occurred or not), and the percentage of phagocytosis was determined by comparing the APC⁻ to the APC⁺ populations. Where indicated, cytochalasin D was included to inhibit actin polymerization, and hence particle uptake. The results show that cells expressing the chimeric receptor were able to internalize zymosan particles in an actin dependent manner. However, mutation of the cytoplasmic tyrosine significantly reduced the ability of these cells to internalise zymosan particles (Fig. 3.6C). Similar results were obtained when these chimeric receptors were expressed in RAW264.7 macrophages and zymosan uptake was analysed by confocal microscopy (Fig. 3.6D).
Following a similar approach used recently to demonstrate the phagocytic potential of the C-type lectin, CD302, I used anti-CLEC-2 coated FITC-labelled Dynabeads to investigate CLEC-2 mediated phagocytosis in primary cells [301]. I first confirmed by flow cytometry that anti-CLEC-2 coated beads were specifically bound by NIH3T3 cells expressing full length CLEC-2 (Fig. 3.7A). To examine whether these beads were internalised, I incubated samples with a PE-conjugated antibody. Using flow cytometry and following a similar approach to that used previously, FITC+ cells were gated and the percentage of phagocytosis was determined by comparing the PE+ to the PE- cell populations. Cytochalasin D was again included to inhibit actin polymerization. The results showed that anti-CLEC-2 coated beads were internalised by CLEC-2 expressing cells in an actin dependent fashion (Fig. 3.7B). Confocal images of these cells show the presence of actin-based phagocytic cups around ingested beads (Fig. 3.7C).

I then investigated whether anti-CLEC-2 coated beads were bound and internalised by primary cells expressing CLEC-2. To do this I added antibody-coated beads to PBLs in the presence or absence of cytochalasin D, and incubated the cells at 37°C to allow phagocytosis to occur. Subsequent staining and analysis by flow cytometry showed that anti-CLEC-2 coated beads bound specifically to peripheral blood granulocytes, as compared with isotype-coated beads (Fig. 3.7D). Anti-Dectin-1 coated beads were included as a positive control and also bound to peripheral blood granulocytes as expected [290]. Upon incubation at 37°C, anti-CLEC-2 and anti-Dectin-1 coated beads were internalised by the granulocytes in an actin dependent fashion, as uptake could be inhibited by the addition of cytochalasin D (Fig. 3.7E). Collectively these results demonstrate that CLEC-2 can function as a phagocytic receptor.
Figure 3.5: Cartoon representation of Dectin-1, CLEC-2 and the Dectin-1/CLEC-2 chimeric constructs used in this study. Note the presence of the extracellular and transmembrane regions of Dectin-1 and the cytoplasmic tail of CLEC-2 in the chimeras. In the Dectin-1/CLEC-2 Y7F chimera, the cytoplasmic tyrosine has been mutated to a phenylalanine.
Figure 3.6: A) Anti-HA staining of transduced NIH3T3 fibroblasts, as determined by flow cytometry, showing expression of HA-tagged constructs at the cell surface as indicated. Primary antibody binding was detected using PE-donkey anti-mouse IgG. B) Quantitation of FITC-zymosan (zy) binding (25 particles/cell) by transduced NIH3T3 fibroblasts, in the presence or absence of glucan phosphate (GP, 100 µg/ml), as indicated. The amount of FITC-zymosan associated with the cells was quantified after lysis, using a Thermo Fluoroskan Ascent FL. RFU, relative fluorescent units. The data shown are the mean ± SD and are representative of at least three independent experiments, p < 0.05 vs control (Student’s t test). C) FACS-based analysis of phagocytosis, showing the extent of FITC-zymosan internalisation (grey histograms) by NIH3T3 cells expressing the constructs, as indicated. Cytochalasin D (10 µM, unfilled histograms) was used to inhibit actin polymerization and served as a control in this assay. External zymosan was detected by the addition of a zymosan opsonising antibody, followed by an APC-conjugated goat anti-rabbit antibody. FITC+ cell populations which had bound or internalised zymosan particles were gated and the percentage of phagocytosis was determined by comparing the APC to the APC+ cell populations. The histograms shown are representative of at least three independent experiments, and the bars indicate the percentage of cells with internalised particles. D) Confocal image demonstrating FITC-zymosan uptake by RAW264.7 cells expressing the chimera, but not by cells expressing the Y7F chimera.
Figure 3.7: A) FACS-based analysis of binding of isotype coated or anti-CLEC-2 coated FITC-dynabeads to NIH3T3 cells expressing mCLEC-2. B) FACS-based analysis demonstrating uptake of anti-CLEC-2 coated dynabeads (grey histogram) by NIH3T3 cells expressing mCLEC-2. Cytochalasin D treated cells (10µM, black lined histogram) were included as a control. For the detection of external beads, cells were incubated with a PE-conjugated anti-rat antibody. FITC⁺ cell populations which had bound or internalised beads were gated and the percentage of phagocytosis was determined by comparing the PE⁺ to the PE⁻ cell populations. C) Confocal image showing an NIH3T3 cell expressing mCLEC-2 internalising a FITC-labelled anti-CLEC-2 coated dynabead. Inset shows the presence of an actin-based (red) phagocytic cup around the particle. D) In comparison to isotype coated beads, peripheral blood granulocytes (identified by forward and side scatter profiles) specifically recognised anti-CLEC-2 and anti-Dectin-1 coated dynabeads. External beads were detected by incubation with a PE-conjugated anti-rat antibody. FITC⁺ cell populations which had bound or internalised beads were gated and the percentage of phagocytosis was determined by comparing the PE⁺ to the PE⁻ cell populations. E) Cells binding the anti-CLEC-2 coated dynabeads internalise these particles in an actin dependent fashion, as uptake could be inhibited by the addition of Cytochalasin D. *, p < 0.05 vs control (Student’s t test).
3.2.6 CLEC-2 induces production of cytokines

The cytoplasmic ITAM-like motif of Dectin-1 can also induce the production of cytokines, including TNFα [157, 178, 208] and I therefore decided to explore whether signalling via CLEC-2 could similarly induce cytokine production in neutrophils, as has been reported for other neutrophils receptors [302]. I found that the CLEC-2 ligand rhodocytin and LPS (included as a positive control), both induced the production of TNFα from purified peripheral blood neutrophils (Fig. 3.8). Although this suggests that CLEC-2 can mediate cytokine production from primary neutrophils, rhodocytin is known to be recognised by other receptors which may have contributed to the observed cytokine production [268]. I therefore attempted to stimulate cells using antibody crosslinking, and antibody-coated Dynabeads, but specific responses could not be demonstrated in this manner due to high background levels of cytokine production in control samples (data not shown).

![Graph](image)

**Figure 3.8:** Addition of rhodocytin or LPS to purified neutrophils induces the production of TNFα. *, p < 0.05 vs control (Student's t test). ND: not detected.
Therefore, to specifically demonstrate that signalling from CLEC-2 is able to induce cytokine production, I used the chimeric CLEC-2/Dectin-1 receptor constructs expressed in RAW264.7 macrophages. As described previously, these constructs were engineered to include a C-terminal HA-tag to allow for convenient detection of the receptors on the surface of transduced cells. Anti-HA staining and analysis by flow cytometry confirmed that the full length chimera and the Y7F mutant chimera were expressed at comparable levels on the surface of RAW264.7 macrophages (Fig. 3.9A). Cells transduced with Dectin-1 were also included as a positive control for the experiments (Fig. 3.9A). Expression of these receptors conferred an enhanced ability to bind zymosan that was inhibited by the addition of glucan phosphate which, as explained before, indicated the specificity of binding and functionality of the chimeric receptors (Fig. 3.9B). Furthermore, in response to zymosan, the full length chimeric receptor induced high levels of TNFα, comparable to those induced by Dectin-1 (Fig. 3.9C). In contrast, the level of TNFα produced from cells expressing the Y7F mutant chimera in response to zymosan, was comparable to the levels from the vector-only transduced cells (Fig. 3.9C). Thus these data demonstrate that CLEC-2 can induce cytokine production and that this activity is dependent on the ITAM-like motif in the cytoplasmic tail of the receptor.

3.2.7 CLEC-2 can signal via Syk kinase in myeloid cells

CLEC-2 signals via Syk kinase in platelets [268] and I therefore decided to explore whether it signals via this pathway in other cell types. I first performed immunoprecipitations from RAW264.7 lysates using tyrosine phosphorylated or unphosphorylated peptides, corresponding to the cytoplasmic tails of mCLEC-2 or Dectin-1. Subsequent Western blot analysis demonstrated that the phosphorylated CLEC-2 and Dectin-1 peptides could associate with Syk from the macrophage cell extracts (Fig. 3.10A). Anti-phosphotyrosine probing also revealed that the phosphorylated CLEC-2 peptide associated with another tyrosine phosphorylated protein with a molecular weight of approximately 68kDa (Fig. 3.10A). Further analysis carried out by Dr. Kevin Dennehy demonstrated that this 68kDa protein was in fact Shp2, (src homology 2 domain containing tyrosine phosphatase) (data not shown). I also wanted to investigate whether cellular activation results in phosphorylation of the single tyrosine in the cytoplasmic tail of CLEC-2, as is known to occur with Dectin-1. I explored this by immunoprecipitating the receptor from pervanadate stimulated or unstimulated transduced A20 cells expressing full-length CLEC-2. Subsequent Western blot analysis using anti-phosphotyrosine as a probe, demonstrated the CLEC-2 was indeed tyrosine phosphorylated (Fig. 3.10B).

Since I had now shown that CLEC-2 is an activating receptor with the ability to induce cytokine production and to associate with Syk, the next step was to determine if CLEC-2 could induce intracellular signalling via Syk. To explore this possibility, Syk-sufficient (WT8) and Syk-deficient (C35) B-cell lines were transduced with the HA-tagged chimeric receptor and the production of IL-2 in response to zymosan stimulation was examined. This strategy has been successfully used to examine Syk-signalling in other receptors [253]. Anti-HA staining and analysis by flow cytometry confirmed that the chimeric receptor was expressed at comparable levels on the surface of these
cells (Fig. 3.10C). Although addition of zymosan to the Syk-sufficient cells expressing the chimera induced significant production of IL-2, the Syk-deficient cells did not show any response to zymosan (Fig. 3.10D), indicating that the cytoplasmic tail of CLEC-2 can induce intracellular signalling via Syk.

Finally, to demonstrate CLEC-2 mediated recruitment of phospho-Syk to the phagosome, RAW264.7 macrophages expressing the chimeric receptor were examined by confocal microscopy following a short exposure to zymosan. The activation of Syk around the phagosome, as measured by staining for phospho-Syk, was clearly detected (Fig. 3.11). This activation of Syk was absent in the cells expressing the Y7F mutant chimeric receptor (Fig. 3.11). Collectively, these data demonstrate that CLEC-2 signals via Syk kinase in myeloid cells.
Figure 3.9: A) Anti-HA staining of transduced RAW264.7 macrophages, as determined by flow cytometry, showing expression of HA-tagged constructs at the cell surface as indicated. Primary antibody binding was detected by PE-donkey anti-mouse IgG. B) Quantitation of zymosan (zy) binding and C) zymosan induced TNFα production, by transduced RAW264.7 macrophages, in the presence or absence of glucan phosphate (GP, 100μg/ml), as indicated. The amount of FITC-zymosan associated with the cells was quantified after lysis, using a Thermo Fluoroskan Ascent FL. RFU, relative fluorescent units. The data shown are the mean ± SD and are representative of at least three independent experiments. *, p <0.05 vs control (Student’s t test).
Figure 3.10: A) Western blotting of immunoprecipitates from RAW 264.7 macrophages using phosphorylated (YP) and unphosphorylated peptides (Y), corresponding to the cytoplasmic tail of mCLEC-2 or Dectin-1. B) Western blotting of anti-HA immunoprecipitates from A20 cells expressing HA-tagged mCLEC-2. Cells were either unstimulated (US) or stimulated with pervanadate (S). Blots were probed with anti-phosphotyrosine (aPY) and anti-Syk as indicated. C) Anti-HA staining of transduced B cell lines, as determined by flow cytometry. Bold black lined histograms represent anti-HA staining and grey lined histograms represent isotype control staining. Primary antibody binding was detected using PE-donkey anti-mouse IgG. D) IL-2 production following zymosan stimulation of Syk-deficient (Syk-) and Syk-sufficient (Syk+) B-cells, transduced with the chimeric receptor. The data shown are the mean ±SD and are representative of at least three independent experiments. *, p <0.05 versus C35 Syk- cells (Student’s t test).
Figure 3.11: The upper panel shows a confocal image demonstrating the recruitment of phospho-Syk (red) by RAW264.7 macrophages expressing the chimeric receptor following stimulation with FITC-zymosan. Conversely, in RAW264.7 macrophages cells expressing the Y7F receptor, shown in the lower panel, confocal images demonstrate a lack of recruitment of phospho-Syk following stimulation with FITC-zymosan.
3.3 Discussion
The study of the 'Dectin-1 cluster' of NK-like C-type lectin receptors has provided important insights into mechanisms underlying homeostasis and immunity. Arguably one of the most important discoveries has been the identification of receptors containing cytoplasmic ITAM-like motifs which can trigger cellular activation. These motifs possess only a single tyrosine, yet are able to recruit and signal via Syk kinase through a process which is not yet fully understood. One receptor possessing this motif is CLEC-2, a molecule previously thought to be exclusively expressed on platelets and capable of triggering the activation of these cells [268]. Here I have shown that CLEC-2 is also expressed on murine peripheral blood neutrophils. As in platelets [181, 268], I also demonstrated that the cellular functions of CLEC-2 are mediated through its ITAM-like motif and that the receptor can induce intracellular signalling via Syk kinase.

The ITAM-like motif of CLEC-2 shows a striking similarity to that of Dectin-1, suggesting that CLEC-2 may possess some of the functions of Dectin-1 [45, 181]. Indeed, it was shown here that CLEC-2 can trigger phagocytosis and the induction of TNFa, and it is possible that the receptor may also be able to induce the production of a number of other cytokines and chemokines in neutrophils [302]. Furthermore, it is possible that CLEC-2 mediated cytokine production may be amplified by co-stimulation through the TLR pathway, as has been shown for Dectin-1 [45, 178, 208, 303]. Thus like Dectin-1 and CLEC9A, the data here suggests that CLEC-2 functions as an activation receptor on myeloid cells. These conclusions were based partly on the results of experiments using cell lines transfected with a chimeric receptor consisting of the extracellular and transmembrane regions of Dectin-1, fused to the cytoplasmic tail of CLEC-2. This approach was initially used in the absence of a commercially available CLEC-2 ligand. However there are potential drawbacks to using a chimera-based approach for studying the functions of a given receptor. In this case I used the defined Dectin-1 ligands, zymosan or particulate β-glucan, to trigger signalling via the CLEC-2 cytoplasmic tail. Therefore these Dectin-1 ligands directed the chimeric receptor clustering which may have imposed steric constraints or lead to interactions that are significantly different from those that occur when full-length CLEC-2 interacts with natural ligands. Nevertheless, the phagocytic capacity of CLEC-2 was confirmed by the uptake of anti-CLEC-2 coated beads by NIH-3T3 cells transduced with full length CLEC-2. This function was further confirmed by demonstrating the uptake of anti-CLEC-2 coated beads by peripheral blood neutrophils which express the receptor. The ability of CLEC-2 to induce cytokine production was also demonstrated using peripheral blood neutrophils stimulated with the CLEC-2 ligand rhodocytin. As mentioned previously this suggests that CLEC-2 can mediate cytokine production from primary neutrophils, however rhodocytin is known to be recognised by other receptors which may have contributed to the observed cytokine production [268]. For this reason, the chimera-based approach was used to specifically investigate the ability of CLEC-2 to trigger cytokine responses.

Cross-linking antibody experiments are an alternative to using a chimera-based approach to investigate the functions of receptors in the absence of ligands. For example, cytokine production
by CLEC-2 could potentially have been investigated by stimulating primary neutrophils or RAW264.7 macrophages expressing full-length CLEC-2, with Fab fragments of anti-CLEC-2 antibodies. Although not detailed here, I made several unsuccessful attempts to express full-length mCLEC-2 in RAW264.7 macrophages and was therefore unable to take this approach. Furthermore, as mentioned in the results section I also attempted to stimulate peripheral blood neutrophils using antibody crosslinking, and antibody-coated Dynabeads, but specific responses could not be demonstrated in this manner due to high background levels of cytokine production in control samples. Due to time constraints I therefore relied on the chimera-based approach and the stimulation of peripheral blood neutrophils with rhodocytin to demonstrate the ability of CLEC-2 to induce cytokine production.

The ability of CLEC-2 to mediate phagocytosis is likely to involve the highly charged cytoplasmic triacidic cluster (DED), in addition to the ITAM-like motif. This cluster is conserved in Dectin-1, but not CLEC9A, and mutation of these residues in Dectin-1 has been shown to abolish particle uptake [180]. Furthermore, although possessing an ITAM-like motif, CLEC9A does not mediate phagocytosis [253]. However, it is still unknown if CLEC-2 mediated uptake requires Syk in neutrophils. For Dectin-1, the requirement for Syk is cell-type specific; in DCs Dectin-1-mediated uptake involves Syk, but in macrophages this process is independent of Syk and occurs through uncharacterised, and possibly novel, pathways [177, 304]. In neutrophils, phagocytosis mediated by the unrelated activation receptor, CEACAM3, which possesses a traditional ITAM motif, was shown to involve Syk, but the requirement for this kinase was shown to be dependent on the nature of the ligand [305]. Although this study clearly demonstrates that CLEC-2 has the potential to mediate phagocytosis, the physiological relevance of this activity remains to be determined.

Analysis carried out by Dr. Kevin Dennehy confirmed that the tyrosine phosphatase Shp2 also associates with the CLEC-2 cytoplasmic tail. Shp2 has been previously been co-isolated with Syk, and furthermore it has been shown that when Shp2 is recruited to the plasma membrane, it desphosphorylates numerous substrates, including Syk [306, 307]. Shp2 has also been shown to bind directly, via its SH2 domains, to the tyrosine phosphorylated β-subunit of FcεRI where it becomes activated via tyrosine phosphorylation [308]. Here it is thought to desphosphorylate β and γ subunits of FcεRI thereby functioning in a negative feedback loop that regulates this receptor’s tyrosine phosphorylation [308]. It seems plausible to speculate that in the case of CLEC-2 signalling, the recruitment of Shp-2 may serve a similar regulatory function.

One possibility that I have not addressed here is whether CLEC-2 signalling and phagocytosis can be uncoupled. I have shown for example that the cytoplasmic tyrosine of CLEC-2 is required for phagocytosis. I have also shown that cytokine production is dependent on the cytoplasmic tyrosine of CLEC-2. However, it is possible that particle uptake is required to initiate signalling. In this case, the cytoplasmic tyrosine of CLEC-2 would be required for phagocytosis as shown, but signalling may proceed via an alternative mechanism. This scenario could be investigated by inhibiting phagocytosis using cytochalasin D, and measuring the effects on cytokine production. In the case
of macrophages expressing Dectin-1 however, this type of experiment has shown that uptake is not required for subsequent signalling [157].

The analysis of CLEC-2 expression indicates that high levels of expression of the receptor occurs only on circulating neutrophils, and that the receptor is only weakly expressed on bone marrow or elicited inflammatory cells. This suggests that its expression is tightly regulated and implies a specific function for the receptor on circulating cells. Although not analysed in detail, I also observed that expression of CLEC-2 could be upregulated on murine monocytes, following stimulation with Pam3CSK4, but not other TLR agonists tested, suggesting some specificity in this response. It is possible that CLEC-2 may be similarly regulated on human neutrophils or other leukocytes, which were not examined in this study. Why the expression of this receptor appears to be primarily restricted to circulating cells is unclear, and despite the identification of both endogenous and exogenous ligands, the physiological role of CLEC-2 is still unclear.

Like Dectin-1, CLEC-2 may function as a pattern recognition receptor. The expression of this receptor on neutrophils certainly implies a role in innate immunity, as these short-lived cells provide a first-line of defence against infection and are essentially required for the control of bacterial and fungal infections [302]. A role in immunity is also suggested by the ability of specific TLR agonists to induce CLEC-2 expression on monocytes, and the phagocytic capacity of this receptor may be important for the clearance of blood-borne pathogens, although such interactions have yet to be documented. However, other than rhodocytin, only HIV has been identified to possess an exogenous CLEC-2 ligand and rather than being protective, the interaction with CLEC-2 may promote transfer of infectious HIV-1 particles [275].

The primary function of CLEC-2 may be in the regulation of homeostasis through the recognition of endogenous ligands. One endogenous ligand that has been identified is podoplanin, a mucin like glycoprotein which is expressed on a variety of cell types including osteoblasts, keratinocytes, fibroblasts, airway epithelia, renal tubular epithelial cells, lymphatic endothelial cells and certain tumour cells, although podoplanin is not expressed on blood vessel endothelium [309]. The interaction between CLEC-2 and podoplanin was reported to be carbohydrate-mediated, and that a di-sialylated core O-glycan sequence on Thr52 of podoplanin is essential for recognition by CLEC-2 [273]. However, as I mentioned in Chapter 1, Kato et al used recombinant glycopeptides to study the interaction between CLEC-2 and podoplanin [273] and it therefore remains unclear whether the oligosaccharide sequence alone is sufficient for CLEC-2 binding, or whether any of the flanking peptide region is also involved.

During this thesis, podoplanin expression was identified on inflammatory macrophages, inflammatory neutrophils and stimulated bone marrow derived macrophages (see Chapter 5). The significance of this expression in terms of CLEC-2 function remains unclear, but will be considered further in Chapter 5. However, the interaction of CLEC-2 with podoplanin following tumour invasion has been proposed to promote platelet activation and aggregation, which may be associated with
tumour metastasis [273]. Metastasis is also promoted by inflammation, induced in part by neutrophils [310], and it is tempting to speculate that the interaction of podoplanin with neutrophil expressed CLEC-2 may contribute to this process.

It is also possible that simultaneous recognition of CLEC-2 ligand(s) by both platelets and neutrophils contributes to the interactions between these two cell types and to the activation and cross-talk of the inflammatory and coagulation pathways. These interactions are known to be important for the control of infection, and for limiting inflammatory pathology, but are also involved in the development of disease [311, 312].

In summary I demonstrated here that murine CLEC-2 is expressed by peripheral blood neutrophils as well as on monocytes activated with selected TLR ligands. I also demonstrated that CLEC-2 can function as an activation receptor on these cells, inducing phagocytosis and inflammatory cytokine production. Furthermore, I established that CLEC-2 can recruit and signal via Syk kinase in myeloid cells. These data therefore demonstrate that CLEC-2 expression is not restricted to platelets as previously thought and that it functions as an activation receptor on neutrophils.
4 Investigation of the role of CLEC-2 in the respiratory burst and a preliminary examination of the mechanisms underlying this response

4.1 Introduction

4.1.1 Aims of this chapter

Investigations using zymosan and Candida albicans have shown that Dectin-1 signalling induces a respiratory burst in macrophages, neutrophils, DCs and mast cells [162, 178, 180, 313, 314]. Furthermore, studies in Syk deficient bone marrow derived macrophages revealed an absolute requirement for Syk in Dectin-1 mediated induction of the respiratory burst in these cells [180]. Considering the similarity between the cytoplasmic tails of CLEC-2 and Dectin-1, and that I have shown that CLEC-2 is a Syk-coupled activatory receptor expressed on neutrophils [269], I decided to investigate whether like Dectin-1, CLEC-2 could also mediate induction of the respiratory burst. During my work described in the forthcoming chapter, I determined that signalling via CLEC-2 is not involved in the generation of reactive oxygen species. This suggested that additional unidentified components, distinct from Syk, are required for Dectin-1 mediated induction of the respiratory burst in response to microbes, and I therefore performed some preliminary investigations to investigate the underlying mechanism. Before proceeding, it is appropriate to first give a brief overview of the respiratory burst and its regulation.

4.1.2 The respiratory burst

Superoxide, and other oxygen-derived intermediates that can modify organic molecules are referred to as 'reactive oxygen species' (ROS). Nicotinamide adenine dinucleotide phosphate (NADPH)- oxidase is the most characterised cellular source of ROS, however a number of other enzymes can also produce ROS including nitric oxide synthase, lipoxygenases, cyclo-oxygenases, xanthine oxidase and cytochrome P450 enzymes [315]. The generation of ROS is critical for host defence against microbial infections. The prevailing model for their role in innate immunity is that these radicals attack and kill bacteria through their chemical reactivity [316]. The vital nature of this mechanism is demonstrated in chronic granulomatous disease, where inherited defects in this response are associated with enhanced susceptibility to bacterial and fungal infections [317]. In phagocytic cells, ROS are produced via the multicomponent NADPH oxidase which assembles on cellular membranes [318]. ROS may be generated intracellularly at the phagosomal membrane after pathogen ingestion, or extracellularly in response to soluble agonists such as formylated bacterial peptides (e.g. fMLP) or activated complement. ROS are produced in a vigorous 'respiratory burst' which is characterised by a rapid increase in oxygen uptake, an increase in glucose consumption and the abrupt release of ROS. In addition to their role in host defence, ROS
may also be pathophysiological and have been implicated in tissue injury and inflammation in several disease settings [319].

4.1.3 Components of the NADPH oxidase complex

In unstimulated cells, components of the NADPH oxidase are segregated between the membrane and the cytosol [320-322]. The catalytic core of NADPH oxidase is gp91phox (recently renamed Nox-2), a highly glycosylated membrane spanning protein with an apparent molecular mass of approximately 91kDa. gp91phox contains NADPH and FAD binding domains in the C-terminal cytoplasmic region and two hemes in the N-terminal region [320-322]. It is the electron transport chain of the active NADPH oxidase complex which mediates the transfer of electrons from cytosolic NADPH to O₂ to produce the superoxide anion, a precursor of other toxic ROS. gp91phox is complexed with a 22kDa regulatory protein, p22phox, forming a mutually stabilising heterodimer known as flavocytochrome b558 [320-322]. Flavocytochrome b558 becomes activated after exposure of cells to microbes or inflammatory mediators as a result of translocation and assembly of cytosolic subunits with the membrane bound subunits (Fig. 4.1).

The cytosolic components of the NADPH oxidase are p47phox, p67phox, p40phox and the small GTPase Rac [320-322]. p47phox contains a PhoX domain (PX) which is involved in phosphoinositide binding, two tandemly arranged Src homology 3 (SH3) domains that target a proline-rich region in the p22phox subunit, an auto-inhibitory region (AIR) that masks the SH3 domains in the resting state and a proline rich region that mediates binding to an SH3 domain of p67phox [320-322]. The p67phox subunit contains a tetratricopeptide (TPR) region that is a target of Rac-GTP in the active NADPH oxidase complex, an activation domain, two SH3 domains and a PBI (Phox and Bem1) motif that binds to a complimentary motif in p40phox [320-322]. In addition to the PBI motif just mentioned, p40phox contains a PX domain and an SH3 domain [320-322].

4.1.4 Assembly of the NADPH oxidase complex

In resting cells, the cytosolic phox proteins are present in a heterotrimeric complex which is formed by the tail-to-tail SH3-PRR (proline rich region) association between p67phox and p47phox and the PBI-PBI interaction between p67phox and p40phox. In addition, Rac is coupled to GDP dissociation inhibitor (GDI), which maintains it in its inactive form bound to GDP [322, 323]. During cellular activation all of the phox proteins are phosphorylated. Phosphorylation of p47phox at multiple Ser residues causes the SH3 domains to be unmasked and allows interaction with the PRR of the p22phox subunit. In this manner, p47phox transports the entire cytosolic complex to the membrane during NADPH oxidase activation [324] (Fig. 4.1). Furthermore, Rac rapidly converts from a GDP- to a GTP-bound state, dissociates from GDI and migrates to the membrane where it interacts directly with the TPR region of p67phox [322, 323]. p67phox directly participates in the activation of gp91phox by activating electron transfer from NADPH to bound FAD. The precise mechanism of activation is still unclear; however it is believed that binding of Rac to p67phox induces a conformational change in p67phox which may allow its activation domain to act on gp91phox [322]. From FAD, electrons are then transferred to the haem centres of gp91 and finally
to oxygen, which results in the generation of superoxide. This radical can be converted to various other species including hydrogen peroxide, the hydroxyl radical, hypochlorous acid, singlet oxygen or peroxynitrite.

![Diagram of NADPH oxidase complex](image)

**Figure 4.1: The NADPH oxidase complex.** Following cellular activation, p40phox, p67phox and p47phox all become phosphorylated which results in their translocation, together with Rac, to the membrane. The activated complex accepts an electron from reduced nicotinamide adenine dinucleotide phosphate (NADPH) at the cytosolic surface of the membrane and donates it to molecular oxygen on the other side of the membrane generating a superoxide anion, \( \text{O}_2^- \). (Adapted from Hultqvist et al., Trends in Immunology, 2009).
4.1.5 Upstream signalling and activation of NADPH oxidase

Activation of NADPH oxidase can occur via different signalling pathways and is an extremely complex and evolving area of research. For example, different stimuli induce different signalling pathways and different patterns of extra- vs intracellular production of ROS. Furthermore, certain stimuli induce different signalling pathways in different cell types. Many investigations use purified reagents, such as fMLP, in order to investigate signalling pathways resulting in NADPH oxidase activation. It is necessary to take such a reductionist approach when studying a complex system like NADPH oxidase that involves assembly and activation of a number of subunits. However, it should also be considered that it is unlikely that a cell would ever encounter individual stimuli under physiological conditions and several signalling pathways must be integrated to orchestrate an appropriate response to a pathogen.

All pathways that activate NADPH oxidase converge at a point characterised by phosphorylation of p47phox and activation of Rac. However, the upstream signalling varies considerably [325]. It appears that a combination of kinases may participate in the phosphorylation of p47phox in vivo and PKCζ, PKCθ, PKCδ, p21-activated kinase (PAK), ERK1/2 and AKT have all been shown to be involved during fMLP- or PMA- induced NADPH oxidase activation [324]. With regard to fMLP, which signals through G-protein-coupled receptors (GPCR), stimulation of an inhibitory G protein results in activation of PI3K (phosphoinositide 3-kinase) via a complex pathway involving MAP kinases. PI3K phosphorylates membrane lipids leading to the accumulation of PIP3 (phosphatidylinositol (3,4,5)-trisphosphate), which activates the serine kinase AKT which proceeds to phosphorylate p47phox [325]. Signalling induced by angiotensin II (AngII) through its GPCR receptor, AT-II, results in activation of NADPH oxidase via a multitude of different signalling cascades involving the activation of PLA2, PI3K, PLC, PLD and PKC [326]. Investigations have also shown that G-CSF signalling through its receptor resulted in stimulation of the Src kinase Lyn, with subsequent activation of PI3K, AKT and phosphorylation of p47phox [327]. RhoG, a Rho family small GTPase guanine exchange factor (GEF), has been implicated in the regulation of Rac activation in response to soluble agonists [328]. It has also been shown that Vav GEF regulation of Rac is indispensable for Fcer-mediated ROS production in both neutrophils and macrophages, however it is not required for GPCR-mediated induction of ROS [329]. Vav proteins have also been shown to be critical mediators of LPS-induced MyD88-dependent activation of Rac and subsequent production of ROS [330]. As mentioned previously, the activation of Syk by Dectin-1, and in addition Fcer and CEACAM3, is required to induce the respiratory burst in a variety of cell types, including neutrophils [162, 180, 305, 331].

As this very brief discussion shows, the respiratory burst is regulated by multiple factors that can be differentially activated by various immune receptors. Furthermore, the various signalling pathways involved are not sufficiently resolved. Here, I investigated the involvement of CLEC-2 in mediating the respiratory burst response and I further went on to perform some preliminary experiments that would begin to decipher the mechanisms underlying Dectin-1 mediated induction of this response.
4.2 Results

4.2.1 Expression of receptors on the surface of RAW264.7 macrophages

Signalling via Dectin-1 has been shown to activate the respiratory burst in macrophages in a Syk-dependent manner [180]. The respiratory burst is an important anti-microbial mechanism in neutrophils, and as CLEC-2 signals via Syk, I wondered if this receptor could also mediate activation of the respiratory burst. To examine this response, RAW264.7 cells were transduced with Dectin-1 and CLEC-2/Dectin-1 chimeric receptors, all of which had a C-terminal HA tag to enable surface detection by means of flow cytometry. The chimeric receptors were previously used to examine the phagocytic potential of CLEC-2, and consisted of the extracellular and transmembrane regions of Dectin-1 fused to the cytoplasmic tail of CLEC-2 (Fig. 3.5, Chapter 3). The use of such receptors allowed triggering of CLEC-2 signalling using zymosan, a defined particulate ligand for Dectin-1 [290]. Anti-HA staining confirmed that these receptors were expressed at equivalent levels on the surface of the transduced RAW264.7 cells (Fig. 4.2A).

4.2.2 Investigation of CLEC-2 mediated induction of the respiratory burst

Cells successfully transduced with Dectin-1 or the CLEC-2 chimeric receptors were compared with vector-only transduced cells for their ability to mediate activation of the respiratory burst. For this, cells were loaded with DHR 123 and subsequently stimulated with zymosan. DHR is a nonfluorescent ROS indicator that can passively diffuse across membranes where it can be oxidised by H$_2$O$_2$, an end product of the respiratory burst, to rhodamine, which emits a bright fluorescent signal. Flow cytometric analysis revealed that macrophages expressing the CLEC-2 chimeric receptor failed to induce a respiratory burst in response to zymosan. Similarly, macrophages expressing the tyrosine mutant CLEC-2 chimeric receptor, Y7F, also failed to induce a respiratory burst. In contrast, macrophages expressing Dectin-1 induced a robust respiratory burst, as expected (Fig. 4.2B) [180]. I also examined this response in peripheral blood neutrophils which I have shown express CLEC-2 (see Chapter 3, fig 3.2B). As a positive control, these cells were stimulated with PMA which induced the respiratory burst as expected. However, stimulation with the CLEC-2 ligand rhodocytin, did not elicit an equivalent response (Fig. 4.2C). It is important to point out here that rhodocytin elicited a response from peripheral blood neutrophils in terms of cytokine production (see Figure 3.8, Chapter 3), although the possibility that it may have been signalling through other receptors in addition to CLEC-2 could not be excluded.
Figure 4.2: A) Anti-HA staining of transduced RAW264.7 macrophages, as determined by flow cytometry, showing expression of HA-tagged constructs at the cell surface as indicated. Primary antibody binding was detected using PE-donkey anti-mouse IgG. B) The respiratory burst in various RAW264.7 transfectants, measured by assessing the conversion of dihydrorhodamine 123 to rhodamine by flow cytometry, following stimulation with zymosan. The data are expressed as mean fluorescent intensity (mfi). The data shown are the mean ±SD and are representative of at least three independent experiments. *, p < 0.05 vs pFB vector control (Student's t test). C) The respiratory burst in peripheral blood neutrophils, determined as in A, following stimulation with rhodocytin or PMA. Number indicates mean fluorescent intensity.
4.2.3 CLEC-2 does not induce a respiratory burst in response to particulate β-glucan, nor does it collaborate with TLRs to induce such a response

During my investigations there was a period where I observed a weak, but significant respiratory burst response mediated by CLEC-2 (Fig. 4.3). I suspected that this could be due to the use of different batches of zymosan. It is known that there is batch to batch variability in the composition of zymosan (G. Brown, personal communication), and to eliminate this confounding factor, I decided to examine the respiratory burst response using highly purified particulate β-glucan as a stimulus. The data generated using particulate β-glucan as a stimulus correlated with the results from the original set of experiments i.e: macrophages expressing the CLEC-2 chimeric receptor failed to induce a respiratory burst, whereas cells expressing Dectin-1 induced a robust response (Fig. 4.4, upper panel). This suggested that some component of the second batch of zymosan may have been acting as a co-stimulatory molecule which in combination with β-glucan ligation of the CLEC-2 chimera resulted in the production of ROS. I therefore took a two pronged approach to investigate the possibility of collaboration between TLR and CLEC-2 signalling to induce the respiratory burst. Firstly, cells were stimulated with either zymosan, particulate β-glucan or depleted zymosan. Commercially available depleted zymosan is obtained by treating zymosan with hot alkali to remove all TLR-stimulating properties. The inclusion of depleted zymosan in this experiment would reveal whether a TLR-activating component of zymosan was contributing to the respiratory burst response. All of the stimuli elicited a response from the Dectin-1 transduced cells. In contrast, the respiratory burst from CLEC-2 chimeric cells was not enhanced in response to any of the stimuli used (Fig. 4.4). The second approach to investigate whether CLEC-2 may function in collaboration with TLRs to induce the respiratory burst, involved the stimulation of cells with particulate β-glucan in the presence of a TLR4 ligand (LPS), or a TLR2/6 ligand (Pam3CSK4). In this case, it was observed that there was no enhanced response when CLEC-2 chimeric cells were stimulated with both β-glucan and TLR stimuli (Fig. 4.5).

In conclusion, collectively these data show that CLEC-2 signalling via Syk kinase does not mediate the respiratory burst. Nor does it appear that co-stimulation of TLR and CLEC-2 signalling induces the production of ROS. The intermediate response mediated by CLEC-2 in response to zymosan observed in one set of experiments is not due to differences in TLR ligand composition and may be caused by as of yet unidentified zymosan components which vary from batch to batch.
Figure 4.3: The respiratory burst in various RAW264.7 transfectants, measured by assessing the conversion of dihydrorhodamine 123 to rhodamine by flow cytometry, following stimulation with zymosan for various times as indicated. The data are expressed as mean fluorescent intensity (mfi). The data shown are the mean ±SD and are representative of at least three independent experiments. *, p < 0.05 vs pFB vector control (Student’s t-test).
Figure 4.4: The respiratory burst in various RAW264.7 transfectants, measured by assessing the conversion of dihydrorhodamine 123 to rhodamine by flow cytometry, following stimulation with particulate β-glucan, zymosan or depleted zymosan (all at 50μg/ml). The data are expressed as mean fluorescent intensity (mfi). The data shown are the mean ±SD and are representative of at least three independent experiments. *, p < 0.05 vs pFB vector control; NS, non significant (Student’s t-test).
Figure 4.5: The respiratory burst in RAW264.7 cells expressing the mCLEC-2/Decin-1 chimera, measured by assessing the conversion of dihydrorhodamine 123 to rhodamine by flow cytometry, following stimulation with particulate β-glucan (pβG, 50μg/ml) in the presence or absence of Pam3CSK4 (P3, 0.1ng/ml) or LPS (1μg/ml). The data are expressed as fold difference in mean fluorescent intensity (mfi), and are representative of two independent experiments.
4.2.4 The membrane proximal tyrosine of Dectin-1 is required for induction of the respiratory burst in response to β-glucan

The activation of Syk by the Fcy receptor, Dectin-1, and CEACAM3 has been shown to induce the respiratory burst in a variety of cell types, including neutrophils [162, 180, 305, 331]. However, studies have shown that zymosan induced Dectin-1 signalling in macrophages resulted in the activation of Syk and the subsequent respiratory burst only in a subset of cells, yet assembly of the NADPH oxidase on zymosan phagosomes occurred in all cells [180]. This data, along with my demonstration that CLEC-2 signals via Syk yet does not induce the respiratory burst, suggests that additional unidentified components, distinct from Syk, are required for the induction of the respiratory burst in response to microbes. I therefore sought to investigate the mechanism underlying Dectin-1 mediated activation of the respiratory burst.

The most likely explanation to account for the differences in the ability to trigger the respiratory burst that I observed between CLEC-2 and Dectin-1, is that there are differences in the downstream signalling pathways induced by these receptors. Although there is a degree of similarity in their cytoplasmic tails, the full tail of CLEC-2 is actually significantly shorter than that of Dectin-1 (Fig. 4.8 and see Chapter 3, fig. 3.5). I therefore performed some preliminary experiments to investigate what specific components of the Dectin-1 cytoplasmic tail are required to induce the respiratory burst response. For these experiments I used previously generated RAW264.7 macrophages expressing various mutant Dectin-1 constructs (Fig 4.6) [179]. Dectin Y15F cells were transduced with a Dectin-1 receptor construct in which the membrane proximal tyrosine was mutated to a phenylalanine. The Dectin Y3F cells were transduced with a Dectin-1 receptor construct in which the membrane distal tyrosine was mutated to a phenylalanine. Examination of the respiratory burst in these cells revealed that production of ROS still occurred when the membrane distal tyrosine was mutated, indicating that this residue is dispensable for induction of the response. In contrast, the membrane proximal tyrosine was required as expected (Fig. 4.7).

I next generated three different chimeric constructs (ROS chimera 1, 2, 3), as represented in figure 4.8, and verified their integrity by sequencing. These constructs consisted of the basic mCLEC-2/Dectin-1 chimera described earlier, but with various sections of the Dectin-1 cytoplasmic tail incorporated. Assessing the ability of these constructs to induce the respiratory burst in RAW264.7 cells would theoretically pinpoint the areas of the Dectin-1 cytoplasmic tail that confer the ability to induce this response. Due to time constraints, I did not complete the retroviral transduction of RAW264.7 cells before thesis submission. However, this work is ongoing and will form a major aspect of the research I will pursue in the future.
Figure 4.6: Schematic representation of the Dectin-1 constructs used in these experiments demonstrating the position of the various cytoplasmic tail mutations.

Transduced RAW cells + particulate β-glucan (50μg/ml)

Figure 4.7: The respiratory burst in various RAW264.7 transfectants, measured by assessing the conversion of dihydrorhodamine 123 to rhodamine by flow cytometry, following stimulation with particulate β-glucan. The data are expressed as mean fluorescent intensity (mFI). The data shown are the mean ±SD and are representative of at least three independent experiments. *, p < 0.05 vs pFB vector control (Student's t-test).
Figure 4.8: Amino acid sequences of the cytoplasmic tails of Dectin-1 and CLEC-2 with boxed similarities, and the chimeras incorporating sections of the Dectin-1 cytoplasmic tail (boxed) into the Dectin-1/CLEC-2 chimeric receptor which will be used to study the respiratory burst.

4.2.5 Preliminary identification of proteins that associate with Dectin-1 following cellular activation

To identify potential signalling candidates that may be crucial for Dectin-1 mediated induction of ROS, I looked for a protein or proteins that associated directly or indirectly with the cytoplasmic tail of Dectin-1 during cellular activation, but did not associate with the cytoplasmic tail of CLEC-2. This investigation was carried out by performing immunoprecipitations from stimulated RAW264.7 cells using tyrosine phosphorylated or unphosphorylated peptides, corresponding to the cytoplasmic tails of murine CLEC-2 or Dectin-1. Immunoprecipitations from the lysates were resolved by SDS-PAGE and stained with coomassie blue (Fig. 4.9). Subsequent MS/MS analysis of separated proteins was performed by Dr. Caroline Jeffreys (Royal College of Surgeons, Dublin, Ireland). From this analysis, a number of proteins which associated specifically with Dectin-1 were identified (Table 4.1), and work is ongoing to investigate whether any of these candidates are involved in the respiratory burst in a regulatory capacity.

Figure 4.9: Immunoprecipitations from RAW264.7 macrophages using the indicated phosphorylated (P) or unphosphorylated peptides were resolved by 10% SDS-PAGE and proteins were stained with coomassie blue prior to MS/MS analysis. The CLEC-2 peptides corresponded to the MQDEDGYITLNKPR region of the CLEC-2 cytoplasmic tail. The Dectin-1 peptides corresponded to the MKYSHIENLDEDGYITQLDFSTQ region of the Dectin-1 cytoplasmic tail and have been described previously [177].
Table 4.1: Proteins that associate with Dectin-1, but not with CLEC-2, during cellular activation

<table>
<thead>
<tr>
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<th>Protein Name</th>
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<tbody>
<tr>
<td>1</td>
<td>TIP120 protein</td>
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<tr>
<td>2</td>
<td>Ras GTPase-activating protein</td>
</tr>
<tr>
<td>3</td>
<td>Heat shock 70 kDa protein 4</td>
</tr>
<tr>
<td>4</td>
<td>Polypyrimidine tract binding protein (PTB)–associated splicing factor</td>
</tr>
<tr>
<td>5</td>
<td>Moesin</td>
</tr>
<tr>
<td>6</td>
<td>Far upstream element binding protein 2 (FUSE binding protein 2)</td>
</tr>
<tr>
<td>7</td>
<td>Dead-box protein 17</td>
</tr>
<tr>
<td>8</td>
<td>Succinate dehydrogenase flavoprotein subunit</td>
</tr>
<tr>
<td>9</td>
<td>T-plastin</td>
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<tr>
<td>10</td>
<td>Lamin A/C</td>
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<tr>
<td>11</td>
<td>EH-domain containing protein 4</td>
</tr>
<tr>
<td>12</td>
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</tr>
<tr>
<td>13</td>
<td>Tyrosine-protein kinase LYN</td>
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<td>19</td>
<td>S-adenosylmethionine synthetase</td>
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<tr>
<td>20</td>
<td>Hsp90 co-chaperone Cdc37</td>
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4.3 Discussion

On the basis of the similarity between the cytoplasmic tails of CLEC-2 and Dectin-1, and because CLEC-2 is a Syk-coupled activatory receptor expressed on neutrophils, I was interested in investigating whether this C-type lectin is involved in mediating the respiratory burst. I found that despite signalling via Syk kinase, CLEC-2 does not mediate induction of this response, either alone or in collaboration with TLRs. There was a period when I observed a weak, but significant respiratory burst response mediated by CLEC-2, which I suspected could be due to the use of different batches of zymosan whose components can vary. I performed some experiments which eliminated the possibility of collaboration with TLRs. However due to limited amounts of the various batches, I was unable to compare them directly and furthermore could not rule out the possibility that the batch which elicited a CLEC-2 response was contaminated. It should also be noted that these conclusions were drawn from experiments using a mCLEC-2/Dectin-1 chimera. As discussed in the previous chapter there are potential drawbacks to using a chimera-based approach and further experiments using full length CLEC-2 should ideally be performed to confirm that it does not induce the respiratory burst.

The activation of Syk by the Fcy receptor, Dectin-1, and CEACAM3 has been shown to induce the respiratory burst in a variety of cell types, including neutrophils. In fact, this response is completely absent following stimulation of these receptors in Syk-deficient cells [180, 331]. However, in macrophages stimulated with zymosan, the Dectin-1 mediated activation of Syk and the subsequent respiratory burst occurred only in a subset of cells, yet the assembly of the NADPH oxidase on the zymosan phagosomes occurred in all cells [180]. Taken together with the observation that CLEC-2 does not mediate the generation of ROS, these data therefore suggest that there is another component / pathway stimulated by these receptors, in addition to Syk, that is required for the induction of the respiratory burst.

The most likely explanation to account for the differences in the ability of CLEC-2 and Dectin-1 to trigger the respiratory burst, is that there are differences in the downstream signalling pathways induced via these receptors. Although there is a degree of similarity in their cytoplasmic tails, the full tail of CLEC-2 is actually significantly shorter than that of Dectin-1. I determined that the tyrosine within the ITAM-like motif of Dectin-1 is crucial for the respiratory burst response, whereas the membrane distal tyrosine is not required. This is not unexpected, as the ITAM-like motif has been shown to mediate Dectin-1 signalling and is required for other cellular responses [179, 180]. Future work will involve the generation of various other CLEC-2/Dectin-1 chimeric receptors which will help to identify other components of the Dectin-1 cytoplasmic tail which are critical for induction of the respiratory burst. As mentioned, this work is in progress and the initial constructs have been generated and their sequences verified.

To identify potential signalling candidates that may be crucial for Dectin-1 mediated induction of ROS, I performed another preliminary experiment which was designed to identify proteins that associated directly or indirectly with the cytoplasmic tail of Dectin-1 during cellular activation, but
did not associate with the cytoplasmic tail of CLEC-2. This investigation was carried out by performing immunoprecipitations from stimulated RAW264.7 cells using tyrosine phosphorylated or unphosphorylated peptides, corresponding to the cytoplasmic tails of murine CLEC-2 or Dectin-1. Subsequent MS/MS analysis revealed a number of proteins which associated with Dectin-1 but not with CLEC-2. Investigations are underway to determine whether any of these proteins are critically involved in Dectin-1 mediated induction of the respiratory burst. Some have previously being implicated in this response, for example the Src family kinase, Hck, has been shown to regulate the fMLP-induced respiratory burst response in neutrophils [332].

It should be noted that it is also possible that Dectin-1 may function in collaboration with other cell surface receptors to induce the respiratory burst response. However, this is unlikely as the chimeric receptor that I used contained the extracellular and transmembrane regions of Dectin-1 and would therefore in all likelihood, participate in any collaborative receptor interactions that Dectin-1 may be involved in.

In summary, I determined that CLEC-2 is not involved in mediating the generation of ROS. This finding raised questions about how other Syk-coupled ITAM receptors regulate their activation of the respiratory burst. A greater understanding of the underlying signalling mechanisms will ultimately advance our global understanding of antimicrobial immunity and the pathogenesis of disease.
5 Characterisation of the expression of the CLEC-2 ligand, podoplanin, on macrophages

5.1 Introduction

5.1.1 Aims of this chapter

When I started this project in 2006 there were no known ligands for CLEC-2. I therefore set out to identify endogenous and/or exogenous CLEC-2 ligands and I used two strategies to pursue this objective. The first approach involved screening carbohydrate microarrays with a soluble protein which contained the extracellular region of CLEC-2. The second approach involved the use of a reporter cell system. Using the reporter cell system a CLEC-2 ligand was identified on inflammatory macrophages. In the intervening period, two other research groups identified a transmembrane sialoglycoprotein called podoplanin as an endogenous CLEC-2 ligand [272, 274]. I subsequently determined that the CLEC-2 ligand on macrophages, was in fact podoplanin. Podoplanin expression on macrophages had not been described previously and I therefore went on to examine its expression on various macrophage populations. It is appropriate at this point to give a brief introduction and overview of podoplanin, in terms of its expression and function.

5.1.2 Identification, structure and nomenclature of podoplanin

Podoplanin is a type I transmembrane protein which contains a short cytoplasmic tail, a single transmembrane region and an extensively O-glycosylated, sialylated extracellular domain [333, 334]. Rat, mouse, human and canine forms of podoplanin have been described, although several different names have been used in the literature. The first description of the murine form was as an early-response protein called OTS-8, that was inducible by phorbol ester treatment in an osteoblast cell line [335]. The protein was also identified as a cell surface antigen called gp38, expressed by murine stromal cells and a subset of thymic epithelial cells [336, 337]. In rats, podoplanin was initially described as E11, expressed on mature osteoblasts and newly formed osteocytes [338]. A further rat homologue, T1α, was identified as a developmentally regulated gene which was translated in high abundance in adult type 1 alveolar cells [339, 340]. The protein was also described as gp38P and podoplanin in the glomerular epithelial cells (podocytes) of mice and rats respectively [333, 341]. In humans, podoplanin was initially reported as a marker of lymphatic epithelial cells [342], and was independently cloned by several groups who assigned various names to the protein including aggrus, gp36, PA2.26 and T1α-2 [343-345]. Although, various names are still in use for this protein, for the purposes of clarity and continuity, I will use the term 'podoplanin' only.
5.1.3 Expression of podoplanin

Podoplanin is broadly expressed in normal tissues (for details refer to Table 5.1) and is widely used as a specific marker for lymphatic endothelium and lymphangiogenesis (formation of lymphatic vessels) in many species [342]. Expression of podoplanin in normal tissues is regulated by Prox1, a master control gene in the development of lymphatic endothelium [346]. Podoplanin expression is also upregulated in a number of different human cancers, including squamous cell carcinomas of the lung [347], the oral cavity [345] and the cervix [348]; mesotheliomas [349], dysgerminomas of the ovary and granulosa cell tumours [350], subsets of tumors of the central nervous system [351], chondrosarcomas [352], colorectal tumours [343], Kaposi's sarcomas [342], ameloblastomas [353], follicular dendritic cell sarcomas [354-356] and a subset of angiosarcomas [342].

5.1.4 Function of podoplanin

Mice that are deficient in podoplanin die at birth as a result of respiratory failure [357]. Compared to wild-type mice, these animals displayed abnormally high expression of proliferation markers such as PCNA (proliferating cell nuclear antigen) in the distal lung, fewer type I cells, narrower and irregular air spaces, and defective formation of alveolar saccules [357]. This phenotype suggests that podoplanin is required for normal lung cell proliferation and alveolus formation at birth. In addition, these mice showed defects in lymphatic vessel pattern formation which resulted in diminished lymphatic transport, congenital lymphedema and dilation of cutaneous and intestinal lymphatic vessels [350]. This suggests that podoplanin is also required for the correct formation and function of the lymphatic vascular system. Podoplanin may function in this role by way of its interaction with galectin-8, a soluble protein which was identified as a glycosylation dependent binding partner of podoplanin [358]. Recent investigations suggested that galectin-8 and podoplanin play a role in supporting the connection of the lymphatic endothelium to the surrounding extracellular matrix [358]. Studies in podoplanin knockout mouse embryos also showed cardiac malformations and myocardial abnormalities, indicating a role for this protein in normal cardiac development [359-361]. A number of reports have also suggested that podoplanin is involved in mediating cell motility by promoting cell-surface extensions and reorganisation of the actin cytoskeleton [345, 350, 362, 363]. Interestingly, in squamous cell carcinomas, podoplanin expression is restricted to the outer cell layer of the invading tumour front [345, 363]. It has also been shown to interact with the cytoplasmic linkers ezrin and moesin through a cluster of basic amino acids within its cytoplasmic tail [364]. These observations have led to the suggestion that podoplanin may induce collective tumor invasion [363].

It has long been known that some tumour cells have the capacity to induce platelet aggregation and that this ability is associated with the tumours metastatic potential [365]. Podoplanin was eventually identified as a platelet aggregation-inducing factor on tumours [343, 366, 367], and further studies described CLEC-2 as its physiological target on platelets [272, 274]. In vitro studies have suggested that podoplanin aggregation through CLEC-2 is an important mechanism of tumour metastasis [273]. As mentioned previously, it was concluded that the interaction between podoplanin and CLEC-2 was mediated by a di-sialylated core O-glycan sequence on Thr52 of...
podoplanin [273]. However, recombinant glycopeptides were used in this study and it remains unclear whether the oligosaccharide sequence alone is sufficient for CLEC-2 binding, or whether any of the flanking peptide region is also involved.

Notwithstanding the expression of podoplanin on follicular dendritic cells [354-356], whose hematopoietic origin remains controversial [368, 369], this protein has not been previously described on any other hematopoietic derived cell types. Here I examine the expression of podoplanin on various macrophage populations.

Table 5.1 Summary of podoplanin expression (M: mouse, R: rat, H: human)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Location/Organ</th>
<th>Species</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular epithelial cells (podocytes)</td>
<td>Glomerulus, Kidney</td>
<td>M/R</td>
<td>[333, 341]</td>
</tr>
<tr>
<td>Parietal epithelial cells</td>
<td>Bowman’s capsule, Kidney</td>
<td>R/M/H</td>
<td>[333, 362, 370]</td>
</tr>
<tr>
<td>Type 1 alveolar epithelial cells</td>
<td>Lung</td>
<td>R/H/M</td>
<td>[339, 356]</td>
</tr>
<tr>
<td>Follicular dendritic cells</td>
<td>Spleen/Lymph node</td>
<td>M</td>
<td>[356]</td>
</tr>
<tr>
<td></td>
<td>Thymus/Tonsils/Lymph node</td>
<td>H</td>
<td>[344-356]</td>
</tr>
<tr>
<td>Pleural mesothelial cells</td>
<td>Lung</td>
<td>M</td>
<td>[362]</td>
</tr>
<tr>
<td>Cells of the choroid plexus</td>
<td>Brain</td>
<td>R/M</td>
<td>[340, 356]</td>
</tr>
<tr>
<td>Meningeal cells</td>
<td>Brain</td>
<td>M</td>
<td>[356]</td>
</tr>
<tr>
<td>Ependymal cells lining ventricles</td>
<td>Brain</td>
<td>M</td>
<td>[356]</td>
</tr>
<tr>
<td>Ciliary epithelial cells</td>
<td>Eye</td>
<td>R</td>
<td>[340]</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>Thymus</td>
<td>M/R</td>
<td>[336]</td>
</tr>
<tr>
<td>Lymphatic capillary endothelial cells</td>
<td>Lymph vessels</td>
<td>M/R/H</td>
<td>[342, 356]</td>
</tr>
<tr>
<td>Osteoblasts</td>
<td>Bone</td>
<td>R/H</td>
<td>[338, 371]</td>
</tr>
<tr>
<td>Osteocytes</td>
<td>Bone</td>
<td>R/M/H</td>
<td>[338, 371, 372]</td>
</tr>
<tr>
<td>Myoepithelial cells</td>
<td>Salivary gland</td>
<td>M/H</td>
<td>[373]</td>
</tr>
<tr>
<td></td>
<td>Breast gland</td>
<td>H</td>
<td>[374]</td>
</tr>
<tr>
<td>Fibromyocytes</td>
<td>Testis</td>
<td>M</td>
<td>[356]</td>
</tr>
<tr>
<td>Perineural cells</td>
<td>Spinal nerve roots</td>
<td>M</td>
<td>[356]</td>
</tr>
<tr>
<td>Stromal reticular cells</td>
<td>Spleen/Lymph node</td>
<td>M</td>
<td>[337]</td>
</tr>
<tr>
<td></td>
<td>Thymus/Tonsils/Lymph node</td>
<td>H</td>
<td>[356]</td>
</tr>
<tr>
<td>Germinal epithelial cells</td>
<td>Primary &amp; secondary ovarian follicles</td>
<td>M</td>
<td>[356]</td>
</tr>
<tr>
<td>Granulosa cells</td>
<td>Primary &amp; secondary ovarian follicles</td>
<td>M</td>
<td>[356]</td>
</tr>
<tr>
<td>Odontoblasts</td>
<td>Tooth</td>
<td>M</td>
<td>[375]</td>
</tr>
<tr>
<td>Enamel epithelial cells</td>
<td>Tooth</td>
<td>M</td>
<td>[375]</td>
</tr>
<tr>
<td>Basal epithelial keratinocytes</td>
<td>Skin/Cervix/Oesophagus</td>
<td>H</td>
<td>[356]</td>
</tr>
<tr>
<td>Myofibroblasts</td>
<td>Prostate</td>
<td>H</td>
<td>[350, 356]</td>
</tr>
</tbody>
</table>
5.2 Results

5.2.1 Carbohydrate microarray analyses do not reveal ligands for CLEC-2

As there had been no CLEC-2 ligands described at the outset of this project, the recently
developed neoglycolipid-based oligosaccharide microarray technique, a unique approach for
constructing microarrays of lipid-linked oligosaccharide probes from desired sources, was used to
screen for CLEC-2 ligands. The soluble Fc-CLEC-2 fusion protein was used to probe the
microarray for CLEC-2 ligands. This approach was previously used to successfully determine the
precise carbohydrate specificity of Dectin-1 [155]. CLEC-2 lacks the residues involved in the
ligation of calcium that are usually required for carbohydrate binding in classical lectins. However,
there is still a precedent for carbohydrate binding as exemplified by Dectin-1, which has
carbohydrate binding capabilities even though it lacks the residues involved in the ligation of
calcium. Nevertheless, the results here show that none of the probes included in the microarrays
elicited significant binding signals with FcCLEC-2 (Fig. 5.1). Included as a positive control was
FcDectin-1 which gave strong and selective binding signals with β1-3 linked glucose sequences
(11 mer and 13 mer) as before (Fig. 5.1) [155].

5.2.2 Generation of a reporter cell line to screen for CLEC-2 ligands

As an alternative to the oligosaccharide microarray approach described above, it was also
attempted to identify CLEC-2 ligands using a reporter cell system. The reporter assay was based
on CLEC-2 chimeric receptors triggering the expression of IL-2 and β-galactosidase (LacZ) in
BWZ.36 cells (Fig 5.2A). BWZ.36 cells are a T-cell line which have been transduced with a
construct consisting of the NFAT enhancer element of the IL-2 promoter. They were then further
transduced with a CLEC-2 chimeric receptor which consisted of the extracellular and
transmembrane regions of CLEC-2, fused to the cytoplasmic region of the CD3ζ chain. In this
system, if the extracellular region of CLEC-2 interacts with a ligand, the ITAMs in the CD3ζ chain
become phosphorylated and induce intracellular signalling. As a consequence, cytoplasmic NFAT
proteins translocate to the nucleus and bind NFAT sites in the promoter of the LacZ gene. This
induces β-galactosidase expression whose level of induction can be quantitated through its
enzyme activity. Endogenous IL-2 production from these cells is also an indication of cellular
activation. This approach had been successfully used in our laboratory, and by others, to identify
ligands for other NK gene complex receptors [137, 376-379]. Using flow cytometry, it was initially
established that the reporter construct was expressed at the surface of transduced cells by staining
with anti-CLEC-2 (Fig. 5.2B).
Figure 5.1: Microarray analyses of mCLEC-2- and mDectin-1-human Fc chimeras. Binding signals at 7 fmol/spot (with error bars) are shown for 326 oligosaccharide probes arrayed. None of the probes included in the microarrays elicited significant binding signals with mCLEC-2 (the signal at position 146 has a large error bar and is not significant, NS). This is in contrast with Dectin-1, which gave binding to α1-3-linked glucose sequences (11 and 13mers) derived from a glucan polysaccharide, in accordance with earlier data. The probes are sorted according to their backbone sequence and are divided into 10 main groups. Their annotation is indicated at the bottom of the figure. Abbreviations are as follows: Lac, lactose; LacNAc, N-acetyl-lactosamine; LNnT, lacto-N-neo-tetraose; LNT, lacto-N-tetraose; PolyLac, polyactosamine; GAGs, glycosaminoglycans; and Misc., miscellaneous.
Figure 5.2: A) Cartoon representation of the mCLEC-2/CD3ζ chimeric receptor and signal transduction pathway in BWZ.36 reporter cells. Ligand binding to the receptor induces phosphorylation of the cytoplasmic tyrosines of the CD3ζ chain, leading to recruitment of Zap-70 and further downstream signalling resulting in the activation of NFAT and expression of β-galactosidase and IL-2. B) Anti-CLEC-2 staining of BWZ.36 cells transduced with the mCLEC-2/CD3ζ chimeric receptor construct, showing expression of the receptor at the cell surface (filled histogram). Isotype control staining is indicated by the unfilled histogram. Primary antibody binding was detected with PE-donkey anti-rat IgG.
5.2.3 Preliminary ligand screen using reporter cells

To identify ligands using the reporter system described above, murine primary cells or single cell suspensions isolated from various murine tissues were co-cultured with the reporter cells for 20 hours at 37°C. The concentration of IL-2 in the culture supernatants was then determined by ELISA. By comparison with reporter cells which were incubated with media only, IL-2 induction was observed following co-culture with inflammatory peritoneal macrophages, and cells from various other tissues (Fig. 5.3A). Cell which were stimulated with PMA-ionomycin were included as a positive control. These results indicated that a ligand for CLEC-2 was present on inflammatory macrophages as well as in various other tissues. To further explore ligand expression on macrophages, reporter cells were co-cultured with RAW264.7 macrophages that had been pre-treated with LPS or left untreated. These LPS-activated macrophages also elicited a positive response from the reporter cells in terms of IL-2 production (Fig. 5.3B). The induction of β-galactosidase following co-culture of reporter cells with various cell types was also examined. By comparison with Dectin-1 reporter cells which were used as a control, induction of β-galactosidase was observed following co-culture of the CLEC-2 reporter cells with thioglycollate elicited inflammatory macrophages and cells from various tissues (Fig. 5.4A). Similarly, co-culture of reporter cells with LPS-treated RAW264.7 macrophages also resulted in expression of β-galactosidase, whereas untreated RAW264.7 macrophages did not induce expression of this gene in the reporter cells (Fig. 5.4B). These data further suggest that mCLEC-2 recognises an endogenous ligand on activated macrophages and on various other mouse tissues.

5.2.4 Podoplanin is the CLEC-2 ligand expressed on inflammatory macrophages and activated RAW264.7 macrophages

During the preliminary ligand screening investigations described above, podoplanin was reported as a ligand for CLEC-2 by two other groups [272-274]. I subsequently examined whether the macrophage expressed CLEC-2 ligand that was revealed during the reporter cell screening, was in fact podoplanin. Staining with a podoplanin specific antibody and analysis by flow cytometry established that podoplanin is indeed present on the surface of these cells (Fig. 5.5). Additionally, I probed these cells using the FcCLEC-2 fusion protein to confirm the presence of a CLEC-2 ligand. Using this approach I detected binding of FcCLEC-2 to inflammatory macrophages and activated RAW264.7 cells, confirming the presence of a CLEC-2 ligand on these cells. This interaction appeared to be specific, as similar binding was not obtained with FcDectin-1 (Fig. 5.5). As podoplanin expression had not been previously described on macrophages. I decided to investigate its expression on other macrophage populations. Analysis by flow cytometry revealed that podoplanin expression was negligible on resident peritoneal macrophages and on resident alveolar macrophages (Fig. 5.5). This was in agreement with staining using FcCLEC-2 which indicated that these cells did not express a CLEC-2 ligand (Fig. 5.5).
Figure 5.3: A) Fold differences in IL-2 production from reporter cells following coculture with various cell types as indicated. The data were generated from two independent experiments and were normalised to the media only control value. Error bars represent the SEM. (Thio-pM0: thioglycollate elicited peritoneal macrophages) B) IL-2 production from reporter cells following coculture with RAW264.7 cells or RAW264.7 cells which had been pretreated with LPS. This data is representative of two individual experiments.
Figure 5.4: X-gal staining of reporter cells following co-culture with A) various murine primary cells and B) RAW264.7 macrophages which had been untreated or pretreated with LPS. These data are representative of several experiments. (Thio-pMØ: thioglycollate elicited peritoneal macrophages)
Figure 5.5. Anti-podoplanin and FcCLEC-2 staining of various macrophage populations, as indicated. In the left column, filled histograms represent podoplanin staining (light grey) and IgG2a isotype control staining is represented by unfilled histograms. Primary antibody binding was detected by PE-donkey anti-rat IgG. In the right column, filled histograms represent FcCLEC-2 staining (dark grey) and FcDectin-1 control staining is represented by unfilled histograms. Primary antibody binding was detected by PE-donkey anti-human IgG.
5.2.5 Signalling through certain TLR's causes upregulation of podoplanin expression

As podoplanin expression was upregulated on inflammatory macrophages, I investigated whether stimulation of macrophages with microbial agonists could also induce regulation of this protein. To do this I examined expression of podoplanin by flow cytometry following a 20 hour stimulation of bone marrow derived macrophages with a variety of TLR agonists. Bone marrow derived macrophages were chosen for this investigation as they showed equivalent levels of podoplanin expression as alveolar and resident peritoneal macrophages (Fig. 5.6A). Stimulation with a TLR4 agonist resulted in upregulation of podoplanin expression that corresponded to an approximately 14 fold increase over unstimulated cells. Although treatment with TLR2/1 and TLR2/6 agonists caused an apparent increase in podoplanin expression, statistical analysis revealed that this upregulation was not significant. The addition of TLR3 and TLR5 agonists did not result in any upregulation of podoplanin expression and its levels on these cells were comparable to untreated macrophages (Fig. 5.6B). Concentrations of TLR agonists ten fold less than those shown in Fig. 5.6B were also tested and resulted in similar effects on podoplanin expression (data not shown).

5.2.6 Cytokine regulation of podoplanin expression on bone marrow derived macrophages

As macrophage phenotypes are strongly affected by the action of cytokines, I also investigated the regulation of podoplanin expression on macrophages in response to various cytokine treatments. To do this I examined expression of podoplanin by flow cytometry following a 20 hour stimulation of bone marrow derived macrophages with a variety of cytokines. I found that TNF, and to a lesser extent IL-10, were involved in podoplanin regulation and caused a modest increase in its expression. Treatment with IFNγ and GM-CSF resulted in an apparent but non-significant increase in podoplanin expression. Macrophages which were treated with IL-4 had equivalent levels of podoplanin expression as untreated cells (Fig. 5.6C).

5.2.7 Podoplanin expression on neutrophils and during systemic inflammation

As CLEC-2 is expressed on neutrophils, I wondered whether podoplanin may also be present on these cells. In contrast to CLEC-2, there was no evidence of podoplanin expression on peripheral blood neutrophils. However, I found that podoplanin was expressed on inflammatory neutrophils (Fig. 5.7). It appears then that podoplanin is upregulated on neutrophils when these cells are recruited to sites of inflammation from the bloodstream.

To evaluate podoplanin expression during systemic inflammation, a preliminary experiment was performed in which mice were challenged with LPS (1μg, i.v) to induce a low-grade inflammatory response [380]. Using flow cytometry, splenocytes and lymph node cells were analysed for podoplanin expression 20 hours after challenge. It was found that LPS challenged mice exhibit similar levels of podoplanin expression as untreated mice which suggests that this protein is not upregulated during systemic inflammatory responses (Fig. 4.8). However, this experiment was preliminary and must be repeated with various LPS doses.
Figure 5.6: A) Anti-podoplanin staining of bone marrow derived macrophages, showing negligible expression of the protein at the cell surface (filled histogram). IgG2a isotype control staining is represented by the unfilled histogram. Primary antibody binding was detected by PE-donkey anti-rat IgG. B) The changes in podoplanin expression following stimulation with TLR agonists as indicated, quantified as described in Methods. The data were generated from two independent experiments and were normalised to the untreated control value. Error bars represent the SEM. C) The changes in podoplanin expression following stimulation with various cytokines as indicated, quantified as described in Methods. The data are representative of two independent experiments and were normalised to the untreated control value. Error bars indicate the SD. *, p<0.05 vs untreated control (Student’s t-test).
Figure 5.7: Anti-podoplanin or rlgG2a staining of indicated cells. Neutrophils were identified by their expression of CD11b and Gr-1 and density plots depicted here were gated on said cells. Primary antibody binding was detected by PE-donkey anti-rat IgG. Percentages shown are indicative of the percentage of gated cells in the upper right quadrant.

Figure 5.8: Anti-podoplanin staining of indicated cells. Primary antibody binding was detected by PE-donkey anti-rat IgG. Data is representative of one experiment in which 4 mice were untreated and 4 mice were LPS challenged.
5.3 Discussion

I was interested in identifying CLEC-2 ligands and my initial approach was to use an oligosaccharide microarray, a technique which had been successfully applied to determine the precise carbohydrate specificity of Dectin-1 [155]. None of the carbohydrates included in the array elicited a positive response in terms of CLEC-2 binding. This is perhaps not surprising as CLEC-2 lacks the residues conserved in other C-type lectins that mediate Ca\(^{2+}\) coordination and carbohydrate binding. However, as mentioned previously, this approach was taken as there are examples of C-type lectins (such as Dectin-1) that lack these residues yet still bind carbohydrates.

In the interim, other researchers reported that the interaction between CLEC-2 and its ligand podoplanin, is mediated by a di-sialylated core O-glycan sequence (NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAcα-1) on Thr52 of podoplanin [273]. Interestingly, this oligosaccharide was present as a neoglycolipid probe in the microarray used here, but was not recognised by FcCLEC-2. This highlights an apparent discrepancy between my array result and previously published work. This may be accounted for by the fact that in the microarray used here, the probe in question was conjugated to the lipid tag by reductive amination, which results in the ring-opening of the GalNAc core. Further analysis with the ring-closed structure would be helpful in elucidating whether the oligosaccharide alone is sufficient for binding, or as mentioned previously whether any of the flanking peptide region is also required.

As an alternative approach to identify CLEC-2 ligands, a reporter cell system was used which had been previously employed to identify ligands for other NK gene complex receptors [137, 376-379]. Using this method, an endogenous CLEC-2 ligand was identified on activated macrophages and further exploration indirectly showed that this ligand was podoplanin. A subsequent review of the literature revealed that this was the first demonstration of podoplanin expression on macrophages. Although interactions between podoplanin and CLEC-2 have now been clearly shown elsewhere, future immunoprecipitation experiments using FcCLEC-2 and macrophage lysates, followed by probing of associated proteins for podoplanin should be performed to directly demonstrate an interaction between macrophage expressed podoplanin and CLEC-2.

Macrophages are a highly heterogenous population of cells with well-defined roles in the innate immune response, but also in inflammation, tissue homeostasis, resolution, repair and as antigen presenting cells [1]. Generally, monocytes derived from bone marrow progenitors circulate in the blood for several days before entering tissues to differentiate into macrophages [381, 382]. Macrophages in different anatomical locations exhibit various functional specialisations, suggesting that the tissue microenvironment is involved in the macrophage differentiation process [381]. It is therefore expected that the range of expressed proteins differs between the various macrophage populations. I explored the expression of podoplanin on various macrophage populations and other myeloid cells. Although I found that podoplanin was highly expressed on inflammatory macrophages; alveolar macrophages, resident peritoneal macrophages and bone marrow derived macrophages showed negligible expression of the protein. As tissue macrophages are known to rapidly respond to external factors such as cytokines and microbial ligands resulting in significant
changes in gene and protein expression, I investigated whether the expression of podoplanin on macrophages was regulated following stimulation with TLR agonists and cytokines. I found that podoplanin expression was upregulated following stimulation via TLR4 but not other TLR agonists, suggesting some specificity in this response. In terms of cytokines, TNF, and to a lesser extent IL-10, resulted in an increase in podoplanin expression. Although not examined in this study, it is possible that podoplanin may be similarly regulated on human macrophages or other leukocytes.

Considering the expression of its binding partner on neutrophils, and to simply investigate its presence on other myeloid cells, I also examined podoplanin expression on neutrophils. The results indicate that podoplanin is not expressed on peripheral blood neutrophils but is upregulated when these cells migrate to inflammatory sites. This is in direct contrast to CLEC-2, which is expressed on peripheral blood neutrophils but not on inflammatory neutrophils. It would appear then that CLEC-2 and podoplanin are reciprocally regulated in terms of their expression. The mechanism and functional significance of this is unclear; one possibility is that this regulation may be in place to avoid inappropriate cellular activation via CLEC-2 signalling in inflammatory neutrophils. Although not detailed here, I made several unsuccessful attempts to express full-length mCLEC-2 in RAW264.7 macrophages. This reciprocal regulation may explain why I was unsuccessful; perhaps there are mechanisms in place that prohibit the expression of both CLEC-2 and its ligand podoplanin, by the same cell.

Alternatively, it is possible that CLEC-2 is expressed on inflammatory neutrophils but that its detection by antibody staining is masked by cis interactions with podoplanin on these cells. Indeed, in the case of Dectin-1, binding of soluble β-glucan ligands masked detection of the receptor by a Dectin-1 specific monoclonal antibody [290]. The possible expression of both CLEC-2 and podoplanin on the same cell is reminiscent of the Siglecs (sialic-acid-binding immunoglobulin-like lectins), whose binding sites are typically masked by cis interactions with other glycan ligands expressed on the same cell [383]. This possibility could be explored by exposing cells to sialidase, a treatment which would presumably cleave the cis-interacting podoplanin from CLEC-2 and allow its detection by flow cytometric methods. Other approaches, such as Western blotting, could also be used to explore the possibility of CLEC-2 expression on inflammatory neutrophils.

The significance of podoplanin expression on macrophages remains unclear. Transfection studies with cultured normal and cancer cells showed that podoplanin promotes cell spreading, migration and invasion [345, 350, 362, 363]. Furthermore, podoplanin is upregulated in the invasive front of certain tumors. Given this role in cell motility, it is tempting to speculate that podoplanin could be involved in directing inflammatory macrophage migration. In the future, I intend to investigate whether podoplanin has a role in inflammatory cell recruitment by using podoplanin deficient cells. For this I will attempt to generate podoplanin deficient radiation chimeras by reconstituting the immune system of lethally irradiated recipient mice with podoplanin deficient fetal liver cells. This model will bypass the developmental defects by limiting the deletion of podoplanin to hematopoietic cells and the immune cells that they give rise to.
It has also been shown that the induction of platelet aggregation by podoplanin promoted the establishment of pulmonary metastasis in both experimental and spontaneous metastasis models and decreased survival of the mice [384]. This suggests that tumors which express podoplanin have more malignant characteristics and indeed podoplanin expression has been associated with poor clinical outcome in oral cancer [385]. On the other hand, there are also a number of studies which have shown that the expression of podoplanin on certain tumors and tumor associated cells, is associated with a good disease prognosis [386-388]. The differences in tumor origin most likely account for these apparent contradictions. What would be interesting to explore further is whether podoplanin is expressed on tumor associated macrophages (TAMs). TAMs are a major inflammatory leukocyte population present in tumors and have been shown to promote tumor growth through the promotion of angiogenesis, matrix remodelling and suppression of adaptive immunity [389, 390]. Considering the expression of podoplanin on certain inflammatory macrophages, it is certainly possible that the protein is also expressed on TAMs. This is an aspect of podoplanin expression on macrophages and its function in tumor growth that it worthy of further attention.

Finally, as mentioned previously, the simultaneous recognition of podoplanin on macrophages by both platelets and neutrophils may contribute to the interactions between these two cell types and to the activation and cross-talk of the inflammatory and coagulation pathways. These interactions are known to be important for the control of infection, and for limiting inflammatory pathology, but are also involved in the development of disease [311, 312]. A further indication that podoplanin may be involved in immunity is its recent identification on follicular dendritic cells [354-356]. These cells reside in primary B cell follicles and germinal centres of lymphoid tissues and assist in B cell maturation by the presentation of intact antigen to the B cells [391, 392].

In conclusion, I have shown here that the CLEC-2 ligand podoplanin is expressed on inflammatory macrophages and inflammatory neutrophils, but not on tissue resident alveolar or peritoneal macrophages, or bone marrow derived macrophages. I have also shown that certain TLR agonists and cytokines cause upregulation of podoplanin on bone marrow derived macrophages. Further studies will elucidate the physiological and/or pathophysiological significance of this expression of podoplanin on inflammatory cells.
6 Summary and future directions

6.1 Review of the aims

The three principal areas of investigation of this thesis were

- The characterisation of CLEC-2 in terms of its functionality.
- An investigation of the mechanisms underlying Dectin-1 mediated induction of the respiratory burst.
- The characterisation of the expression of the CLEC-2 ligand, podoplanin, on macrophages.

In summary I found that CLEC-2 is a Syk-coupled phagocytic activation receptor expressed on murine peripheral blood neutrophils. I also determined that it is not involved in the induction of the respiratory burst. I showed that its ligand, podoplanin is expressed at high levels on inflammatory macrophages and neutrophils. Finally, I performed preliminary investigations into the mechanisms underlying Dectin-1 mediated induction of the respiratory burst and generated tools which will be used for future research in this area.

Here I will give a final summary of the key findings which have been previously discussed within the context of the relevant chapter. I will also briefly discuss and speculate on future directions.

6.2 Summary of key findings

6.2.1 CLEC-2 is a Syk-coupled phagocytic activation receptor expressed on murine peripheral blood neutrophils.

Using specific antibodies, I confirmed that CLEC-2 was expressed on the surface of platelets as described, and furthermore showed that there are high levels of its expression on circulating neutrophils. In contrast, I found that CLEC-2 is only weakly expressed on bone marrow or elicited inflammatory cells. I also observed that expression of CLEC-2 could be upregulated on murine monocytes following stimulation with Pam3CSK4, but not other TLR agonists tested, suggesting some specificity in this response.

Having identified CLEC-2 on neutrophils, I next wished to determine the function of this receptor on these cells. I first explored the possibility that CLEC-2 could mediate particle uptake, similar to Dectin-1. I initially examined the phagocytic potential of CLEC-2 using a chimeric receptor that allowed triggering of CLEC-2 signalling using zymosan. Expression of the chimeric receptor in NIH3T3 cells conferred an ability to internalise zymosan particles in an actin-dependent manner.
To evaluate whether the cytoplasmic tyrosine of CLEC-2 contributes to this activity, a chimeric receptor construct in which the cytoplasmic tyrosine was mutated to a phenylalanine was expressed in NIH3T3 cells. I found that mutation of the cytoplasmic tyrosine significantly reduced the ability of these cells to internalise the zymosan particles. Similar results were obtained when these chimeric receptors were expressed in RAW264.7 macrophages. I also examined the phagocytic potential of CLEC-2 in primary cells and showed that CLEC-2 antibody coated beads bound specifically to peripheral blood granulocytes and that upon incubation at 37°C, these beads were internalised in an actin dependent fashion. Collectively these results demonstrate that CLEC-2 can function as a phagocytic receptor.

I next investigated whether signalling via CLEC-2 can induce cytokine production. Using cell lines expressing the chimeric constructs referred to above, I demonstrated that CLEC-2 can induce TNFα production and that this activity is dependent on the ITAM-like motif in the cytoplasmic tail of the receptor. I also showed that CLEC-2 on peripheral blood neutrophils can mediate production of TNFα in response to the CLEC-2 ligand rhodocytin, although the possibility that it may have been signalling through other receptors in addition to CLEC-2 could not be excluded.

CLEC-2 signals via Syk in platelets, and I wanted to confirm that this pathway was being used in other cell types. I performed immunoprecipitations from RAW264.7 using tyrosine phosphorylated or unphosphorylated peptides, corresponding to the cytoplasmic tail of CLEC-2. Subsequent analysis demonstrated that the phosphorylated CLEC-2 peptide could associate with Syk from the macrophage cell extracts. I also confirmed that cellular activation results in phosphorylation of CLEC-2, by immunoprecipitating this receptor from pervanadate stimulated or unstimulated transduced A20 cells expressing full-length CLEC-2. The expression of the chimeric receptor in Syk-sufficient and Syk-deficient B cell lines and quantification of the production of IL-2 in response to zymosan stimulation, offered further evidence that CLEC-2 signals via Syk. Finally, confocal microscopy analysis of zymosan stimulated RAW264.7 macrophages expressing the chimeric receptor showed clearly the activation of Syk around the phagosome. Collectively these data demonstrate that CLEC-2 signals via Syk kinase in myeloid cells.

6.2.2 CLEC-2 does not induce the respiratory burst

One of the most surprising discoveries was the inability of CLEC-2, either alone or in collaboration with TLRs, to induce the respiratory burst despite signalling via Syk. This highlighted the likelihood that there is another component / pathway stimulated by Dectin-1, in addition to Syk, that is required for the induction of the respiratory burst. I performed some preliminary experiments to investigate the underlying mechanism of Dectin-1 mediated induction of the respiratory burst. I found that the membrane proximal tyrosine is crucial for induction of this response. I also generated chimeric constructs which will allow for specific identification of critical components of the Dectin-1 cytoplasmic tail. Furthermore, using proteomic techniques I identified a number of proteins whose involvement in the regulation of the respiratory burst is currently being explored.
6.2.3 The CLEC-2 ligand, podoplanin, is expressed on inflammatory macrophages and neutrophils

During the course of ligand screening attempts, the presence of a CLEC-2 ligand on inflammatory macrophages was determined. This ligand turned out to be podoplanin, a sialoglycoprotein which was identified by others as a CLEC-2 ligand. This was the first demonstration of podoplanin expression on macrophages and I therefore proceeded to explore its expression on various macrophage populations and other myeloid cells. Although I found that podoplanin was highly expressed on inflammatory macrophages; alveolar macrophages, resident peritoneal macrophages and bone marrow derived macrophages showed negligible expression of the protein.

I investigated whether the expression of podoplanin on macrophages was regulated following stimulation with TLR agonists and cytokines. I found that podoplanin expression was upregulated following stimulation via TLR4 but not other TLR agonists. In terms of cytokines, TNF, and to a lesser extent IL-10, resulted in an increase in podoplanin expression. I also examined podoplanin expression on neutrophils and found that the protein is not expressed on peripheral blood neutrophils but is upregulated when these cells migrate to inflammatory sites. This is in direct contrast to CLEC-2, which is expressed on peripheral blood neutrophils but not on inflammatory neutrophils. It would appear then that CLEC-2 and podoplanin are reciprocally regulated in terms of their expression.

6.3 Future directions

6.3.1 CLEC-2 and podoplanin

The work described in this thesis forms the basis for a more comprehensive analysis of the true physiological role of CLEC-2, which remains unclear at present. An important tool not yet available is a CLEC-2 deficient mouse. When such a mouse becomes available it will allow for the in vivo investigation of the role of CLEC-2 in disease settings and/or homeostasis. There are several areas which could direct future investigations which have become apparent during this thesis. The significance of the expression of the CLEC-2 ligand, podoplanin, on macrophages is unclear at present. As mentioned, perhaps the simultaneous recognition of podoplanin on macrophages by CLEC-2 on both platelets and neutrophils contributes to the interactions between these two cell types and to the activation and cross-talk of the inflammatory and coagulation pathways. These interactions are known to be important for the control of infection, and for limiting inflammatory pathology, but are also involved in the development of disease. Whether CLEC-2 is involved in these processes is an area that warrants further attention. It is also tempting to speculate that podoplanin could be involved in directing inflammatory macrophage migration and I intend to investigate whether podoplanin has a role in inflammatory cell recruitment by using podoplanin deficient cells. For this I will attempt to generate podoplanin deficient radiation chimeras by reconstituting the immune system of lethally irradiated recipient mice with podoplanin deficient fetal liver cells. This model will bypass the developmental defects by limiting the deletion of podoplanin to hematopoietic cells and the immune cells that they give rise to. The reciprocal regulation of podoplanin and CLEC-2 on inflammatory neutrophils is certainly intriguing and implies that this receptor-ligand system is tightly controlled and may be in place for a very specific reason. The
functional significance of this reciprocal regulation is not understood, and this is an area where both the CLEC-2 deficient and podoplanin deficient mice may be useful for future investigations.

Future work will also involve the precise determination of the expression of human and mouse CLEC-2. There is some evidence that CLEC-2 is present on DCs (Caetano Reis e Sousa, personal communication). The capacity of DCs to induce and regulate adaptive immunity has potential therapeutic implications; for example, in the design of vaccines to treat and prevent infectious disease by enhancing immunogenesis, similarly in tumor immunotherapy and in situations where tolerance needs to be increased such as autoimmunity, allergy and transplantation [393]. Future studies may explore the precise function of CLEC-2 on DCs and assess its potential as a targeting molecule in immunotherapeutic settings. It will also be interesting to determine if CLEC-2 expression is regulated under conditions of infection and disease, and to understand its function in these settings.

Preliminary studies carried out directly prior to submission indicate that CLEC-2 has fungal-binding capabilities. Future work will involve the specific identification of the fungal component that CLEC-2 recognises, and the exploration of the ability of CLEC-2 to recognise a range of fungal pathogens. The availability of a CLEC-2 deficient mouse will allow the determination of whether CLEC-2 plays an essential role in anti-fungal immunity in vivo. Initially, the in vitro phagocytic and cytokine inducing capabilities of CLEC-2, could be examined in a physiological setting.

6.3.2 Respiratory burst
As outlined, the observation that CLEC-2 is not involved in the induction of the respiratory burst raised interesting questions about the mechanisms underlying this cellular response. This is an area which I will focus on following submission of this thesis. Future work, which is already underway, will involve the generation of various other CLEC-2/Dectin-1 chimeric receptors which will help to identify whether there are specific components of the Dectin-1 cytoplasmic tail that are required to induce this response. Using biochemical and proteomic techniques I have also identified a number of proteins whose involvement in downstream pathways will be explored. Based on the findings and to further explore the proximal signalling events induced by Dectin-1, computational methods based on protein-protein interactions will be used to construct an activation model. This will allow the determination of how exactly Syk associates with the atypical ITAM of Dectin-1, an aspect that is currently not fully understood. Particular attention will also be paid to the regulation of assembly of the NADPH oxidase complex at the phagosome membrane following triggering of ITAM-coupled receptors, as this complex is ultimately responsible for the production of ROS and previous studies indicate that it is likely to be an area of regulation for the respiratory burst. Ultimately the determination of the mechanisms underlying Dectin-1 mediated induction of the respiratory burst will advance our global understanding of antimicrobial immunity and the pathogenesis of disease.
References


Appendix I: Publications

Publications contributed to during this thesis are included here and are entitled as follows:


- **Kerrigan, A.M.** and Brown, G.D. *Syk-coupled C-type lectin receptors that mediate cellular activation via single-tyrosine based activation motifs.* Immunological Reviews, 234 (1) 335-52 (2010)


CLEC-2 Is a Phagocytic Activation Receptor Expressed on Murine Peripheral Blood Neutrophils

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CLEC-2 is a member of the "dectin-1 cluster" of C-type lectin-like receptors and was originally thought to be restricted to platelets. In this study, we demonstrate that murine CLEC-2 is also expressed by peripheral blood neutrophils, but only weakly by bone marrow or effluxed inflammatory neutrophils. On circulating neutrophils, CLEC-2 can mediate phagocytosis of Ab-coated beads and the production of proinflammatory cytokines, including TNF-α, in response to the CLEC-2 ligand, rhodocytin. CLEC-2 possesses a tyrosine-based cytoplasmic motif similar to that of dectin-1, and we show using chimeric analyses that the activities of this receptor are dependent on this tyrosine. Like dectin-1, CLEC-2 can recruit the signaling kinase Syk in myeloid cells, however, we demonstrate that this pathway does not induce the respiratory burst. These data therefore demonstrate that CLEC-2 expression is not restricted to platelets and that it functions as an activation receptor on neutrophils.

Article citation:

Mature myeloid cells express an extensive collection of cell-surface receptors that are involved in a diverse range of functions ranging from microbial recognition and activation of cellular responses to cell development, migration, proliferation, maturation, and survival. Of specific interest are the Group V C-type lectin-like receptors, which are type II transmembrane proteins, with a single extracellular carbohydrate recognition domain (CRD), a transmembrane region, and a cytoplasmic tail that may contain signaling motifs (1, 2). Within this group is a subgroup of receptors known as the "dectin-1 cluster," which is encoded in a distinct genetic locus within the NK gene complex (3, 4). This subgroup consists of dectin-1, LOX-1 (dectin-like oxidized low-density lipoprotein-1), MBL, and MCLL (myeloid inhibitory C-type lectin-like receptor).

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CLEC-2 was originally identified through a computational screen for C-type lectin-like receptors (10). To date, surface expression of CLEC-2 has only been shown on platelets, although RT-PCR analysis has shown transcripts in PBMC, bone marrow cells, monocytes, dendritic cells, and granulocytes (10, 11). Podoplanin, a slit/glycoprotein involved in tumor cell-induced platelet aggregation, tumor metastasis, and lymphatic vessel formation, has recently been identified as a physiological ligand for CLEC-2, and it has been suggested that their interaction may be involved in tumor growth and/or metastasis (12, 13). Exposure of platelets to rhodocytin, a snake venom toxin that is also a ligand for CLEC-2, leads to tyrosine phosphorylation of a cytoplasmic ITAM-like motif and Syk-dependent platelet activation (11). In addition to Syk, Src and Tec family kinases, PLCβ, and RAC1 are also involved in the signaling pathway activated by CLEC-2 (15, 16). Furthermore, CLEC-2 has been identified as an HIV-1 attachment factor that may capture and transfer infectious HIV-1 in cooperation with DC-SIGN (17). There are two further splice variants of murine CLEC-2 (mCLEC-2), with different expression profiles and subcellular localization compared with full-length CLEC-2 and in addition, full-length CLEC-2 can be cleaved into a soluble homodimeric form (18).

We are interested in the dectin-1 cluster of receptors and wondered whether CLEC-2, like the other receptors in the cluster, was also expressed on myeloid cells. We show in this study that in addition to platelets, mCLEC-2 is expressed on peripheral blood neutrophils as well as on monocytes activated with selected TLR ligands. We demonstrate that this receptor can function as an activation receptor on these cells, inducing phagocytosis and proinflammatory cytokine production, but not the respiratory burst.
Materials and Methods

Cell lines and growth conditions

NIH3T3 and HER293T fibroblasts, RAW264.7 macrophages, EL4 T cells, A20 B cells, HER293T-based dual-luciferase reporter packaging cells (a gift from Professor Kisawü, University of Tokyo, Tokyo, Japan), and Syk-deficient (C53Y) and Syk-reconstituted (WT18) B cell lines (19), were maintained in DMEM or RPMI 1640 (Cambrex) supplemented with 10% FCS (Invitrogen), 2 mM glutamine, 100 U/ml penicillin, and 1 mg/ml streptomycin (Cambrex) and cultured at 37°C with 5% CO2.

Generation of constructs and transduced cell lines

The complete murine CLEC-2 and a hemagglutinin (HA)-tagged version of the receptor were amplified from mouse splenic cDNA by PCR. The CLEC-2-deleted chimera was also generated by PCR, such that the chimeric sequence translated as CLEC-2:177/327:1:246 generating a chimeric receptor consisting of the cytoplasmic tail of CLEC-2 and the transmembrane, stalk, and CTD of dock-11. We also generated a chimeric ITAM mutant in which the syrosine in the DvTyck 11 cytoplasmic motif was mutated to a phenylalanine (referred to in the text as Y11F). All constructs were cloned into the pBSSK (Stratagene) retroviral vector, packaged into viruses using Polybrene-enhanced packaging cells, and the various cell lines were transduced as previously described (20). All cell lines were used as non-tumoral populations in all functional assays and were generated at least twice to confirm phenotype. A purified soluble fusion protein, Mc-LEC-2, containing the CTD and stalk region of CLEC-2, fused to human IgG1Fc, was generated by PCR and cloned into the piTag vector, essentially as described for dock-11 (21, 22).

Primary cells and stimulations

Six- to 12-wk-old BALB/c, 129/Sv, or C57Bl/6 mice, obtained from the specific pathogen-free animal unit at the University of Cape Town (UCT), were used in these experiments. All procedures were approved by the UCT animal ethics committee. Bone marrow and peripheral blood was collected as described previously (23). Thymocyte-cleaved peritoneal neutrophils were isolated by standard procedures and identified by their high expression of Gr-1. To isolate neutrophils, peripheral blood was collected by cardiac puncture into a final concentration of 10 mM EDTA, and leukocytes were separated by centrifugation over a 3-layer (78, 56, and 38%) Percoll Plus (GE Healthcare) gradient at 4°C. Neutrophils were harvested from the 78%/56% interface and the remaining erythrocytes lysed using Gey's solution. Cell viability was generally >95% as determined by trypan blue staining.

To test the effect of TLR agonists on CLEC-2 surface expression, PBMCs were plated in 24-well plates and stimulated for 6 h with Pam3CSK4 (TLR2/1, 100 ng/ml), LPS (TLR4, 100 ng/ml), flagellin (TLRS, 20 ng/ml), and FSL-1 (TLR2/6, 20 ng/ml) (all TLR agonists fromInvivogen). Cells were then analyzed by flow cytometry, as described below.

Generation and purification of polyvalent and mAbs against CLEC-2

A purified polyclonal Ab, specific for CLEC-2, was affinity purified from the serum of Winter rats after immunization with FC CLEC-2. mAb production was performed at Cancer Research U.K. Winter rats were immunized three to four times with RHL-2B3 cells expressing mouse CLEC-2 fused to an HA epitope. Fusion of oligonucleotides with the ras myeloma cell line Y3 was conducted using standard procedures. Hybridoma screening was conducted as previously reported (19).

FACS analysis

FACS was performed on live cells according to conventional protocols at 4°C in the presence of 2 mM NaN3, as previously described (27). The Abs used in these studies (includes: anti-CD11b/biotin (BD Pharmingen), P3 donkey anti-rat IgG (Jackson ImmunoResearch Laboratories), anti-HA (clone 12B12, Covance), 2A11 (anti-dock-11 T/24), 5C8-FITC (anti-CD11b) (25), Gr-1-biotin, 7E4-FITC, and R4-6A2-mAb produced in house, isotype-control Abs were obtained from the same suppliers or produced in house. Biotinylated Abs were detected using streptavidin-allophycocyanin (BD Pharmingen).

Phagocytosis assay

Phagocytosis was quantified in NIH3T3 and RAW264.7 cells as previously described (26, 27). In brief, transduced cells were seeded at 2.5 × 105 cells/well the day before the assay. To inhibit phagocytosis, some cells were preincubated with 10 μM Cytochalasin D (Calbiochem) for 20 min before and throughout the assay. After washing, FITC-rhodamine (Molecular Probes, 1:5000) or anti-CLEC-2 coated Dyneabeads (Invitrogen, 1 bead/cell) labeled with FITC, were added and allowed to settle for 1 h at 4°C. After washing to remove unbound particles, cells were incubated at 37°C for various times, as indicated. External rhamnoside was detected with anti-rhamnoside Abs, as described (27). For the detection of external anti-CLEC-2 coated FITC-labeled Dynabeads, cells were incubated with PMA-pretreated neutrophils. After FITC-ITAM cell populations which had bound or internalized beads were gated, and the percentage of phagocytes was determined by comparing the FC to the PE to the P+ cell populations.

For microscopy, cells were fixed and permeabilized, and acini were stained with 1 μM tetramethylrhodamine isothiocyanate-labeled phalloidin (Sigma-Aldrich). Syk reconstituted cells were detected with anti-phospho Syk (Cell Signaling Technology, followed by cyanin-3-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Coverslips were mounted on slides containing containing Hoechst nuclear dye and cells were observed by confocal laser scanning microscopy on a Zeiss LSM 510 META confocal microscope. Images were processed using Adobe Photoshop version 6.0.

Phagocytosis in PBLs was determined similarly, except, FITC-labeled Dynabeads, coated with anti-CLEC-2, 2A11 or isotype control Abs, were added at a ratio of two beads/cell. Following incubation with reaction for 1 h at 4°C, unbound beads were removed by centrifugation over a two-layer Percoll gradient (40 and 70%) and the cells harvested following centrifugation. Phagocytes, in the presence or absence of 5 μM Cytochalasin D, was allowed to occur at 37°C for 45 min. External beads were detected with a PMA-pretreated anti-rat Ab, as described above, and granulocytic FITC-ITAM cell populations which had bound or internalized beads were gated, and the percentage of phagocytes was determined by comparing the FC to the PE to the P+ cell populations.

Fluorescent rhamnoside binding, respiratory burst, and cytokine production assays

Rhamnoside binding and TNF-α production by transduced RAW264.7 cells in the presence of absence of soluble β-glucan was determined as previously described (24). To examine cytokine production from primary cells, murine neutrophils were purified as described and plated in 3 × 105 cells/well. Cells were left unstimulated or stimulated with 15 μg/ml rhodocytin, purified as described previously (28), or 1 μg/ml LPS (Sigma-Aldrich), for 6 h at 37°C. TNF-α in supernatants was measured using the OptEIA mouse TNF-α ELISA set (BD Bioscience). For the analysis of the Syk-sufficient and Syk-deficient B-cells, 2 × 106 transduced cells were stimulated with various concentrations of anti-β-glucan rhamnoside (Sigma-Aldrich) for 10 h at 37°C. The supernatant was quantified by OptEIA mouse IL-2 ELISA set (BD Bioscience).

For analysis of the respiratory burst, cells were loaded with dihydrodihydrofluorescein diacetate (Sigma-Aldrich) at a final concentration of 2 μM. After incubation for 1 h at 37°C with rhamnoside (25 particles), 15 μg/ml rhodocytin, or 1 ml/ml PMA, the conversion of dihydrodihydrofluorescein 1232 in rhodamine was assessed by flow cytometry.

Immunoprecipitations

Immunoprecipitations from RAW macrophages were performed as previously described (6), except pervanadate-stimulated RAW264.7 cell lysates were added to streptavidin beads precomplexed with cyanine phophorylated or unphosphorylated biotinylated CLEC-2 signaling peptides (25 μM, MOOCQ Tigerskin; Cancer Research U.K. Peptide Synthesis Laboratory). The dock-11 peptides have been described previously (23). For immunoprecipitations from A20 cells expressing a HA-tagged version of CLEC-2, 1 × 106 cells were preincubated with anti-HA Ab and then stimulated with pervanadate and lysed as previously described (6). Cell lysates were added to anti-rat IgG coated Dynabeads and rotated for 2 h at 4°C. Beads were washed extensively before analysis by Western blotting. Protein in the immunoprecipitations were detected with anti-phosphotyrosine (clone 4G10) or anti-Syk (Santa Cruz Biotechnology), followed by appropriate HRP-linked secondary Abs (Jackson ImmunoResearch Laboratories).

Results

Marine CLEC-2 is expressed on peripheral blood neutrophils.

To explore the expression of CLEC-2, we generated affinity purified polyclonal and a mAb to this receptor. The specificity of these
CLEC-2 is a phagocytic receptor expressed on neutrophils. Abs were obtained in all mouse strains examined (data not shown). Thus, these results suggest that expression of CLEC-2 appears to be up-regulated upon neutrophil emigration from the bone marrow into the peripheral blood, but down-regulated again following recruitment to sites of inflammation.

Regulation of CLEC-2 expression

As CLEC-2 expression was down-regulated on recruited inflammatory neutrophils (Fig. 1F), we determined whether stimulation of peripheral blood neutrophils with microbial agonists could also induce regulation of this receptor, as has been described for other NOD-like receptors (11, 12). We examined CLEC-2 expression by flow cytometry following a 6 h stimulation of PBLs with various TLR agonists, but did not observe any significant regulation of surface expression of neutrophil-expressed CLEC-2 (Fig. 1G). However, CLEC-2 expression was observed to increase on monocytes defined by FSC and SSC profiles (30), following stimulation with Pam3CSK4, a TLR2/TLR1 agonist (Fig. 1G). Thus, these results suggest CLEC-2 is not directly regulated on neutrophils following microbial stimulation, but that these conditions can induce up-regulation of the receptor on other leukocytes.

CLEC-2 mediates phagocytosis

Having identified CLEC-2 on neutrophils, we next wished to determine the function of this receptor on these cells. As CLEC-2

FIGURE 1. CLEC-2 is expressed on the surface of peripheral blood neutrophils. A, Anti-HA staining of EL4 cells, as determined by flow cytometry, showing expression of HA-tagged mCLEC-2 (filled histogram) at the cell surface. The vector-only control cells are indicated by the unfilled histogram. B, The monoclonal and affinity-purified polyclonal Ab raised against CLEC-2 specifically recognized EL4 cells transduced with HA-tagged mCLEC-2 (filled histograms), but not vector-only control cells (unfilled histogram) as determined by flow cytometry. C, mCLEC-2 is expressed on the surface of CD11b<sup>Gr-1</sup><sup>+</sup> neutrophils (filled histogram) on CD11b<sup>Gr-1</sup><sup>+</sup> peripheral blood neutrophils from BALB/c, C57Bl/6, and 129/Sv mice, as indicated. D, mCLEC-2 is only weakly expressed on CD11b<sup>Gr-1</sup><sup>+</sup> bone marrow cells and F, elicited inflammatory neutrophils. C–F, The polyclonal Ab was used for staining and isotype control staining is indicated by the unfilled histogram. The data shown are representative of at least three independent experiments. G, Regulation of mCLEC-2 expression on neutrophils and monocytes following stimulation with various TLR agonists, as detected with monoclonal anti-CLEC-2 by flow cytometry. The data show results of PBLs pooled from 18 mice.

Abs was demonstrated by staining EL4 cells transduced with HA-tagged CLEC-2 or vector-only controls (Fig. 1A). Analysis by flow cytometry demonstrated that both the polyclonal and mAbs specifically recognized CLEC-2 expressed on these cells (Fig. 1B).

Using these Abs, we first confirmed CLEC-2 expression on platelets, and could detect expression of this receptor on the surface of CD62<sup>Gr-1</sup><sup>+</sup> cells, as previously described (11) (Fig. 1C). To determine whether CLEC-2 was also expressed on other cell types, we then examined PBLs from BALB/c mice using a variety of markers to distinguish the various cellular populations (30), and could clearly detect expression of CLEC-2 on the surface of CD11b<sup>Gr-1</sup><sup>+</sup> neutrophils (Fig. 1D and data not shown). The expression of this receptor on these cells was not dependent on the mouse strain, as similar levels of expression were also detected in other strains including C57Bl6 and 129/Sv mice (Fig. 1D). We did not detect CLEC-2 on any other cell population in the blood (data not shown). Thus, these data demonstrate that expression of CLEC-2 is not restricted to platelets, and that this receptor is also expressed by peripheral blood neutrophils.

Under normal conditions, the majority of neutrophils are located in the bone marrow, and only a small fraction of these cells is released into the blood, from where they can be recruited to sites of inflammation (31). However, when we characterized CLEC-2 expression in the bone marrow or on 18-h thioglycollate elicited peritoneal neutrophils, we found that expression of this receptor was much lower on these cells (Fig. 1 E and F). Similar findings were obtained in all mouse strains examined (data not shown). Thus, these results suggest that expression of CLEC-2 appears to be up-regulated upon neutrophil emigration from the bone marrow into the peripheral blood, but down-regulated again following recruitment to sites of inflammation.
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441. We also confirmed that cellular activation results in the phosphorylation of CLEC-2, by immunoprecipitating this receptor from pervanadate stimulated or unstimulated transduced A20 cells expressing full-length CLEC-2 (Fig. 4F). In addition, as we had done for other receptors (6, 29), we expressed the chimeric receptor at comparable levels in Syk-sufficient and Syk-deficient B cell lines (data not shown), and examined the production of IL-2 in response to zymosan stimulation. Although addition of zymosan to the Syk-sufficient cells expressing the chimera induced the production of IL-2, the Syk-deficient cells did not show any response to this particle (Fig. 4C). Finally, we also examined RAW264.7 macrophages expressing the chimeric receptor by confocal microscopy, following a short-2 min exposure to zymosan, and could clearly detect the activation of Syk around the phagosome, as measured by staining for phospho-Syk (Fig. 4D). Similar activation of Syk was absent in the cells expressing the Y7F mutant chimeric receptor (data not shown). Thus, these data demonstrate that CLEC-2 signals via Syk kinase in myeloid cells.

CLEC-2 does not induce the respiratory burst

Signaling via dec1-1 has been shown to activate the respiratory burst in macrophages in a Syk-dependent manner (39). The respiratory burst is an important antimicrobial mechanism in neutrophils, and as CLEC-2 signals via Syk, we wondered whether this receptor was also mediating this activity. We examined this response in the RAW264.7 macrophages transduced with the various chimeric receptors following stimulation with zymosan. Somewhat surprisingly however, macrophages expressing the chimeric receptors failed to induce a respiratory burst in response to these particles (Fig. 5A). In contrast, macrophages expressing dec1-1 induced a robust respiratory burst, as expected (39). Furthermore, this response was also absent in peripheral blood neutrophils stimulated with rhodocytin (Fig. 5B). Thus, these data show that despite signaling via Syk kinase, CLEC-2 does not induce the respiratory burst.

Discussion

The study of the dec1-1 cluster of NK-like C-type lectin receptors has provided important insights into mechanisms underlying homeostasis and immunity (4, 7). Arguably one of the most important discoveries has been the identification of receptors containing cytoplasmic ITAM-like motifs which can trigger cellular activation. These motifs possess only a single tyrosine, yet are able to recruit and signal via Syk kinase through a process which is not yet fully understood (29, 40). One receptor possessing this motif is CLEC-2, a molecule previously thought to be exclusively expressed on platelets and capable of triggering the activation of these cells (11). In this study, we show that CLEC-2 is also expressed on murine peritoneal blood neutrophils. As in platelets (11, 15), we show that the cellular functions of CLEC-2 are mediated through its ITAM-like motif and that the receptor can induce intracellular signaling via Syk kinase.

The ITAM-like motif shows a striking similarity to that of dec1-1, suggesting that CLEC-2 may possess many of the functions of dec1-1 (15, 41). Indeed, we have shown that CLEC-2 can trigger phagocytosis and the induction of TNF-α, and it is possible that the receptor may also be able to induce the production of a number of other cytokines and chemokines in neutrophils (38). Furthermore, CLEC-2 mediated cytokine production may be amplified by costimulation through the TLR pathway, as has been shown for dec1-1 (36, 37, 41, 42). Thus, like dec1-1 (29, 41) and CLEC9A (6), CLEC-2 functions as an activation receptor on myeloid cells.

The ability of CLEC-2 to mediate phagocytosis is likely to involve the highly charged cytoplasmic tricladic cluster (DED), in addition to the ITAM-like motif. This cluster is conserved in dec1-1, but not CLEC9A, and mutation of these residues in dec1-1 has been shown to abolish particle uptake (39). Furthermore, although possessing an ITAM-like motif, CLEC9A does not mediate phagocytosis (6). However, it is still unknown if CLEC-2-mediated uptake requires Syk in neutrophils. For dec1-1, the requirement for Syk is cell-type specific; in dendritic cells dec1-1-mediated uptake involves Syk, but in macrophages this process is independent of Syk and occurs through uncharacterised, and possibly novel, pathways (26, 29). In neutrophils, phagocytosis mediated by the unrelated activation receptor, CEACAM3, which possesses a traditional ITAM motif, was shown to involve Syk, but the requirement for this kinase was shown to be dependent on the nature of the ligand (43). Although we have clearly demonstrated that CLEC-2 has the potential to mediate phagocytosis, the physiological relevance of this activity remains to be determined.

One of the most surprising discoveries was the inability of CLEC-2 to induce the respiratory burst, despite signaling via Syk kinase. The activation of Syk by the FcyR, dec1-1, and CEACAM3 has been shown to induce the respiratory burst in a variety of cell types, including neutrophils (35, 39, 43, 44). In fact, this response is completely absent following stimulation of these receptors in Syk-deficient cells (39, 44). In macrophages stimulated with zymosan, however, the activation of Syk by dec1-1 and the subsequent respiratory burst occurred only in a subset of cells, yet the assembly of the NADPH oxidase on the zymosan phagosomes occurred in all cells (39). Taken together, these data therefore suggest that there is another component/pathway stimulated by these receptors, in addition to Syk, that is required for the induction of the respiratory burst.

Our analysis of CLEC-2 expression indicates that high levels of expression of the receptor occurs only on circulating neutrophils, and that the receptor is only weakly expressed on bone marrow or elicited inflammatory cells. This suggests that its expression is tightly regulated and implies a specific function for the receptor on circulating cells. The expression of CLEC-2 on neutrophils, however, may only occur in mice, as we did not detect expression on human peripheral polymorphonuclear leukocytes using commercially available Abs (data not shown). Although not analyzed in detail, we also observed that expression of CLEC-2 could be up-regulated on murine monocytes following stimulation with Pam3CSK4 but not other TLR agonists tested, suggesting some specificity in this response. It is possible that CLEC-2 may be similarly regulated on human polymorphonuclear leukocytes or other leukocytes, which were not examined in this study. Why the expression of this receptor appears to be primarily restricted to circulating cells is unclear, and despite the identification of both endogenous and exogenous ligands, the physiological role of CLEC-2 is still unknown.

Like other receptors in the dec1-1 cluster (4), CLEC-2 may function as a pattern recognition receptor. The expression of this receptor on neutrophils certainly implies a role in innate immunity, as these short-lived cells provide a first-line of defense against infection and are essentially required for the control of bacterial and fungal infections (38). A role in immunity is also suggested by the ability of specific TLR agonists to induce CLEC-2 expression on monocytes, and the phagocytic capacity of this receptor may be important for the clearance of blood-borne pathogens, although such interactions have yet to be documented. However, other than rhodocytin, only HIV has been identified to possess an exogenous CLEC-2 ligand and...
CLEC-2 is a PHAGOCYTIC RECEPTOR EXPRESSED ON NEUTROPHILS

rather than being protective, the interaction with CLEC-2 may promote transfer of infectious HIV-1 particles (17).

The primary function of CLEC-2, however, may be in the regu-

lation of homostasis through the recognition of endogenous lig-

ands. One endogenous ligand that has been identified is podo-

plastin, a mucin-like protein that is expressed on a variety of cell types

including osteoclasts, keratinocytes, fibroblasts, airway epithelia,

renal tubular epithelial cells, lymphatic endothelial cells, and cer-

tain tumor cells, although podoplanin is not expressed on blood

ed vessels endothelium (145). Thus, under normal circumstances, cir-

culating neutrophils and platelets would not come into contact with

this ligand. However, the interaction of CLEC-2 with podoplanin

following tumor invasion has been proposed to promote platelet

activation and aggregation, which may be associated with tumor

metastasis (11, 13). Metastasis is also promoted by inflammation,

induced in part by neutrophils (46, 47), and it is tempting to specu-

late that the interaction of podoplanin with neutrophil-expressed

CLEC-2 may contribute to this process.

Finally, the simultaneous recognition of CLEC-2 ligands by

both platelets and neutrophils may contribute to the interactions

between these two cell types and to the activation and cross-talk of

the inflammatory and coagulation pathways. These interactions

are known to be important for the control of infection, and for limit-

ing inflammatory pathology, but are also involved in the develop-

ment of disease (124, 491). We are currently determining whether CLEC-2

is involved in these processes, and the possibility that this receptor

recognizes other endogenous and exogenous ligands.

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References


Syk-coupled C-type lectin receptors that mediate cellular activation via single tyrosine based activation motifs

Summary. Different dendritic cell (DC) subsets have distinct specialized functions contributed in part by their differential expression of pattern recognition receptors (PRR). C-type lectin receptors (CLRs) are a group of PRRs expressed by DCs and other myeloid cells that are able to recognize endogenous motifs as well as a wide range of exogenous structures present on pathogens. Dual roles in homeostasis and immunity have been demonstrated for some members of this receptor family. Largely due to their endocytic ability and subset specific expression, DC-expressed CLRs have been the focus of significant antigen targeting studies. A number of CLRs function on the basis of signaling via association with immune-complex transmembrane-based activation motif (ITAM)-containing adaptors. Other contain ITAM-related motifs or immune-complex transmembrane-based inhibitory motifs (ITIMs) in their cytoplasmic tails. Here we review CLRs that induce intracellular signaling via a single tyrosine-based ITAM-like motif and highlight their relevance in terms of DC function.

Keywords: pattern recognition receptors, C-type lectins, myeloid cells, Syk, immune-complex transmembrane-based activation motif, DCs

Introduction

Dendritic cells (DCs) are a heterogeneous family of leukocytes that are centrally involved in the translation of innate into adaptive immunity. Their functional diversity is dependent on various DC subtypes which differ in location, developmental history, and specialized function. In general, DCs function primarily to capture, process, and present antigens to T cells. The particular DC type and its activation state determine whether antigen presentation to naive T cells results in immune suppression or immune activation (1). For example, self-antigen presented by DCs in the absence of other signals usually induces tolerance and consequently limits autoreactivity. Conversely, in certain contexts such as pathogen invasion or massive cell death, simultaneous signals stimulate DC maturation, activation, and migration to draining lymph nodes where they present antigens to naive T cells and induce antigen-specific immune responses (1–3).
DCs express a large repertoire of cell surface receptors which help them to perform their various functions. In particular, pattern recognition receptors (PRRs) are fundamental to DC biology. According to Janeway's original hypothesis, PRRs evolved to recognize microbial ligands and functioned to distinguish infectious non-self from non-infectious self with the resultant initiation of adaptive immunity (4, 5). For a long time this theory seemed to hold true, and reports emerged that various PRRs, such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-oligomerization domain (Nod) like receptors (NLRs), and retinoic acid-inducible gene I (RIG-I) like receptors (RLRs), did recognize pathogen-associated molecular patterns (PAMPs) and could induce immune responses. TLRs were the first class of cellular PRRs described and are present at the cell surface or within endosomes. They recognize specific microbial components and play a crucial role in immunity that has been reviewed extensively elsewhere (6, 7). CLRs comprise both soluble and transmembrane receptors and can recognize a wide range of carbohydrate structures on pathogens. NLRs and RLRs are intracellular cytosolic receptors involved primarily in the detection of bacteria and viruses respectively (8–10). As our understanding of these receptors has deepened, it has become necessary to reconsider Janeway's original theory. For instance, it is now known that the bacterial ligands recognized by TLRs are not unique to pathogens but are also present on commensal microorganisms. For example, studies have revealed that TLR signaling induced by commensal bacteria has an essential role in maintaining epithelial homeostasis, and protection from direct epithelial injury (11–17). Furthermore, growing evidence suggests that PRRs respond to endogenous ligands which are released from, or exposed on the surface of dying cells. For example the CLRs, Mincle and CLEC9A, were both shown to recognize ligands on necrotic cells with the resultant activation of adaptive immunity (18, 19). It has also been demonstrated that TLRs can respond to endogenous ligands which are released during inflammatory responses and tissue damage. One example is the induction of signaling and DC activation via TLR2 and/or TLR4 recognition of hyaluronan (10–12). Finally, it is becoming increasingly apparent that the recognition of endogenous ligands by certain PRRs contributes to immune homeostasis. This is particularly apparent in the case of CLRs (23). A select example is DC-SIGN, which in addition to recognizing several pathogens, also binds endogenous proteins and in this way mediates adhesion between DCs and endothelial cells during DC migration, and between DCs and T cells during antigen presentation (24). We have learned a tremendous amount about the immune system in the 20 years since Janeway proposed the idea of PRRs and it has become clear that his hypothesis does not encompass all scenarios. However, as Ruslan Medzhitov recently put it, 'no theory is ever complete and (the) pattern recognition concept will continue to evolve and eventually integrate with other concepts into a more general theory' (25).

C-type lectin receptors

The receptors on which we will focus in this review are members of the C-type lectin superfamily. The term 'C-type lectin' was originally used to describe a group of Ca²⁺-dependent carbohydrate binding proteins containing a conserved carbohydrate recognition domain (26, 27). Other proteins containing the same domain were subsequently identified, but carbohydrate ligands have not been identified for many of these proteins. To clarify this ambiguity, the term C-type lectin-like domain (CTLD) was introduced to refer to such domains (28, 29). The CTLD is a structural motif which was originally identified as a protein fold in the carbohydrate recognition domain of mannose-binding lectin (30). The C-type lectin superfamily is now defined as a large group of proteins that are characterized by the presence of one or more CTLDs. Both soluble and transmembrane CLRs exist and the superfamily is divided into 17 groups based on their phylogeny and domain organization (31).

Despite the presence of a highly conserved domain, CLRs are functionally diverse and have been implicated in various processes including cell adhesion, tissue integration and remodeling, platelet activation, complement activation, pathogen recognition, endocytosis, and phagocytosis (32–36). A prominent role for CLRs in immunity was originally identified in natural killer (NK) cells, where they were implicated in controlling NK function in response to transformed and virally infected cells (37). The functions of NK cell were found to be controlled by paired CLRs which delivered activation and inhibitory signals, the balance of which controlled NK cell function (38). The identification of CLRs on myeloid cells raised the possibility that they may serve similar functions in controlling cellular activation in these cells. However, CLR function on myeloid cells now appears to be much more complex and these receptors have been implicated in both immunity and homeostasis. This duality in function is based on the capacity of many CLRs to recognize both endogenous and exogenous ligands. It raises interesting questions about the evolution of such receptors and Stanton Gordon, among others, has speculated that the further identification of endogenous ligands and an increasing appreciation of homeostatic requirements will result in the rationalization of the innate
immune system as 'primarily a self-regulating, physiologic system, with defense as a secondary, albeit vital, by-product' (39).

Here we focus on CLRs that are expressed on DCs. For example, DEC-205 is expressed on a number of subsets: at high levels on thymic medullary DCs (CD11c' CD8'), and subsets of peripheral DCs (CD11c' CD8' splenic/lymph node DCs, dermal/intestinal DCs, and Langerhans cells (40, 41). DEC-205, and several other C-type lectins such as Decin-1, MCL, and the MB to name a few, are also expressed on other myeloid cell types apart from DCs (35). However, some CLRs are more restrictively expressed and are considered markers for particular DC subsets. For example, BDCA-2 is widely accepted as a specific marker for human plasmacytoid DCs (pDCs) (42). CLEC9A is selectively expressed by the CD8α' DC subset in mice and in the BDCA3' putative human equivalent DCs (43–45). Langerin, which was initially believed to be unique to Langerhans cells, is now known to be expressed by a population of dermal DCs (46–49).

**Signaling via CLRs**

**ITAM signaling**

In several instances, CLR function has been linked to the presence of a signaling motif in its cytoplasmic tail or to an ability to couple with signaling chains. For example, Mincle, Decin-1, BDCA-2 and DCAR, which are members of the ‘Decin-1 family’ of CLRs (50), have all been shown to associate with Fc receptor y chain (FcRy), an immunoreceptor tyrosine-based activation motif (ITAM) containing receptor (18, 51–54). An ITAM consists of a short amino acid sequence containing a duplicate of the sequence YxxL/I with six to twelve intervening residues [YxxL/I-(6-12) YxxL/I, where Y is tyrosine, L is leucine, I is isoleucine and x is any residue] (55–57). The conserved tyrosines and leucine/isoleucine residues, and the spacing between them are critical for the biological function of an ITAM (58). Immunoreceptors which activate intracellular signaling through ITAMs include T-cell and B-cell antigen receptors, and Fc receptors, as well as the myeloid receptors mentioned above. Signaling via ITAMs is mediated by ligand binding which induces receptor clustering and phosphorylation of the ITAM tyrosines by Src family kinases. This phosphorylation promotes recruitment of members of the Syk family of protein tyrosine kinases (ZAP-70 or Syk), which initiates a series of downstream signaling events (56, 57).

ITAM signaling often results in the activation of cellular responses, including cell migration and adhesion (59), proliferation and differentiation (60, 61), and phagocytosis and degradation (62). This activation may be kept in check by immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptors (56, 57). An ITIM is a cytoplasmic sequence consisting of the amino acid residues L/V/L/XxxL/V, where L is leucine, V is valine, I is isoleucine, and x is any residue (63, 64). Generally, ITIM containing receptors are tyrosine phosphorylated by Src kinases in response to immunoreceptor engagement. This results in recruitment and activation of protein tyrosine phosphatases such as SHP-1, which inhibit cellular activation by dephosphorylation of various immunoreceptor-regulated substrates (63, 64).

The concept of paired receptors, one activating and the other inhibitory, working in collaboration to ensure controlled cellular responses was prevalent for a long time and seems a plausible model of regulation (65). This model certainly appears to hold true when one considers the tight regulation of NK cell function (38). However, in recent years a number of examples have emerged which have questioned this paradigm (56, 57, 66). For instance, it has been shown that ITAM signaling under certain conditions can induce negative responses. One example is TREM-2 (triggering receptor expressed on myeloid cells 2) which associates with the ITAM containing adapter DAP12 (DAX Activating Protein of 12 kDa). TREM-2/DAP12 signaling was shown to trigger nitric oxide production by macrophages (67), cytokine production, antigen uptake and maturation in DCs (68, 69), and differentiation of osteoclasts (70). However, it was also shown that in certain contexts, TREM-2/DAP12 signaling can negatively regulate TLR and FcR signaling in macrophages and inhibit inflammatory cytokine production (71, 72). There are also examples of ITAM-containing receptors that function to activate cellular responses. For instance, TREM-like transcript-1 (TLT-1) was shown to enhance FcR-mediated calcium signaling, an outcome which was dependent on its association with SHP-2 (73). To add further complexity to ITAM/ITIM signaling models, ITAM-like motifs which vary from the traditional consensus sequence have now been identified in a number of receptors.

**ITAM-like signaling**

Decin-1 exemplifies an ITAM-like motif containing receptor. It contains two tyrosines in its cytoplasmic tail that are positioned in an arrangement similar to the traditional ITAM but not conforming exactly to the consensus sequence. The two tyrosines are appropriately spaced, but the N-terminal tyrosine resides in a YXXL sequence (YxxL for mouse), as opposed

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as the lung, gut, and spleen (133). One investigation concluded that Dectin-1 was expressed exclusively on CD11c+ DCs (112), but a number of other reports have demonstrated its presence on both CD11c+ and CD11c- (77, 133–135). Furthermore, Dectin-1 was identified on skin-derived migratory DCs in lymph nodes draining the skin (48, 112, 135). Although not specifically examined in DCs, it has been shown that the expression of Dectin-1 can be influenced by various cytokines and microtubal factors (130, 133, 136, 137). For example, IL-4, IL-13, and GM-CSF caused Dectin-1 expression to be highly upregulated whereas IL-10, IPS, and dexamethasone caused downregulation of Dectin-1 expression (136).

Signaling from Dectin-1 following ligand binding can induce many cellular responses including the respiratory burst, DC maturation, ligand uptake through phagocytosis and endocytosis, the production of arachidonic metabolites and various cytokines and chemokines such as TNF, CXC12, IL-23, IL-6, IL-2, and IL-10 (138). In recent years, there have been several studies which have sought to decipher the Dectin-1 induced signaling pathways that underlie various cellular responses. Before discussing the findings below, it is worth noting that as evidence of cross-talk and branching in various systems is emerging, the traditional visualization of a linear signaling pathway is giving way to the realization that these pathways are in fact much more intricate (139). This holds true for Dectin-1 induced signaling, which in some cases can trigger a response directly but in other cases may drive cooperative signaling with MyD88-coupled TLRs. A further layer of complexity was revealed by recent evidence which shows variability in Dectin-1 signaling in different myeloid cell types (140, 141).

Signaling initiated via Dectin-1 results in activation of NF-kB through both the classical and alternative pathways (142, 143). The classical or canonical pathway is mediated by activation of an IκB (inhibitor of κB kinase) complex which mediates the phosphorylation of IκBα resulting in their ubiquitylation and subsequent degradation. The released NF-kB dimers translocate to the nucleus and activate gene transcription. In DCs, this pathway is initiated by ligation of Dectin-1 which triggers Syk that further activates phospholipase-Cycl (PLCy2), leading to the engagement of the capase recruitment domain (CARD) containing protein, CARD9, which together with Bcl10 and Malt1 signal for activation of the transcription factor NF-kB (142, 144, 145). The signaling events that link PLCy2 activation to CARD9 recruitment are not yet known. However in lymphocytes, signaling through ITAM-containing proteins leads to recruitment of Syk tyrosine kinases that activate PLCy, which in turn catalyzes the hydrolysis of membrane phospholipids generating diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3) (146). DAG activates PKC isoforms which then activate the CARD9 related protein, CARMA1, allowing the formation of a signaling complex consisting of CARMA1, Bcl10, and Malt1 (146). It is likely that a pathway analogous to this one culminating in CARMA1 activation in lymphocytes results in CARD9 activation in myeloid cells (144). How signals are transmitted from the CARD9/Bcl10/Malt1 complex to NF-kB is also not yet described. However, if one looks again to defined pathways in other cells, it seems reasonable to consider that the direct interaction of Malt1 with the adapters Tnfb and Taf6 may lead to activation of IKK, which in lymphocytes results in translocation of NF-kB to the nucleus where it influences gene transcription (146, 147).

It has recently come to light that Dectin-1 signaling via the CARD9 pathway described above is differentially regulated in myeloid cells (140, 141). For example, bone marrow derived DCs, resident peritoneal cells and alveolar macrophages activate NF-kB and drive TNFα production as described above, whereas bone marrow derived macrophages, thiglycolate elicited macrophages and Fl5L-derived DCs fail to do so. The failure to drive proinflammatory cytokine production is in spite of Dectin-1 induced activation of the Card9/Bcl10/Malt1 signaling complex (140). This variability in Dectin-1 signaling in different cell populations is potentially a novel mechanism by which myeloid cells can be tweaked to regulate immune responses and may have implications for therapeutic uses (140, 141).

In addition to activating NF-kB via the classical pathway, Dectin-1 is also the first PRR shown to induce Syk-dependent activation of NF-kB in DCs via the alternative pathway (143). In the alternative or non-canonical pathway, RelB-p52 dimers translocate to the nucleus following the successive activation of NF-kB-inducing kinase (NIK) and IκB kinase-α (IKKα) (148). Previously, alternative activation of NF-kB had been reported for only a few members of the TNF receptor superfamily which do so by a Syk-independent mechanism (149, 150). The intricacies of the pathway which relays signals from Syk to the activation of RelB-p52 dimers remain undefined at present.

Apart from activation of NF-kB in DCs, ligation of Dectin-1 also triggers activation of the transcription factor NFAT in macrophages and DCs (145, 151). The role of NFAT and the sequence of events leading to its activation are most well defined in T cells. The signaling pathway induced by ITAM containing T-cell receptors in lymphocytes (discussed above) branches after the generation of DAG and IP3. As described,
DAG initiates a cascade of events resulting in the activation of NF-κB. The action of D1 initiates another signaling pathway by triggering the release of calcium ions (Ca^{2+}) from intracellular stores which, in turn triggers the opening of Ca^{2+} channels in the plasma membrane resulting in the maintenance of the increased levels of intracellular Ca^{2+}. This results in the activation of the phosphatase calcineurin which dephosphorylates NFAT transcription factors, leading to their nuclear translocation and regulation of gene expression (152, 153). Although the signaling events linking Dectin-1 signaling with NFAT activation have yet to be elucidated, it is possible that NFAT activation in myeloid cells occurs via a pathway analogous to the one described for T cells. This is the most likely since zymosan has been reported to trigger calcium flux and Dectin-1 is known to signal via Syk in macrophages and DCs (145, 154, 155). Dectin-1-mediated activation of NFAT regulates cyclooxygenase-2 and prostaglandin production in macrophages, and the induction of IL-1, IL-10, and IL-23 in DCs (156).

It has also been shown that certain cellular responses controlled by Dectin-1/Syk signaling are not dependent on CARD9. For example, in DCs there is Syk-dependent activation of ERK, a mitogen-activated protein kinase (MAPK), through a CARD9-independent pathway (156). Another example of this type of signaling is the induction of phagocytosis via Dectin-1 in DCs. Although this pathway is not fully understood, it is known that it is Syk dependent but does not require CARD9 (77, 142). In macrophages, however, Dectin-1-mediated phagocytosis is induced in a Syk-independent manner (see below). An artificial system, which demonstrated the phagocytic capacity of Dectin-1 by expressing the receptor in normally non-phagocytic fibroblasts, showed that Dectin-1 signaling caused activation of Cdk42 and Rac-1 triggering actin polymerisation and pseudopod extension around the particle (76). It also showed that protein kinase C (PKC) was required. The precise role of PKC in phagocytosis is unclear; however, one of its substrates, MARCKS (myristoylated alanine-rich C-kinase substrate), possesses actin cross-linking activity that is involved in zymosan phagocytosis (157).

Dectin-1 is also capable of inducing signaling via Syk-independent pathways. In macrophages, ligand of Dectin-1 results in phagocytosis, and this response requires the ITAM-like motif and a triadric DEF sequence but does not require Syk and is possibly propagated by a novel kinase (74, 76, 77). This is in contrast to the Syk requirement for Dectin-1-mediated phagocytosis by DCs, discussed above. Recently, it was found that Dectin-1 induced a signaling pathway through the kinase Raf1 that was independent of the Syk pathway but integrated with it at the level of NF-κB activation for regulation of cytokine production (143).

Interactions between Dectin-1 and other receptors

Continuing research in the field of pathogen recognition and the receptors involved in mediating immune responses has highlighted that receptor collaboration as opposed to receptor functioning in isolation is an important facet of controlling infection (158, 159). This holds true for Dectin-1, which collaborates with various TLRs to promote anti-fungal and anti-mycobacterial responses. In addition to β-glucans, there are a number of PAMPs in the cell walls of fungi that are recognized by several PRRs, whose cooperation is required for efficient anti-fungal immune responses (160). Dectin-1 has been shown to collaborate with TLRs for optimal cytokine production and enhancement of the respiratory burst. This collaboration depends on cell type. For example, in macrophages, collaborative signaling by TLRs and Dectin-1 is required for TLR5 production (75, 79). In DCs, Dectin-1 signaling alone can trigger TLRs and IL-10 production, although collaborative signaling with TLRs enhances these responses (158, 161). In macrophages, it was also shown that the Syk kinase pathway triggered by Dectin-1 is required for collaborative signaling with TLRs, which induced sustained iNOS degradation, resulting in enhanced nuclear translocation of NF-κB (158). In fact, Dectin-1 can interact with several MyD88-coupled TLRs (TLR2, TLR4, TLR5, TLR7, TLR9) to induce production of multiple cytokines (138, 158, 162). In addition to Syk, integration of TLR and Dectin-1 signaling requires the adapter Raf-1 (143). Functional cooperation between Dectin-1 and TLR7 has also been established during phagocytosis, ERK1/2 MAPK activation and the secretion of pro-inflammatory cytokines by macrophages challenged with mycobacteria (121–123). Indeed, a study which examined how many different myeloid derived macrophages challenged with Mycobacterium abscessus or zymosan showed that Dectin-1 physically interacts and co-localises with TLR2 (121).

Apart from the TLRs, Dectin-1 also cooperates with a number of other plasma membrane proteins. In human DCs, co-stimulation of Dectin-1 and DC-SIGN triggers the arachidonic acid cascade (163). In macrophages Dectin-1 also cooperates with SIGNR1, the murine homologue of DC-SIGN, during homocytotopic recognition of yeast (164). In addition to pattern recognition receptors, Dectin-1 can interact with tetraspans, a family of proteins that have been shown to modulate signal transduction by interacting with many other transmembrane proteins to form 'tetraspan microdomains' in the plasma
membrane (165). Dectin-1 associates with the ubiquitous tetraspanin CD63. Furthermore, it was shown that phagocytosis of yeast particles by DCs was accompanied by a decrease in CD63 expression, which was inhibitable by the soluble β-glucan, laminarin (166). Although the functional significance of a Dectin-1-CD63 interaction has not yet been elucidated, it may represent part of a signaling complex that could influence phagocytosis. Dectin-1 also interacts with the immune cell specific tetraspanin CD37 resulting in stabilization of Dectin-1 in the cell membrane and inhibition of Dectin-1 mediated IL-6 production in response to zymosan (167, 168). Whether the tetraspanin-Dectin-1 interactions are involved with Dectin-1 collaboration with TLRs is not known, although there has been speculation that tetraspanins may directly link the Dectin-1 and TLR signaling pathways (167).

Dectin-1 and innate immunity

β-glucans can constitute up to 50% of fungal cell walls and as mentioned, Dectin-1 recognizes a number of fungal species by way of its β-glucan specificity including Candida, Pneumocystis, Saccharomyces, Aspergillus, Cryptococcus and Penicillium (79, 169-176). Many studies have shown that detection of various fungal species by Dectin-1 results in the initiation of protective immune responses such as fungal uptake and killing by timely reactive oxygen intermediates, and the production of protective inflammatory cytokines and chemokines, including TNFa, CXCL2, IL-1β, IL-1α, CCL3, GM-CSF, G-CSF, and IL-8 (74, 177). Dectin-1 also induces the production of IL-2 and IL-10, cytokines whose role in fungal infection is less clear. IL-10 in particular has been shown to have inhibitory effects on anti-fungal immune responses (178). These cytokines also contribute to the development of regulatory T cells (Tregs), the implications of which are discussed later.

In vivo studies, although not wholly consistent, have supported other evidence that Dectin-1 plays a role in antifungal immunity. Loss of Dectin-1 in mice with a 129Sv background resulted in increased susceptibility to systemic infection with C. albicans and invasive pulmonary infection with A. fumigatus as a consequence of inflammatory defects and decreased fungal killing (173, 179). Interestingly, loss of Dectin-1 in mice with a C57BL/6 background showed no increased susceptibility to C. albicans but did exhibit increased susceptibility to Pneumocystis carinii due to defects in the respiratory burst (170). This disparity is most likely because of the genetic backgrounds of the deficient mice, but could also be due to differences in the fungal strains and/or routes of infection used. Further evidence to support a role for Dectin-1 in fungal infections is the recent identification of a polymorphism in human Dectin-1 that is associated with an increased oral and gastrointestinal colonization with Candida species (180).

Dectin-1 and adaptive immunity

In addition to triggering innate immune responses, Dectin-1 signaling can also induce adaptive immunity and was the first non-TLR PRR shown to do so. Dectin-1 activated dendritic cells can 'instruct' the differentiation of T helper 17 (Th17) and T helper 1 (Th1) CD4+ T cells independently of TLR signaling in response to Calichrome and Mycobacterium tuberculosis (161, 181). It has been shown that Rafl signaling by Dectin-1 is a crucial determinant in the induction and regulation of the relevant polarizing cytokines (143, 161). While it is generally accepted that the generation of a Th1-cell response is required for protection against fungal infection in healthy hosts (182), the role of Th17 cells in anti-fungal immunity is rather more controversial. A number of studies have shown a detrimental role for IL-17 in anti-fungal immunity (183, 184). In contrast, mice that are unable to mount Th17 responses are very susceptible to opharyngeal candidiasis (185, 186). Furthermore, Th17 deficiency in humans is also associated with increased susceptibility to C. albicans infection (187, 188). Conditions such as route of infection and inocula used may account for the observed differences; however, IL-17 like other cytokines can have opposing roles in disease (189). For example, in periodontal disease IL-17 may be either host-protective or destructive, depending on how chronic and severe the disease is (190, 191). It has also been shown that vaccination-induced IL-17-producing T cells are critical for the rapid recruitment of protective CD4+ T cells upon challenge with L. tuberculosis (192). This finding suggests that the Th17 responses induced by targeting Dectin-1 may be a means of enhancing the efficacy of future vaccines to this pathogen.

Dectin-2 was recently identified as another Syk/CARD9-coupled CLR that mediates DC activation and induction of Th17 immunity in response to C. albicans (193). Dectin-2 has a positively charged residue in its transmembrane domain that mediates association with the ITAM-containing FcβR chain and allows coupling to the Syk pathway (193). Despite this distinct difference in Syk recruitment, Dectin-2 and Dectin-1 may be representative of a novel class of myeloid expressed receptors which signal via Syk and CARD9 to initiate immunity, as the authors pointed out (193).

Activation of DCs via Dectin-1 can also convert selected populations of Treg cells into IL-17-producing T cells that cannot be classified as either Threg or Th17 cells (194). Furthermore, costimulation of Dectin-1 and TLR2 contributes
in Treg expansion and function (195, 196). Tregs suppress
T effector cells and have protective effects in certain infections
by means of their anti-inflammatory function (197). They
have also been shown to be essential components of the mem-
ory-protective immune system in certain pathogens (198, 199). On
the other hand, Tregs were demonstrated as immunosuppres-
sive during disseminated candidiasis (200). These adaptive
immune responses mediated via Dectin-1 remain somewhat
unclear in terms of their contribution to host protection or
immunopathology, however, it may turn out that Dectin-1
has a central role in balancing the pro-inflammatory and anti-
-inflammatory responses during fungal infection (138).

In addition to driving CD4+ T-cell differentiation, Dectin-1
stimulation can also induce CD8+ T-cell responses. DCs which
were stimulated with curdlan, a selective β-glucan agonist,
were found to promote the expansion and differentiation
of cytotoxic T lymphocyte (CTL) precursors in vitro (135). The
precise role of CTLs in direct antifungal response is unclear;
however, it has been shown that CD8+ T cells are activated
during fungal infections and can play a protective role in some
cases (201-204). Curdlan was also found to act as a potent
adjuvant for CTL co-stimulating in vivo, eliciting a cytotoxic
response that was sufficient to protect against tumor challenge
(135). This may explain the antitumor activity of β-glucans,
which are used in cancer therapy in some countries (205),
and this study suggests that Dectin-1 agonists may be benefi-
cial as immunotherapeutic agents. Antibody-mediated target-
ing of Dectin-1 also resulted in the induction of CTL
response as well as CD4+ T-cell and antibody responses
(112). This study demonstrated that targeting to different DC
subsets using receptors can induce qualitatively different
immune responses, with anti-Dectin-1 preferentially triggering
a CD4+ T-cell response but anti-DC-105 favoring induction
of a CD8+ T-cell response (112).

Dectin-1 and autoimmunity
Dectin-1-mediated responses have also been implicated in
driving autoimmunity. SKG mice, which are genetically sus-
ceptible to develop autoimmune arthritis, did not develop
arthritis symptoms in a pathogen-free environment.
However, treatment with synovial or purified β-glucans induced
severe chronic arthritis in these animals which could be inhib-
ited by blocking Dectin-1 (206). This may be as a result of
dysregulated generation of IL-17, a cytokine which has been
shown to be involved in the pathogenesis of various autoim-
une diseases in mice (207). Furthermore, blockade of Dectin-
1 could prevent experimental autoimmune uveitis, a Th1/Th17
disease and it has been suggested that Dectin-1 will
be implicated in various other autoimmune diseases in future
(138).

Dectin-1 significance in terms of DC biology
The expression of Dectin-1 on DC subsets allows for the rec-
ognition of fungal and mycobacterial species and the subse-
quent induction of all branches of adaptive immunity.
Dectin-1 was the first Syk-coupled non-TLR PRR shown to
drive such responses, and it may be the prototype of a new
d class of receptor. Furthermore, Dectin-1 was the first example
of a PRR that induces intracellular signaling via a single cyto-
osome-based ITAM-like motif. Further research into the role
of Th17 and Treg responses in fungal and mycobacterial infec-
tion is required before we will fully understand the role
of Dectin-1 in these immune responses, but studies suggest
that Dectin-1 is potentially an immunotherapeutic target.

CLEC9A

CLEC9A is a type II transmembrane protein with a single
CTLD, a transmembrane region and a cytoplasmic tail contain-
ing an ITAM-like motif similar to that of Dectin-1 (44, 45). It
has also been described as DNGR-1 (DC, NK lectin group
receptor-1) (44, 45). In mice, alternative splicing generates at
least five isoforms of CLEC9A. Similar to related CDRyls one of
these isoforms contains six exons, and this form is expres-
sed as a non-glycosylated monomer at the cell surface (44).
Conversely, a seven exon version which contains an additional
exon in the CTLD is expressed as a dimer at the cell surface
(43, 45). The various murine isoforms may display different
physiological functions and cellular distribution, although this
has not yet been examined. Investigation of cellular distribu-
tion has only been carried out for the seven exon version of
murine CLEC9A. It was found to be expressed at low levels on
plasmacytoid DCs and on CD8α+ DCs in the spleen, lymph
nodes, and thymus (43, 45). The CD8α2+ DCs are specialized
for and are by far the most efficient at presenting exogenous
peptides on MHC class I molecules, a process known as cross-
presentation (111, 113, 208, 209).

In humans, CLEC9A (hCLEC9A) is present as a glycylated
dimer at the cell surface and RT-PCR analysis showed that it is
broadly expressed as a single transcript in most organs, with
highest expression in brain, thymus and spleen (44). In
peripheral blood, hCLEC9A expression is restricted to BDCA3+
DCs, a small subset of CD14+ CD16+ monocytes, an unidenti-
fied population of CD14+ CD11b+ CD64+ cells, and B cells
(43-45). Little is understood about the BDCA3+ population,
although they are known to express multiple TLRs and are

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thought to be immature precursors of interstitial DCs (210–212). These cells also express high levels of CD8a and it has been suggested that this may mediate the migration of BDCa37 DCs from the blood into lymphoid tissues (212). Based on the expression of CLEC9A and several other surface markers, it is believed that BDCa37 DCs may represent a DC lineage equivalent to the murine CD8α− DC subset (43–45, 92, 213). CD14−CD16− monocytes are considered ‘classical monocytes’ and are believed to migrate to sites of inflammation where DC differentiation occurs (214, 215).

As mentioned, CLEC9A contains an ITAM-like motif in its cytoplasmic tail, YXXL, and it induces intracellular signaling via recruitment of Syk (44, 45). It is possible that Syk associates with CLEC9A in a similar way to that which has been proposed for Dectin-1; via a bridging interaction between two CLEC9A molecules (77, 80) (Fig. 1). Specific ligands for CLEC9A have yet to be identified. However, it was recently demonstrated that CLEC9A acts as a receptor for necrotic cells and it is thought that this is mediated by recognition of a ubiquitous preformed acid-labile ligand(s) that is normally sequestered in healthy cells but becomes exposed following disruption of membrane integrity during necrosis (19). CLEC9A also regulates the cross-presentation of dead cell-associated antigens in a Syk-dependent manner (19). This investigation found that although CLEC9A is not required for uptake of necrotic cell material, it is necessary for efficient cross-presentation of dead-cell-associated antigens by CD8α− DCs (19).

In addition to CLEC9A, another Syk-coupled C-type lectin, Mincl, has also been shown to recognize necrotic cells (48). As mentioned, Mincl is a member of the Dectin-2 family of CLR, and like Dectin-2, it mediates Syk signaling by association with the FcγIR chain (48). It may be that CLEC9A and Mincl are part of a family of Syk-coupled proteins that are involved in sensing and mediating the response to dead cells (19). Furthermore, Mincl has recently been described as a receptor for the fungus Malassezia and was shown to play a crucial role in the immune response to this pathogen (216).

In light of recent discoveries regarding CLEC9A, Dectin-1, Dectin-2 and Mincl, it appears that a paradigm is emerging of Syk-coupled myeloid expressed CLRs that mediate recognition and initiate cellular responses to pathogens and necrotic cells.

Because CLEC9A is an endocytic receptor that is largely restricted to a specific DC subset, its expression is common to both human and mice, it holds some promise as a candidate for delivery of antigens to DCs. This potential was explored recently by two independent groups. Sandho and colleagues demonstrated that antigen epitopes cotransiently coupled to an anti-CLEC9A monoclonal antibody were selectively cross-presented to CD8" T cells in vivo and induced potent CTL responses in the presence of adjuvant (45). They also determined that targeting of antigen to DCs via CLEC9A did not result in presentation by pDCs, a result which they attributed to the fact that CLEC9A is expressed in very low levels on these cells and that nevertheless pDCs are understood to be inefficient cross-presenting APCs. They then proceeded to explore whether CLEC9A targeting could be used for tumor immunotherapy. In a cancer model, they tested the efficacy of antigen targeting via CLEC9A to either prevent or treat tumors. In their model, tumor derived peptides were covalently coupled to anti-CLEC9A and administered to mice in the presence or absence of adjuvants either before or after tumor challenge. They found that the CTL responses mounted resulted in both tumor eradication and tumor prevention (45). Caminchi and colleagues also showed that targeted delivery of antigens to DCs using anti-CLEC9A monoclonal antibodies induced a striking enhancement of humoral immunity, and enhanced CD4 and CD8 T cell proliferative responses (43). Interestingly, all of the responses in this investigation were observed in the absence of additional adjuvants.

CLEC9A significance in terms of DC biology

As mentioned above, CLEC9A is one of two Syk-coupled myeloid expressed CLRs that mediate recognition of necrotic cells. Similar to Dectin-1, CLEC9A also signals via an ITAM-like motif. It will be interesting to see whether CLEC9A turns out to function in pathogen recognition as has been described for Dectin-1, Dectin-2 and Mincl. The studies described above which use CLEC9A to target antigens to the CD8α− DC subset indicate that CLEC9A is a promising target for therapeutic applications and further research in mouse models could possibly translate to an immunotherapeutic strategy for cancer in clinical settings.

CLEC-2

CLEC-2 is a type II transmembrane receptor displaying an extracellular CTLD, a single transmembrane region and a short cytoplasmic tail that also contains an ITAM-like signaling motif, similar to that described for Dectin-1 and CLEC9A. It was originally identified through a computational screen of myeloid cells for C-type lectin-like receptors homologous to those expressed by NK cells (217). RT-PCR analysis of full length human CLEC-2 showed transcripts in PBMC, bone marrow cells, monocytes, DCs, granulocytes, and in a few NK cells.
cell clones (217). In mice, two further splice variants in addition to the full-length version have been identified. One, named mCLEC-2B, lacks exons 2 and 4, which causes a frameshift and generates an open reading frame of only 252 bp (218). The various isoforms of mCLEC-2 showed different expression profiles and subcellular localization. All three transcripts were expressed in PBMCs and monocytes. However, only mCLEC-2B and mCLEC-2C were detected in a T-cell line, a B-cell line, and a macrophage cell line, and furthermore, only mCLEC-2C m peritoneal macrophages and a fibroblast cell line (218). In transfected fibroblasts, full-length mCLEC-2 was expressed on the cell surface, while mCLEC-2B and mCLEC-2C were retained in the cytoplasm. This is consistent with the cytoplasmic retention of other type II transmembrane receptors, which have been alternatively spliced to generate variants lacking the transmembrane region, for example human Dectin-1 isoform E (134). Surface expression of CLEC-2 has been demonstrated on peripheral blood neutrophils and on platelets where it was shown to undergo differential glycosylation (219, 220). There is also some evidence that CLEC-2 is present on DCs (Bess et al. in press, personal communication), suggesting that it may have a functional role on these cells.

mCLEC-2 can be cleaved by serum proteases releasing a soluble form of the receptor. This soluble version has been shown to exist as disulfide-linked homodimers which led to the suggestion that full-length mCLEC-2 also exists as homodimers, although this has not been directly shown (218). In fact, the structure of the extracellular domain of hCLEC-2 has been solved and analysis revealed that hCLEC-2 appears monomeric (221). However, as is the case with other CLECs, such as MKL, it may be that the murine and human forms differ in terms of their dimerization capacities (222, 223).

Both endogenous and xenogenous ligands of CLEC-2 have been identified. The first endogenous ligand described was rhodocytin (220), a toxin purified from the venom of the Malay pit viper California rufa. Exposure of platelets to rhodocytin leads to tyrosine phosphorylation of the CLEC-2 ITAM-like motifs and Syk-dependent platelet activation (220). It has been speculated that rhodocytin is multimeric, ligand binding may initiate surface clustering of CLEC-2 on platelets causing localization of cytoplasmic signaling domains and augmentation of signal transduction (221). CLEC-2 is likely to recognize a proteinaceous ligand on rhodocytin, as the extracellular predicted protein is not glycosylated which rules out the possibility of a CLEC-2-carbohydrate interaction (221).

Podoplanin, a transmembrane sialophosphoprotein involved in tumor cell-induced platelet aggregation, tumor metastasis, and lymphatic vessel formation, was recently identified as an endogenous ligand for CLEC-2 and it has been suggested that their interaction may be involved in tumor growth and/or metastasis (224–226). Using recombinant glycopeptides to study the interaction of hCLEC-2 with podoplanin, Kato et al. (225) concluded that the interaction was carbohydrate-mediated, and that a glycosylated core O-glycan sequence on Thr-2 of podoplanin was essential for recognition by CLEC-2. This finding is in agreement with a structural study that indicated that an endogenous ligand is likely to be a protein with a predominantly negatively charged binding surface (221). However, it remains unclear whether the oligosaccharide sequence alone is sufficient for CLEC-2 binding, or whether any of the flanking peptide region is also involved.

CLEC-2 was also identified as a HIV-1 attachment factor that may capture and transfer infectious HIV-1 in cooperation with DC-SIGN on platelets (227). The structures recognized by CLEC-2 on HIV-1 particles are so far unidentified. However, binding of HIV-1 to CLEC-2 occurred in the absence of the envelope protein which led to the suggestion that CLEC-2 may recognize a cellular factor which is incorporated into the viral envelope upon budding from infected cells (227). It is well known that a vast array of host proteins can be incorporated by HIV-1 during viral budding (228, 229). It is therefore a plausible idea that CLEC-2 may recognize an HIV-1 incorporated host factor. In fact, there is evidence that HIV-1 can infect renal cells whose podoplanin is expressed and it has been suggested that podoplanin may be incorporated into the budding virion leading to recognition by CLEC-2 (224, 230, 231). The expression of CLEC-2 on DCs raises the possibility that CLEC-2 may be involved in HIV-1 capture by DCs like other CLECs including DC-SIGN and Langerin. Whether the mechanisms are still unclear, there is a general acceptance that DC-SIGN recognition of HIV enhances infection of T cells by DCs in vivo (232, 233), whereas Langerin-mediated recognition of HIV-1 protects against infection (234).

A number of studies have explored the downstream signaling pathways initiated by ligation of CLEC-2. As mentioned previously, it was shown that CLEC-2 signaling is critically dependent on both SH2 domains of Syk, similar to Dectin-1/Syk signaling (80). Furthermore, it was proposed that a single Syk molecule forms a bridge between two CLEC-2 receptors, as has been suggested for both Dectin-1 and CLEC9A (44, 80). It was also shown that in addition to Syk phosphorylation, stimulation of CLEC-2 results in tyrosine phosphorylation of PLCγ2, VavI/3, LAT, SLP-76, and Btk (80, 220, 235). Indeed, platelet

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deficient in Syk, and CLEC-2 transfected T-cells deficient in Syk, Btk or PLCγ2 failed to respond to thromocytin, demonstrating a critical role for these proteins in mediating CLEC-2 signaling in platelets (80, 120). On the other hand, a significant but diminished response of CLEC-2 transfected T-cells to thromocytin was observed in the absence of BLNK or SLP-76, demonstrating that CLEC-2 is only partially dependent on the SLP-76/BLNK family of adapter proteins for signaling (80, 235). Platelets lacking Raci display severely impaired CLEC-2 dependent activation demonstrating a role for this Rac GTPase in CLEC-2 signaling (236).

Overexpression of CLEC-2 in a cell line generated constitutive signaling via Src and Syk kinases that led to NFAT activation (237). Co-expression of the ITIM-containing platelet protein, Gesh B, resulted in inhibition of both constitutive and agonist-induced CLEC-2 signaling. The authors of this study speculated that this may represent an important role of Gesh B and other ITIM-containing platelet receptors in inhibition of platelet activation which is an essential component of physiological blood flow (237). A further study examined the effect of another ITIM-containing platelet protein, PECAM-1, on CLEC-2 signaling using both PECAM-1 deficient mice and antibody cross-linking of PECAM-1. This work demonstrated that PECAM-1 also has an inhibitory effect on CLEC-2 signaling, although the results suggested that this inhibitory effect is mild and unlikely to be physiologically relevant (238). These studies raise the possibility that CLEC-2 signaling in neutrophils and DCs is also regulated by ITIM-containing receptor counterparts.

Dectin-1 and CLEC-2 have an identical sequence consisting of four amino acids residues DEDG, preceding their Tail motifs. Analysis in transfected B cells revealed that mutation of the glycine residue of CLEC-2 resulted in a significant reduction in signaling in response to thromocytin demonstrating that the glycine residue is important for CLEC-2 signaling (80). In a similar vein, mutation of this highly charged cluster in Dectin-1 was shown to abolish particle uptake (78). Given the significance of the glycine residue in CLEC-2 signaling, the requirement of the DED cluster for Dectin-1 mediated phagocytosis, and the demonstration that CLEC9A which lacks such a sequence does not mediate phagocytosis (44), it seems highly likely that the ability of CLEC-2 to mediate phagocytosis involves this highly charged cluster.

Although many of the CLEC-2 signaling studies were carried out in platelets, the importance of CLEC-2 for platelet activation during hemostasis and in the course of thrombotic events was not defined until very recently. May et al. demonstrated that antibody targeting of CLEC-2 resulted in complete and highly specific loss of the receptor in circulating platelets for several days (339). This study demonstrated that CLEC-2 is not required for adhesion of platelets to collagen during thrombus formation, but is necessary for subsequent stable aggregate formation. Furthermore, antibody-induced CLEC-2 deficiency resulted in increased bleeding times of mice indicating that CLEC-2 plays a significant role for normal hemostasis (339). These results suggest that CLEC-2 may represent a novel anti-thrombotic target.

We recently demonstrated that CLEC-2 is a phagocytic receptor expressed on neutrophils which can mediate the production of proinflammatory cytokines (219). Along with the reported expression on DCs, this implies a role for CLEC-2 in immunity. Future studies will undoubtedly explore the precise function of CLEC-2 on DCs and assess its potential as a targeting molecule in immunotherapeutic settings.

Concluding remarks

We speculate that the Syk-coupled ITAM-like CLRs described here are members of a large family of myeloid expressed receptors which signal via Syk and CARD9 to initiate cellular activation. It has been demonstrated that some of these receptors recognize both pathogens and necrotic cells, and it is likely that other endogenous 'danger' ligands will also be revealed. The differential expression of these CLRs on DC subsets may be useful for targeting by antibodies and by specific ligands. Indeed some studies have already yielded promising results with regard to targeting these receptors for immunotherapy. Interesting insights into immune function have been revealed as our understanding of these receptors has expanded, in particular the importance of non-TLR PRRs both in immunity and homeostasis.

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C-type lectins and phagocytosis
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Abstract
To recognise and respond to pathogens, germ-line encoded pattern recognition receptors (PRRs) bind to conserved microbial structures and activate host defence systems, including microbial uptake by phagocytosis. Phagocytosis is a complex process that is instrumental in the control of extracellular pathogens, and this activity is mediated by several PRRs, including a number of C-type lectins. While some of these receptors have clearly been shown to mediate or regulate the uptake of pathogens, others are more contentious and are less well understood in terms of their phagocytic potential. Furthermore, very little is known about the underlying phagocytic mechanisms. Here, we review the phagocytic roles of the mannose receptor, Dectin-1, dendritic cell specific ICAM grabbing non-integrin (DC-SIGN), DCl-1, mannose binding lectin and surfactant proteins A and D.

Keywords: Collectin; DC-SIGN; Dectin-1; Mannose receptor; Pattern recognition receptor

Introduction
Phagocytosis is an actin-dependent mechanism by which cells (phagocytes) ingest large particles that are usually greater than 0.5 μm in diameter (Aderem and Underhill, 1999). Phagocytosis is phylogenetically conserved in mammals and has evolved to a remarkably intricate process. Élie Metchnikoff was the first to describe it in the late nineteenth century and he was awarded the Nobel Prize in Physiology or Medicine in 1908 for his discovery. One hundred years later, Metchnikoff's cellular theory of immunity has stood the test of time, but we are now aware that the phagocytic process is much more complex than originally envisioned and we are only just beginning to decipher its various mechanistic and molecular workings.

Phagocytic cells are involved in a number of biological processes, including the recognition and control of invading microbes. In innate pathogen recognition is mediated by a series of germ-line encoded pattern recognition receptors (PRRs) that are either soluble or membrane-bound. These PRRs recognise conserved microbial structures, such as bacterial lipopolysaccharide or fungal β-glucans, that are known as pathogen associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). Soluble PRRs include the collectins,
mechanism for recognition of carbohydrates by Dectin-1 is still unclear, however, it is known that at least two residues flanking a shallow groove on the protein surface, Trp221 and His223, are crucial for β-glucan binding (Adachi et al., 2004). By way of its β-glucan specificity, Dectin-1 can recognise a number of fungal species, including C. albicans, P. arnini, Saccharomyces cerevisiae, Coccioidoides posadasi and Aspergillus fumigatus (Brown et al., 2003; Gersuk et al., 2006; Saito et al., 2007; Steele et al., 2003, 2005; Taylor et al., 2007; Vrijensbos et al., 2005).

The presence of an ITAM-like motif in the cytoplasmic tail of Dectin-1 initially suggested similarity to FcγRs. During FcγR-mediated internalisation, src kinases phosphorylate the tyrosine residues within the ITAM leading to recruitment and activation of Syk kinase. Syk activation then initiates a flood of signalling events that ultimately result in various cellular responses, including phagocytosis (Garcia-Garcia and Rosales, 2002). Ph-3 kinase is required for pseudopod extension and phagosomal closure during FcγR-mediated uptake, as well as the previously mentioned Rho and Cdc42 GTPases, which are required for actin reorganisation (Fig. 2).

The ligation of Dectin-1 also triggers intracellular signalling resulting in a variety of cellular responses, including phagocytosis. In contrast to uptake by FcγR, however, phagocytosis by Dectin-1 requires phosphorylation of only the membrane proximal tyrosine of the ITAM-like motif (Herre et al., 2004). Src kinases are only partially required for this, as the inclusion of PP2, a src kinase inhibitor, in internalisation experiments, did not cause complete inhibition of uptake. In addition, Syk was not required for Dectin-1 mediated phagocytosis in macrophages, indicating the existence of novel signalling pathway(s) (Herre et al., 2004). On the other hand, Syk is required, at least in part, for Dectin-1 dependent uptake in dendritic cells and in NIH-3T3 cells, suggesting that signalling events initiated from Dectin-1 may differ depending on cell type (Herre et al., 2004; Rogers et al., 2005). Dectin-1 also has a highly charged triadric motif (DED) in the ITAM-like motif which is required for uptake (Underhill et al., 2005). Inhibition studies have shown that Ph-3 kinase was not essential for Dectin-1 mediated phagocytosis, whereas PKC was suggested. In addition, the use of dominant-negative constructs have shown that Cdc42 and Rac-1 are involved, but not Rho (Herre et al., 2004; Ueyama et al., 2004). The route of intracellular processing of Dectin-1 may depend on the molecular nature of the ligand, with the receptor directed to lysosomes during uptake of the particulate ligand, zymosan, but recycled to the membrane during uptake of the soluble ligand, laminarin (Herre et al., 2004).

Other molecules may also associate with Dectin-1 during phagocytosis. For example, immunoprecipitation experiments have shown that the tetraspanin, CD63 associates with Dectin-1 (Mantegazza et al., 2004). In addition, phagocytosis of yeast particles by DCs was accompanied by a decrease in CD63 expression, which was inhibitable by laminarin. Although the functional significance of a Dectin-1-CD63 interaction has not yet been elucidated, it may represent part of a signalling complex that could influence phagocytosis (Brown, 2006). Dectin-1 also recognise opsonised particles. Pentraxin-3 (PTX-3) causes aggregation of zymosan, leading to the Dectin-1 dependent internalisation of an increased number of zymosan particles (Diniz et al., 2004). Moreover, macrophages express higher levels of PTX-3 in the presence of zymosan and it has been suggested that during fungal infection, secreted PTX-3 may enhance clearance by opsonising the pathogen and facilitating its uptake by Dectin-1 (Diniz et al., 2004).

DC-SIGN (CD209)

DC-SIGN is a type II transmembrane protein that is classified as a Group II C-type lectin. It consists of an extracellular C-terminal CTLD, a repetitive stalk region which mediates receptor multimerisation, a single transmembrane region and a cytoplasmic tail within which a number of internalisation motifs are present (Fig. 3). Its expression was initially believed to be restricted to immature dendritic cells, but it is now known to be expressed on endothelium and selected macrophage subpopulations (Geijtenbeek et al., 2003; Krutzik et al., 2005; Lai et al., 2006; Scilleux et al., 2002; Tailleux et al., 2005). DC-SIGN expression is mainly induced by IL-4, and is negatively regulated by IFNγ, TGβ, and dexamethasone (Rellono et al., 2002).

DC-SIGN was originally identified as a receptor for intercellular adhesion molecule-3 (ICAM-3) that facilitates DC-mediated T-cell proliferation and binds HIV-1 (Geijtenbeek et al., 2000a,b). It has since been reported that the receptor interacts with a range of pathogens, including M. tuberculosis, C. albicans, Helicobacter pylori, Schistosoma mansoni and A. fumigatus (Appelmelk et al., 2003; Cambi et al., 2003; Geijtenbeek et al., 2000b, 2003; Serrano-Gomez et al., 2004; Tailleux et al., 2003; van Dde et al., 2003). DC-SIGN recognises both internal mannose branched structures and terminal di-mannoses, and the receptor forms tetramers aiding its specificity for high mannose oligosaccharides (Mitchell et al., 2001).

DC-SIGN is often described as a phagocytic receptor. This is construable given its interactions with pathogens and the presence of internalisation motifs (di-leucine motif, tri-acidic cluster, ITAM motif) in its cytoplasmic tail (Zhou et al., 2006). However, to date, the evidence for the phagocytic potential of DC-SIGN has been
indirect and still needs to be demonstrated conclusively. For example, a study by Serrano-Gomez et al. shows that DC-SIGN mediates efficient binding of *A. fumigatus* conidia to immature and mature MDDC (Serrano-Gomez et al., 2004). In this study, internalisation assays in immature MDDC resulted in conidial uptake in vesicles that stained positively for DC-SIGN indicating a potential role for the receptor in uptake. It has also been shown that DC-SIGN is present in *M. tuberculosis* vacuoles of MDDC (Tallez et al., 2003). Other studies have similarly shown colocalisation of DC-SIGN with phagosomes (Gambi et al., 2008; Geijtenbeek et al., 2003). The demonstration of phagocytosis by usually non-phagocytic cells transduced with DC-SIGN would be a more direct way to examine the phagocytic potential of the receptor. This type of approach has been used by Zhang et al. where they indirectly demonstrated internalisation of a non-pathogenic strain of *Escherichia coli* by HeLa cells transfected with DC-SIGN (Zhang et al., 2006). Interestingly, mutation of the tyrosine in the cytoplasmic domain of DC-SIGN did not affect internalisation of *E. coli* in these assays. In a subsequent study, they used the DC-SIGN transfected HeLa cells to demonstrate uptake of *Yersinia pestis* (Zhang et al., 2008). These studies go some way to implicating DC-SIGN as a phagocytic receptor, however, further work in alternative cell lines is warranted. As *Y. pestis* also binds to heparan sulphate proteoglycan receptor on HeLa cells, which contributes to its internalisation.

There have been no reports of a mechanism for DC-SIGN mediated phagocytosis. However, activation of DC-SIGN triggers Rho-GTPase (Hodges et al., 2007) making it conceivable that Rho could be involved in phagocytosis mediated by this receptor. Further studies are required to confirm the phagocytic ability of DC-SIGN and elucidate the underlying mechanisms.

DCL-1 (CD302)

DCL-1 is a recently described type I transmembrane protein which was identified as a genetic fusion partner of human DEC-205 in Hodgkin’s lymphoma cell lines and is classified as a Group XV C-type lectin (Kato et al., 2003; Zelensky and Greedy, 2005). The name DCL-1 is derived from DEC-205-associated C-type lectin-1 and the receptor consists of a single extracellular CTLD, a short spacer followed by a transmembrane region and a cytoplasmic tail containing a putative tyrosine-based internalisation motif (Fig. 3). Human DCL-1 (hDCL-1) is expressed on monocytes, macrophages, granulocytes and dendritic cells (Kato et al., 2003). The receptor does not contain the amino acids that coordinate Ca^{2+}-dependent sugar binding in other C-type lectins, suggesting that it does not have classic sugar binding capacity, but no endogenous or exogenous ligands have yet been identified. A recent study has shown that cell lines transfected with hDCL-1 efficiently phagocytose microbeads coated with hDCL-1 monoclonal antibodies. However, monocyte derived macrophages displayed relatively inefficient binding and phagocytosis of these antibody-coated microbeads (Kato et al., 2007). Further in vitro and in vivo studies are needed in order to clarify the potential phagocytic role of DCL-1.

Soluble C-type lectins

Mannose-binding lectin (MBL)

Mannose-binding lectin (MBL) is a Group III C-type lectin belonging to the collectins (Holmskov et al., 2003), which are a group of soluble oligomeric proteins containing collagenous regions and C1r/C1s domains. MBL is secreted into the bloodstream as a large multimeric complex and is primarily produced by the liver, although other sites of production, such as the intestine, have been proposed (Uemura et al., 2002). The basic functional unit of MBL is a homotrimer, with each monomer consisting of an amino-terminal cysteine rich domain, a long collagenous domain, an α-helical coiled coil and a single C1r LD (Fig. 3). Oligomerisation of trimeric subunits creates a bouquet-like structure and in serum, MBL consists of oligomers ranging from dimers to hexamers. It recognises carbohydrates such as mannose, glucose, 1-fucose, N-acetyl-mannosamine (ManNAc), and N-acetyl-glucosamine (GlcNAc). Oligomerisation of MBL enables high avidity binding to repetitive carbohydrate ligands, such as those present on a variety of microbial surfaces, including *E. coli*, *Klebsiella aerogenes*, *Neisseria meningitides*, *Staphylococcus aureus*, *P. aeruginosa*, *A. fumigatus* and *C. albicans* (Davies et al., 2000; Neth et al., 2000; Schellenz et al., 1995; Tabona et al., 1995; van Emmen et al., 1994).

MBL has the capacity to modify the efficiency of uptake and the expression of other phagocytic receptors. Activation of the complement system via MBL-associated serine proteases (MASPs) (Dahl et al., 2001; Kawasaki et al., 1989, 1998; Matsuura and Fujita, 1992; Stover et al., 1999; Thiel et al., 1997), results in deposition of complement on the microbial surface that can lead to uptake via complement receptors (Fig. 1) (Kawasaki et al., 1989; Neth et al., 2002). However, inhibition of bacterial growth associated with the MBL-MASP activation of complement has also been observed, without any enhancement of phagocytosis (Ip and Lau, 2004). This indicates that the specific responses induced by MBL may be dependent on the...
nature of the microbial target. MBL can also influence expression of other PRRs, as demonstrated by the ability of MBL to augment the uptake of S. aureus through the up-regulation of scavenger receptor A (SR-A) (Owu et al., 2006) (Fig. 1).

MBL has also been proposed to function directly as an opsonin by binding to carbohydrates on pathogens and then interacting with MBL receptors on phagocytic cells, promoting microbial uptake and stimulating immune responses (Fig. 1). This was first described by Kuhlman et al. who observed that binding of MBL to Salmonella monoviride resulted in an MBL-dependent uptake by monocytes (Kuhlman et al., 1989). Thus MBL can interact directly with receptors on the surface of monocytes and several potential MBL receptors have since been proposed, although their likelihood is still debated in the literature. Calreticulin has emerged as the main candidate (Malhotra et al., 1990), but further studies are required to confirm its interaction with MBL and its role in the phagocytosis of pathogens.

A recent study has shown that MBL modifies cytokine responses through a novel cooperation with TLR2:6 in the phagosome (Ipt et al., 2008). Although the stimulation of the inflammatory response was not caused by enhanced phagocytosis, bacterial engulfment was required. This study therefore demonstrates the importance of phagocytosis in providing the appropriate cellular environment to facilitate cooperation between molecules (Ipt et al., 2008).

Surfactant proteins

Surfactant proteins A and D (SP-A, SP-D) are also collectins within the Group III C-type lectins. Their basic structure is similar to MBL and includes an amino-terminal cysteine rich domain, a collagenous domain, an α-helical coiled coil and a single CTLD. They are also assembled as trimeric subunits which form oligomers. SP-D forms dodecamers that are characterised by a cruciform shape (Fig. 3) (Crouch et al., 1994). SP-A is present mainly as octadecamers with a bouquet-like structure similar to that of MBL oligomers (Hickling et al., 1998). SP-A and SP-D are primarily synthesised in the lungs by type II alveolar and Clara cells (Lu et al., 2002). They are secreted into the alveolar space where they are the main protein constituents of pulmonary surfactant. SP-A and SP-D have been shown to interact with a wide variety of carbohydrates and glycolipids, but both receptors have common and distinct ligand recognition capacities. For example, both proteins bind to glucose and mannose, however, SP-A binds preferentially to α-fucose and N-acetylgalactosamine (Haagsman et al., 1987; Haurum et al., 1993), whereas SP-D displays preferential binding to glucose, maltose and mostol (Lim et al., 1994; Persson et al., 1990). Through these carbohydrate recognition abilities, both proteins have been shown to bind to a wide range of pathogens, including Pseudomonas aeruginosa, P. carini, A. fumigatus, M. tuberculosis, S. pneumoniae and K. pneumoniae (Hartshorn et al., 1998; Khubchandani et al., 2001; Madan et al., 1997; Mariencheck et al., 1999; Ofek et al., 2001; Pasula et al., 1997; Yong et al., 2003).

Unlike MBL, the surfactant proteins do not activate the complement system. However, they can function directly as opsonins by binding to microbial carbohydrates and interacting with surfactant receptors on phagocytic cells (LeVine et al., 1999; Mariencheck et al., 1999; Ofek et al., 2001). A number of candidate receptors, including calreticulin have been described and are reviewed elsewhere (Gardai et al., 2003; Kishore et al., 2008).

Surfactant proteins can also cause aggregation of pathogens, which, depending on the pathogen involved, can either enhance or inhibit microbial uptake. For example, aggregation of S. pneumoniae by SP-D stimulates microbial uptake (Hartshorn et al., 1998), whereas SP-D mediated aggregation of P. carini inhibits phagocytosis by alveolar macrophages (Yong et al., 2003).

Similar to MBL, the surfactant proteins can also indirectly influence phagocytosis by regulating expression of other phagocytic receptors. For example, SP-A increases the expression of scavenger receptor A resulting in an enhancement of uptake of S. pneumoniae (Kurosumi et al., 2004). SP-A can also enhance FcR and CR1-mediated phagocytosis through unknown mechanisms, leading to its description as an activation ligand (Tenner et al., 1989).

Concluding remarks

We have seen that a number of C-type lectins are involved in phagocytosis. These molecules can function as phagocytic receptors, as direct or indirect opsonins, and some can also modulate expression of other receptors (Fig. 1). In certain cases, the individual C-type lectin fulfills more than one of these roles. Although not discussed in detail here, C-type lectins can also cooperate with other molecules, as in the case of MBL and TLR2 mentioned here. In general, the mechanisms of phagocytosis by C-type lectins are not very well characterised but they are clearly complex and appear to be unique to each receptor. Out of necessity, most studies have focused on particular receptors in isolation, but we should remember that the mechanism of uptake in phagocytosis will be a result of the collective contribu-
tion of the several receptors involved in recognition of the specific pathogen.

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18 Signaling through the Fungal β-Glucan Receptor Dectin-1

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18.1 INTRODUCTION

The innate immune response of vertebrates is triggered if a pathogen breaches the physical barriers of the host. It provides a rapid but nonspecific response and is the first line of defense. The functions of innate immunity are based on a variety of germline-encoded receptors, the pattern recognition receptors (PRRs) [1]. PRRs are proteins expressed by cells of the innate immune system that recognize conserved molecules associated with microbial pathogens. These conserved microbial structures are known as pathogen-associated molecular patterns (PAMPs). The recognition of PAMPs enables the immune system to distinguish foreign organisms from cells of the host and initiates a rapid inflammatory response.

Toll-like receptors (TLRs) are a family of PRRs involved in the recognition of a broad range of microbial structures and they are the major molecules through which mammals sense infection and activate inflammatory responses. For example, TLR2 plays an important role in recognition and responses to fungal pathogens [2,3] and TLR4 is critical for detection and clearance of Gram-negative bacteria [4]. There are also several other non-TLR receptors that have roles in microbial detection, including complement receptors, pentraxins, scavenger receptors, classical and nonclassical C-type lectins and collectins. In general, however, the exact function of the non-TLR PRRs in the control of infection remains unclear.

An exception to this is the nonclassical C-type lectin receptor, Dectin-1. Dectin-1 is involved in the innate immune response to fungal pathogens and has been shown to bind many fungal species, including Saccharomyces [2], Candida [5], Cryptococcus [6], Pneumocystis [7,8], and Aspergillus [9-11]. It recognizes β-glucan components of the fungal cell wall [12] and in vitro has been shown
to mediate fungal binding, uptake, and killing [13,14], and the production of numerous cytokines and chemokines [2,7,15,16]. Studies using Dectin-1-deficient mice have established the essential role of β-glucan recognition in the development of an antifungal inflammatory response and the control of fungal dissemination in vivo [5,8]. In fact, to date, Dectin-1 is the only example of a non-TLR PRR that can mediate its own intracellular signaling to induce a protective immune response. This chapter will focus on independent and cooperative signaling by Dectin-1 and how the current knowledge fits into the overall paradigm of immunoreceptor signaling and pattern recognition.

18.2 DECTIN-1: STRUCTURE, EXPRESSION, AND LIGANDS

Dectin-1 was originally identified as a dendritic cell specific receptor that modulates T cell function by recognizing an unidentified ligand on lymphocytes [17,18]. It was reidentified as a β-glucan receptor following a screen of a murine macrophage cDNA expression library with zymosan, a β-glucan-rich extract of *Saccharomyces cerevisiae* [12]. β-Glucans are carbohydrate polymers with well-characterized immunostimulatory activity that are found primarily in the cell walls of fungi, but also in plants and some bacteria [19]. Other receptors such as complement receptor 3 (CR3) have been implicated in β-glucan and fungal recognition [20]. However, studies using specific antagonists, blocking monoclonal antibodies, and knockout mice have clearly shown that Dectin-1 is the major β-glucan receptor on primary macrophages, dendritic cells, and neutrophils [5,8,21].

Dectin-1 is a member of the nonclassical C-type lectin family. It is a type II transmembrane protein consisting of a single extracellular lectin-like domain connected to a single-pass transmembrane region by a stalk and a cytoplasmic tail, which contains an immunoreceptor tyrosine-based activation (ITAM)-like motif (Figure 18.1). It has a similar structure to other members of the nonclassical C-type lectin family, with two significant exceptions. Firstly, there are no cysteine residues in its stalk region, indicating that it does not dimerize. Secondly, the cytoplasmic ITAM-like motif is unusual as most other known activating nonclassical C-type lectin receptors, such as Ly49D (lymphocyte antigen 49D), do not contain cytoplasmic ITAM-like motifs, but rather associate with signaling molecules, such as DAP12, to carry out cellular activation. However, C-type lectin-like receptor 2 (CLEC-2), another

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**FIGURE 18.1** Structure of the β-glucan receptor Dectin-1. Dectin-1 contains an extracellular C-type lectin-like carbohydrate recognition domain (CL), stalk region, transmembrane domain, and cytoplasmic tail that contains an ITAM-like sequence. Alternative splicing can generate an isoform of Dectin-1 that lacks a stalk.
receptor that is encoded within the same gene cluster as Dectin-1, does have a similar ITAM-like motif to Dectin-1, and has also been shown to induce cellular activation [22].

Oligosaccharide microarray technology has shown that Dectin-1 specifically binds β-1,3-linked glucose oligomers [23]. Other members of the nonclassical C-type lectins do not generally recognize carbohydrates, as they lack the conserved residues involved in the ligation of calcium that coordinates carbohydrate binding. Dectin-1 also lacks these conserved residues and its Ca²⁺-independent mechanism for recognition of carbohydrates is still unclear. However, a study, which involved binding analysis of 32 point mutants, revealed that at least two residues, Trp221 and His223 in the extracellular region of Dectin-1, are crucial for β-glucan binding [24].

The murine form of the receptor is expressed by many cell types, including macrophages, dendritic cells, monocytes, neutrophils, and a subset of splenic T cells [25]. There are at least two isoforms of murine Dectin-1, encoding the full-length receptor and a stalkless version. Studies have shown that zymosan binding by the smaller isoform is significantly weakened at lower temperatures and there are also differences in the levels of cytokine production induced by the two isoforms. These studies indicate that Dectin-1 isoforms are functionally distinct and may represent a means whereby cellular responses to fungal infection can be regulated [26]. Structurally and functionally similar human homologs have been identified. The expression of human Dectin-1 differs slightly in that it is also expressed on B cells and eosinophils. Human Dectin-1 mRNA is also alternatively spliced, resulting in eight isoforms, of which only two are functional for β-glucan binding [27]. Of the two functional human isoforms, the most predominant lacks the stalk region [27] (Figure 18.1), and similar to the mouse, there are also temperature-dependent differences in zymosan binding [26]. The two major human isoforms are expressed differently in various cell types although the significance of this remains unclear. It has also been shown that the level of Dectin-1 expression in murine macrophages can be influenced by various cytokines and microbial factors. For example IL-4, IL-13, and GM-CSF cause Dectin-1 expression to be highly upregulated. In contrast IL-10, LPS and dexamethasone cause down-regulation of Dectin-1 expression [28].

18.3 SIGNALING THROUGH ITAM-CONTAINING RECEPTORS

Studies of molecules containing ITAM motifs have contributed largely to the elucidation of patterns of immunoreceptor signaling and are helpful when considering signaling through the ITAM-like motif of Dectin-1. The T cell receptor (TCR)-CD3 complex, the B cell receptor and Ig Fc receptors (FcR) all signal via src and Syk family tyrosine kinases, phosphatases, and a host of adapter proteins that serve as molecular links to downstream signaling pathways.

The activatory Fc receptors are characterized by the presence of an ITAM motif in an accessory chain that associates with the receptor. When Fc receptors interact with their immunoglobulin ligands, the extracellular domains of the receptors aggregate. This results in tyrosine phosphorylation of the ITAM motif by members of the Src-kinase family and subsequent recruitment of SH2-containing kinases including members of the Syk-kinase family. These early events trigger the initiation of downstream signaling cascades that ultimately lead to cellular activation [29]. Such events include phosphorylation and activation of phospholipase C (PLC), which leads to Ca²⁺ flux and activation of nuclear factor of activated T cells (NFAT) transcription factor. In addition, activation of protein kinase C (PKC) isoforms couple ITAM-containing receptors to the caspase recruitment domain (CARD) proteins such as CARD 11. Such proteins form complexes with the adaptors MALT1 and Bcl10, leading to the recruitment and activation of TNF receptor associated factor 6 (TRAF6), which in turn couples to inhibitor of KappaB kinases (IKK). Activation of the IKK complex results in phosphorylation of an inhibitory protein IxB. IxB is bound in a complex with inactive NFkB. Once IxB is phosphorylated, it dissociates from NFkB, allowing the transcription factor to translocate to the nucleus and induce various genes [30]. The TLRs also activate NFkB, but utilize separate pathways either through the adaptor MyD88 or TRIF.
The functional TCR complex is equipped with 10 ITAMs that are phosphorylated by Lck, a Src family kinase, on receptor engagement. This allows recruitment of ZAP70, a Syk family kinase, and phosphorylation of the adaptor molecules LAT and SLP-76, as well as the enzymes Yav and PLCγ1 [31]. PKCθ is subsequently recruited to the membrane and activated. This event appears to be a specific link to the NFκB pathway. PKCθ activates CARD11 by phosphorylating specific serine residues [32]. This in turn leads to recruitment of Bcl10 and MALT1 and activation of NFκB.

The traditional ITAM motif is characterized by a consensus sequence that includes two tyrosines, usually 10–12 amino acids apart YxxILy/Lx/L, Yx/L. The signaling motif of Dectin-1 is only slightly different from other activatory receptors and consists of YxxIL/Lx/y/L. Given this likeness, it was originally thought that Dectin-1 may signal in a similar manner to ITAM-containing receptors such as those described above. Studies have since shown that while parallels can indeed be drawn between signaling by Dectin-1 and other activatory receptors, such as FcγR and TCR, there are also notable differences (Figure 18.2). As previously mentioned, in vitro studies have shown that ligand binding by Dectin-1 can induce many cellular responses, such as phagocytosis, phospholipase A2, COX2, the respiratory burst and cytokine production. These various aspects of Dectin-1 function will be discussed in more detail in the subsequent sections. Signaling from Dectin-1 alone appears to be sufficient for many of these responses, but others, such as the induction of TNF, require cooperation through TLR2 and signaling via MyD88 [2,15,16]. While many activatory receptors are known to associate with Syk kinase, Dectin-1 is presently the sole non-TLR PRR shown to signal via Syk. Furthermore, the association between Dectin-1 and Syk occurs via a novel interaction involving only one tyrosine [15]: However, some cellular responses induced by Dectin-1, such as phagocytosis, do not require Syk. The presence of TLR and Syk-dependent and -independent pathways highlights the complexity of Dectin-1 signaling. The elucidation of the signaling mechanisms of Dectin-1 has potential implications for other non-TLR PRRs that have similar cytoplasmic motifs [33].

![Comparison of NFκB activation pathways through Dectin-1, TLR2, and the TCR. Dectin-1 and the TCR use Syk family kinases to couple to the CARD9 and CARD11 adaptors, respectively, leading to assembly of the TRAF6 complex. Degradation of IkB and NFκB activation. TLR2 utilizes the adaptors MyD88 and MAL and IRAK kinases to couple to the TRAF6 complex and NFκB activation. Dectin-1 and TLR2 collaborative responses may occur by signal integration. Dashed lines represent pathways with multiple steps.](image-url)
18.4 PHAGOCYTOSIS

Dectin-1 triggers cytoskeletal rearrangements leading to particle internalization. Figure 18.3 illustrates a Dectin-1 expressing cell internalizing *Candida albicans*. This phagocytic activity of Dectin-1 is dependent on the ITAM-like motif in the cytoplasmic tail [13]. As previously mentioned, the signaling motif of Dectin-1 differs only slightly from the traditional ITAM motif of activatory receptors. However, this difference appears to be sufficient to warrant an alternative mechanism of internalization. It is widely accepted that the Src-ITAM-Syk signaling pathway results in phagocytosis by FcγR [34]. Studies using macrophages deficient in various Src kinases suggest that there is a high level of redundancy for these enzymes and that all of them are required during activation of phagocytosis by FcγR [29,35,36]. The phagocytic capacity of Dectin-1 depends only on its membrane proximal tyrosine residue within the ITAM-like motif [13]. Dectin-1 becomes tyrosine phosphorylated at this residue upon ligand binding. However, in contrast to FcγR, src kinases appear to be only partially responsible, as was shown by using PP2, a src kinase inhibitor whose inclusion in internalization assays did not cause complete inhibition of uptake [13]. This suggests that other kinases may be involved. Furthermore, in macrophages, Dectin-1-mediated phagocytosis does not require Syk. This indicates the existence of a novel signaling pathway for Dectin-1-mediated phagocytosis in macrophages [13,15]. It has also been shown that phosphoinositide-3 (PI-3) kinase is not essential for Dectin-1-mediated phagocytosis, whereas PKC, ras-related C3 botulinum toxin substrate 1 (Rac-1), and Cdc42 are required [13]. On the other hand, Syk does have some influence on Dectin-1-mediated phagocytosis in dendritic cells where the kinase is recruited to the membrane proximal tyrosine of Dectin-1 [16].

Dectin-1 is also involved in the uptake of opsonized zymosan. Pentraxin-3 (PTX-3) binds to zymosan and it has been shown that internalization of PTX-3 opsonized zymosan is dependent on Dectin-1 [37]. Macrophages actually express higher levels of PTX3 mRNA in the presence of zymosan and it has been suggested that during fungal infection, the PTX3 gene is activated and secreted PTX3 may enhance the clearance of the pathogen [37]. Dectin-1 can also recognize exposed

FIGURE 18.3 (see color insert following page xxx.) Dectin-1 mediates phagocytosis of *Candida albicans*. A NIH-3T3 cell expressing Dectin-1 phagocytosing FITC-labeled *C. albicans*. Actin filaments and phagocytic cups are stained red. (From Brown, G.D. and Gordon, S., *Nature*, 413, 36, 2001. With permission.)
β-glucans on complement opsonized zymosan and it has been shown that Dectin-1 is necessary for inflammatory responses to opsonized particles [38].

Other molecules may also associate with Dectin-1 during internalization of yeast. For example, it has been reported that the tetraspanin CD63 is internalized after yeast phagocytosis, and immunoprecipitation experiments have shown that it associates with Dectin-1 [39]. Although the functional significance of a Dectin-1-CD63 interaction has not yet been elucidated, it may represent part of a signaling complex. A functional interaction between another tetraspanin, CD37, and Dectin-1 has also been reported. This study demonstrated that CD37 inhibits signaling pathways leading to Dectin-1-mediated cytokine production [40]. As cells of the immune system express several PRRs, signals from the various receptors must be integrated to produce an appropriate immune response. This can be achieved by clustering immunoreceptors and signaling molecules into complexes using tetraspanins. Tetraspanins can laterally interact with immunoreceptors to create “tetraspanin microdomains” and consequently regulate downstream signaling [41-43]. The Dectin-1-CD63 or CD37 complexes are therefore quite likely to form part of a larger complex that integrates signaling from other receptors [40].

18.5 CYTOSOLIC PHOSPHOLIPASE A2 ACTIVATION AND CYCLOOXYGENASE 2 EXPRESSION

Zymosan has long been used to investigate arachidonic acid release and metabolism [44,45]. Arachidonic acid is released from cell membranes by cytosolic phospholipase A2 (cPLA2), after which it is available for the production of the inflammatory mediators, leukotrienes, and prostaglandins. Metabolism of free arachidonic acid by the 5-lipoxygenase (5-LO) pathway, or the cyclooxygenase (COX) pathway, results in the production of leukotrienes or prostaglandins, respectively. Recent work has shown that Dectin-1 has a role in cPLA2 activation by zymosan and C. albicans [46,47]. Ligation of Dectin-1 by zymosan and C. albicans also increases expression of COX2 and consequently prostaglandin production. The cytoplasmic tail of Dectin-1 is required for zymosan-induced arachidonic acid release although internalization is not strictly necessary. Arachidonic acid release and leukotriene production stimulated through Dectin-1 ligation is TLR2-independent, but requires Syk, Src kinases, and PI3 kinase [46,47]. In contrast, COX2 expression and consequent prostaglandin production are Syk-dependent and partially TLR2-dependent [46].

18.6 RESPIRATORY BURST

The signaling cascade initiated by FcyR-mediated phagocytosis results in activation of the respiratory burst. The respiratory burst involves production of reactive oxygen species (ROS) that combat invading microorganisms, and it is initiated by the NAPDH oxidase enzyme. FcR signaling in neutrophils causes migration of the various subunits of NAPDH oxidase to the phagosome membranes and subsequent activation of the complex [48]. Various protein kinases have been reported to activate NAPDH oxidase, but the initial signaling events take place along the Src-ITAM-Syk pathway [29]. The generation of intracellular signals by Dectin-1 following ligand binding also result in the activation of NAPDH oxidase and production of ROS [3,15]. It also seems that Dectin-1 can cooperate in some way with TLRs in the production of ROS. For example, ROS production induced by zymosan can be enhanced when cells are prestimulated with TLR ligands, indicating that the Dectin-1-triggered response can be primed by TLR activation [15]. There is also a requirement for Syk in ROS production by macrophages following Dectin-1 ligation [14]. However, this respiratory burst does not occur in all macrophages. Evidence suggests that macrophages can exist in two states, one that is permissive to Syk activation and ROS production and one that is not. These cells that initiate a respiratory burst represent a specialized subpopulation of macrophages that are somehow primed for production of reactive oxygen intermediates [14].
18.7 CYTOKINES

Activation of Dectin-1 results in the production of numerous chemokines and cytokines, such as TNF, IL-10, IL-2, IL-12, IL-6, IL-1α, IL-1β, MCP-1, and MIP-1α [2,5,9,15,49]. The most well studied in relation to Dectin-1 induced production are described below. Cytokine production and other responses induced by Dectin-1 are summarized in Figure 18.4.

18.7.1 TNF

Tumor necrosis factor (TNF) is an essential proinflammatory cytokine required for the successful control of many fungal pathogens [50-53]. Therefore, it is not surprising that Dectin-1 ligation by zymosan and live fungal pathogens induces TNF production [2,15]. In fact, Dectin-1 is the first non-TLR receptor identified as being involved in the generation of a proinflammatory response to fungal pathogens. Studies using cell lines expressing a truncated version of Dectin-1 lacking the cytoplasmic tail demonstrated that the Dectin-1 cytoplasmic tail is required to induce TNF production in response to zymosan [2,15]. However, particle internalization is not required, as cell lines expressing Dectin-1 still show elevated levels of TNF production when phagocytosis is inhibited [2]. TNF production induced by Dectin-1 also requires the recognition of another component of zymosan by TLR2 and signaling through the MyD88 pathway [2,15].

It is well established that stimulation of TLRs leads to activation of NFκB and production of proinflammatory cytokines such as TNF. However, the specificity of these responses is not clearly understood and it is believed that other immunoreceptors contribute to their induction. This appears to be the case for TLR2 and Dectin-1, as the simultaneous engagement of both these receptors greatly enhances TLR2-mediated TNF production [2,15]. Gross et al. have recently shown that bone marrow derived dendritic cells (BMDCs) from CARD 9-deficient mice displayed severely defective TNF production induced by zymosan [54]. CARD 9 is a CARD protein that is related to CARD 11 which is mentioned previously. In the same study, inclusion of specific inhibitors of Syk abolished zymosan-induced production of TNF by wild-type BMDCs. This work shows that zymosan-induced production of TNF is highly dependent on Syk and CARD 9. Gross et al. also provide evidence to support the idea that CARD 9 operates upstream of IκB and Malt1 to transduce signals for NFκB activation and cytokine production [54].

FIGURE 18.4 Responses induced by Dectin-1 and TLR2 signaling pathways. Fungal stimulation of dendritic cells induces IL-10 through the Dectin-1-Syk pathway, and IL-12 through the TLR2-MyD88 pathway. Dectin-1 and TLR2 collaborate to induce IL-2 in dendritic cells and TNF, IL-12 and ROS in macrophages. Phagocytosis in both dendritic cells and macrophages is largely Syk-independent.
18.7.2 IL-12
Dectin-1 also enhances IL-12 production induced by TLR2 stimulation [15]. IL-12 plays a critical role in Th1-based responses, which have been linked to resistance to fungal infection [55]. It has been suggested that Dectin-1 recognition of β-glucans in fungi may serve to focus Th1-type responses through production of IL-12 [15]. Unlike TNF, the production of IL-12 is CARD 9-independent [54] and only partly dependent on Syk, as shown using cells from CARD 9-deficient mice and Syk-deficient mice [16,54].

18.7.3 IL-10 and IL-2
Zymosan also induces significant levels of Dectin-1-mediated IL-10 and IL-2 production [16,49]. The production of IL-10 is somewhat surprising given that IL-10 counteracts the effects of IL-12. IL-10 production has advantages and disadvantages for the host during fungal infection. It impairs the antifungal effector mechanisms of phagocytes and the secretion of proinflammatory cytokines. However, its anti-inflammatory action may be beneficial late in the course of infection to aid in the resolution of the inflammatory response [55]. The production of IL-10 by dendritic cells is TLR-independent [16], but requires the cytoplasmic tail of Dectin-1. Like TNF, Dectin-1-mediated IL-10 production is dependent on Syk and CARD 9 [54]. IL-2 has an essential role in the development and peripheral activity of regulatory T cells [56-58], which are reported to have an essential role in protective memory immunity to fungal infections [34,59]. Production of IL-2 requires the cytoplasmic tail of Dectin-1 and is dependent on Syk and CARD 9 [54]. Maximal IL-2 synthesis also requires signaling via MyD88 [16].

18.8 CONCLUSION AND FUTURE DIRECTIONS
The discovery of Dectin-1 as a receptor for fungal pathogens has changed the way that we think about non-TLR PRRs and opened up a new facet of innate immunology. The TLRs are clearly key components of the innate immune system that recognize a wide range of microbial pathogens. However, the emergence of Dectin-1 as the first example of a signaling non-TLR PRR has shown that TLRs do not function alone. Furthermore, the collaborative signaling between Dectin-1 and TLR2 has provided the skeleton of what may be a valuable model for other receptors.

The immune response is launched in response to microbial recognition by a complex network of receptors. The specific cellular response to this recognition is dependent on functional cooperation of downstream signals initiated by receptor activation. Dectin-1 collaborates with TLR2 to tailor the immune response and induce production of TNF, IL-12, and ROS via a novel interaction involving Syk and other unknown molecules. Future research into the nature of Dectin-1 and TLR2 collaboration will define this functional cooperation and provide insight into collaboration between other immunoreceptors. Dectin-1 is therefore likely to be the first of a number of non-TLR, but Syk-coupled PRRs that are critical for the control of various infectious agents.

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Complement C3 Plays an Essential Role in the Control of Opportunistic Fungal Infections

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The innate recognition of fungal pathogens is a crucial first step in the induction of protective antifungal immunity. Complement is thought to be one key component in this process, facilitating fungal recognition and inducing early inflammation. However, the roles of the individual complement components have not been examined extensively. Here we have used mice lacking C3 to examine its role in immunity to opportunistic fungal pathogens and show that this complement component is essential for resistance to infections with Candida albicans and Candida glabrata. We demonstrate that the absence of C3 impairs fungal clearance but does not affect inflammatory responses. We also show that the presence of C3 contributes to mortality in mice challenged with very high doses of S. cerevisiae, although these effects were found to be mouse strain dependent.

Over the last few decades, modern medical practice and acquired immunodeficiencies have contributed to a substantial increase in the incidence of infections with normally commensal or nonpathogenic fungi (22), prompting a renewed interest in expanding our understanding of the mechanisms underlying protective host immunity. Identification and characterization of the receptors involved in the innate recognition of these organisms are of particular importance, as these "pattern recognition receptors" (14) not only are responsible for mediating the recognition and uptake of fungi by phagocytes but also initiate and direct the resultant immune response (17). While numerous nonopsonic pattern recognition receptors for fungi have been well characterized, including several members of the C-type lectin and Toll-like receptor families (for recent reviews, see references 20, 21, and 32), less is known about the opsonic mechanisms of fungal recognition, such as those mediated by complement, despite the essential role of these systems in antifungal immunity (24).

The complement system consists of more than 30 serum and cellular surface proteins and is activated through three main routes: the classical, alternative, and lectin pathways. Triggering of these three pathways initiates enzymatic cascades which converge on the third complement component, C3, whose activation leads ultimately to microbial opsonization, the release of chemotactic factors, including C5a and C5a, and the generation of a membrane attack complex (MAC) (18). Activation of the classical pathway occurs primarily following the binding of C1q to antibody-antigen complexes, and although involved in the adaptive arm of the immune system, this pathway is also triggered by natural antibodies, thereby contributing to the innate recognition of fungi (16). Initiation of the alternative pathway occurs through the spontaneous activation of C3 on microbial surfaces, and the lectin pathway is initiated by the binding of the mannose-binding lectin to carbohydrates on microbial surfaces (24).

All three of these pathways are induced by opportunistic fungal pathogens, including Candida albicans (13, 15, 24), although the alternative pathway may be the most critical (7, 15). The protective role of complement in immunity to these pathogens has largely been determined in mice treated with cobra venom factor (CVF), which depletes serum complement by forming a potent C3 convertase, or in C5-deficient mouse strains, such as A/J or DHA2, where fungal opsonization remains intact but the animals lack the ability to generate C5a or the MAC (6, 7, 9, 10, 19). Why C5-deficient mice are susceptible is still unclear, but is likely to be due to aberrant inflammatory responses, as the MAC has little effect on the viability of fungal pathogens (15, 19). Although the roles of each of the pathways of complement activation have been relatively poorly examined, recent studies have started to address this issue, looking at the role of factor B, C2, C3b and mannose-binding lectin in the control of systemic candidiasis (11).

C3 is a central component in all of the complement pathways, as described above, but it has not been studied in isolation with respect to fungal infection. Studying the role of this complement component in isolation is particularly relevant, given the alternative route of C3 activation by thrombin, which...
Mice deficient in C3 are susceptible to systemic infection with *C. glabrata*. We also examined the role of C3 in the control of *C. glabrata*, another organism which has been associated with infections in immunocompromised hosts. For these experiments, wild-type and C3-deficient mice were infected i.v. with 7 x 10^7 CFU and survival was monitored over time. In contrast to the wild-type mice, which were fully resistant to infection with *C. glabrata*, as expected (1), nearly all of the C3^-/- mice had succumbed to infection with this organism (Fig. 6). These data therefore demonstrate that C3 is essentially required for resistance to infection with *C. glabrata*.

C3 deficiency promotes mice against high-dose infection with *S. cerevisiae*. Although not normally considered a pathogen, isolates of *S. cerevisiae* have been linked to cause lethal infections in C5-deficient mice (4), and we therefore explored the possibility that C3 would be important for resistance to infection with a nonpathogenic strain of *S. cerevisiae*. For these experiments, we infected wild-type and C3^-/- mice i.v. with a high dose (1 x 10^8 CFU) of a laboratory strain of *S. cerevisiae* and monitored survival over time (Fig. 7). Surprisingly, more than 80% of the C5^-/- wild-type mice died within 24 h following i.v. administration of this organism, whereas the C3^-/- animals all survived the infection. We also examined high-dose infection in DBA/2 mice, which are deficient in the C5 component of complement, and observed that these animals were also fully resistant to high-dose infection with *S. cerevisiae* (Fig. 7). However, we found that infection with this dose of *S. cerevisiae* induced only 14% mortality in wild-type BALB/c mice, suggesting that the level of this response is dependent on the mouse strain background (Fig. 7). Overall, these results demonstrate that C3 can contribute to mortality upon infection with high doses of *S. cerevisiae*.

**DISCUSSION**

Complement plays an important role in immunity to many pathogens: by direct killing through the formation of a MAC, by acting as opsonins and facilitating microbial uptake, and by inducing inflammation. Fungi, in particular, have been known...
to trigger complement activation since the 1990s and have been used to help define the activation pathways (2). Although fungi appear to be resistant to complement-mediated killing, presumably because of their thick cell walls, the opsonic and inflammatory components of complement are thought to play a central role in antifungal immunity (15, 28). The role of complement has, however, largely been determined through the use of CVF, although C5-deficient mice have also been examined. Given the thrombin-dependent, C3-independent, pathway of C5 activation (12, 30), we undertook to examine the role of C3 in immunity to several opportunistic fungal pathogens using C3-sufficient and C3-deficient mice.

*C. albicans* can trigger all three pathways of complement activation, leading to the rapid deposition of C3 on the surface of this organism and facilitating its uptake by phagocytes (15). Animals treated with CVF, or having deficiency in C5, are more susceptible to both systemic and cutaneous candidiasis (15), and here we show that C3 is also an essential component of protective innate immunity to *C. albicans*. Loss of C3 results in increased susceptibility to infection with this organism (Fig. 1 and 2), which is due to defects in fungal clearance (Fig. 5) but not defective inflammatory responses. These results therefore suggest that the increased inflammation seen in the kidneys and blood at 3 days (Fig. 2) is due to higher fungal burdens and not dysregulation of the inflammatory response. Indeed, although not truly representative of systemic infection, using a peritoneal model of candidiasis we could demonstrate that the inflammatory responses in C5-deficient animals were equivalent to those observed in wild-type mice (Fig. 3 and 4). Given that susceptibility due to C5 deficiency results from aberrant inflammatory responses (19), these results suggest that C3 is not essentially required for C5 activation. Under conditions of C3 deficiency, C5 activation is presumably mediated through the thrombin pathway (12, 30).

*C. glabrata* is another species of *Candida* which has been increasingly associated with opportunistic infections in immunocompromised individuals, yet relatively little is known about the host factors which mediate immunity to this organism. Unlike *C. albicans*, *C. glabrata* does not induce a lethal phenotype in immunocompetent mice, even at high inoculums (1). Although this organism can induce the alternative pathway of complement activation (5), the role of complement in the control of *C. glabrata* has not yet been examined. Here we show that mice deficient in C3 are highly susceptible to systemic infection with *C. glabrata*, demonstrating for the first time the essential role of complement in immunity to this organism.

*S. cerevisiae* is not normally considered to be a pathogen, but it has been reported to cause infections under certain settings, such as immunosuppression (15). In mice, the susceptibility of C5-deficient animals following i.v. administration with some clinical isolates has demonstrated the importance of the complement pathway in the control of infection with these micro-
organisms (4). Here we explored the effect of C3 deficiency upon administration of very high doses of a standard laboratory strain of *S. cerevisiae*, with the expectation that the lack of fungal clearance caused by complement deficiency may promote susceptibility, as we had observed for *C. albicans*. Surprisingly, we found that administration of high doses of *S. cerevisiae* caused the rapid death (within 24 h) of C57BL/6 wild-type mice and that deficiency in C3 and C5 was, in fact, protective under these conditions (Fig. 7). Inoculation of a high dose of *C. albicans* has also been shown to induce rapid lethality in mice, and in line with our findings using *Saccharomyces*, the loss of complement function was protective in this model (8). These results therefore suggest that complement 

FIG. 5. C3 deficiency delays fungal clearance. C3−/− and wild-type mice were infected i.v. with 1 × 10^6 CFU of *C. albicans*, and fungal burdens in various organs were determined at 24, 48 and 72 h. *, P < 0.05 (n = 6 animals per group). Bars indicate mean values of the data. Values for 24-h wild type versus C3−/− are as follows: heart, 0.05 × 10^3 ± 0.01 versus 0.17 ± 0.15 CFU/g; lung, 0.02 ± 0.04 versus 0.03 ± 0.05 CFU/g; liver, 0.12 ± 0.07 versus 0.17 ± 0.03 CFU/g; spleen, 1.50 ± 0.05 versus 0.84 ± 0.02 CFU/g; kidney, 10.5 ± 2.5 versus 10.2 ± 2.0 CFU/g. Values for 48-h wild type versus C3−/− are as follows: heart, <100 versus 141 ± 28 CFU/g; lung, <100 versus 3.9 ± 100 CFU/g; liver, 1.9 ± 12 versus 22 ± 22 CFU/g; brain, 0.07 ± 0.05 versus 2.9 ± 1.8 CFU/g; spleen, 503 ± 120 versus 10,000 ± 1,576 CFU/g; kidney, 57,220 ± 26,218 versus 3.8 × 10^3 ± 1.2 × 10^3 CFU/g. Values for 72-h wild type versus C3−/− are as follows: heart, <100 versus 181 ± 81 CFU/g; lung, <100 versus 920 ± 513 CFU/g; liver, 112 ± 12 versus 211 ± 61 CFU/g; brain, 153 ± 54 versus 572 ± 192 CFU/g; spleen, 429 ± 113 versus 1,072 ± 796 CFU/g; and kidney, 9,835 ± 5,412 versus 2.3 × 10^3 ± 9.4 × 10^2 CFU/g.

FIG. 6. C3 deficiency increases susceptibility to infection with *C. glabrata*. C57BL/6 wild-type (○) and C3-deficient (●) mice were infected with 1 × 10^6 CFU of *C. glabrata* i.v. and were monitored for survival over 21 days, as described in Materials and Methods. The data shown are pooled from two independent experiments. *, P < 0.05.

FIG. 7. C3 and C5 deficiency protects against lethality induced upon high-dose infection with *S. cerevisiae*. C57BL/6 wild-type or C3-deficient, DBA/2, and BALB/c mice were infected with 1 × 10^6 CFU of *S. cerevisiae* i.v. and were monitored for survival over 21 days, as described in Materials and Methods. The data shown are pooled from two independent experiments. *, P < 0.05.
itself acts in a deleterious fashion upon high dose inoculation, irrespective of the fungal species; however, the mechanisms for these effects are still unclear. Particularly intriguing is the apparent strain specificity in this response (the effect was far less marked in BALB/c mice than in C57BL/6J mice).

In conclusion, the use of gene-deficient mice has provided invaluable insights into the function of individual components of many biologic processes. We have shown here that C3 plays an essential role in the control of opportunistic fungal infections, but it may also contribute to mortality under certain conditions. Although our studies with C3-deficient mice suggest that the primary function of C3 is in mediating fungal clearance, the exact role of this complement component in the control of other fungal pathogens still needs to be explored.

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SHORT COMMUNICATION:

Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors


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Recognition of microbial components by germ-line encoded pattern recognition receptors (PRR) initiates immune responses to infectious agents. We and others have proposed that pairs or sets of PRR mediate host immunity. One such pair comprises the fungal β-glucan receptor, Dectin-1, which collaborates through an undefined mechanism with Toll-like receptor 2 (TLR2) to induce optimal cytokine responses in macrophages. We show here that Dectin-1 signaling through the spleen tyrosine kinase (Syk) pathway is required for this collaboration, which can also occur with TLR4, 5, 7 and 9. Deficiency of either Syk or the TLR adaptor MyD88 abolished collaborative responses, which include TNF, MIP-1α and MIP-2 production, and which are comparable to the previously described synergy between TLR2 and TLR4. Collaboration of the Syk and TLR/MyD88 pathways results in sustained degradation of the inhibitor of kB (IκB), enhancing NFKB nuclear translocation. These findings establish the first example of Syk- and MyD88-coupled PRR collaboration, further supporting the concept that paired receptors collaborate to control infectious agents.

Key words: C-type lectin - Innate immunity - Macrophage - Syk - TLR

Introduction

Originally identified in Drosophila, the Toll-like receptors (TLR) consist of a family of at least 11 proteins, which recognize a diverse, but receptor-specific range of microbial structures. Ligand recognition leads to TLR homo- or heterodimerization and the initiation of

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indicates that the defect in these animals is due to the lack of collaborative signaling from the Dectin-1 pathway. Receptor collaboration may also explain the previously observed synergy between mannans, which may be TLR ligands, and β-glucan fungal cell wall components in the response to these organisms [7, 20]. These results thus highlight the importance of receptor collaboration during infection as opposed to receptors functioning in isolation, and further support the concept that pairs or sets of receptors collaborate to control infectious agents.

While Syk has been implicated in the modulation of TLR responses in other systems [10, 21–24], the interaction between Dectin-1 and Syk occurs through a novel mechanism [9], which is likely to be representative of other receptors involved in pathogen recognition such as the HIV-1 receptor CLEC-2 and CD66d, a PRR for Neisseria gonorrhoeae and Haemophilus influenzae [25–28]. Recently, it has also been proposed that the adaptor CARD9, which is downstream of Syk in myeloid cells, plays a role in TLR signaling [29]. We propose an alternative explanation in which Syk-and CARD9-coupled PRR collaborate with TLR to control infectious agents.

Materials and methods

Reagents and mice

All TLR ligands were from InvivoGen (San Diego, CA), and the production of highly purified particulate β-glucan and soluble β-glucan (glucan phosphate) have been described elsewhere [15, 29]. BALB/c, C57BL/6 and C57BL/6 MyD88−/−, 129/Sv and 129/Sv Dectin-1−/− [7] mice were obtained from the animal unit of the University of Cape Town. BALB/c Syk−/− chimeric mice were generated by the transfer of Syk−/− fetal liver cells into irradiated BALB/c recipients as described [9]. All procedures were approved by the University of Cape Town animal ethics committee.

Cell stimulation

Murine thioglycollate-elicited macrophages were plated at 106 cells/mL in RPMI medium containing 10% FCS (Gibco) and incubated overnight. Medium was replaced, and cells were stimulated with 10 μg/mL particulate β-glucan or 10 ng/mL Pam3CSK4, unless otherwise indicated, for 3 h. Human peripheral monocytes were prepared as described [20, 31] and stimulated with 10 ng/mL particulate β-glucan and relevant TLR ligands for 20 h. Cytokine secretion was assayed by ELISA using kits from Becton Dickinson (Mountain View, CA), R&D Systems (Abingdon, UK) or KOMA Biotechnology (Korea). Data were analyzed using the Student’s t-test.

Syk and SLP-76 phosphorylation

RAW 264.7 cells expressing Dectin-1 [12] (103 cells in 100 μL HBSS) were stimulated with 10 μg/mL soluble β-glucan (glucan phosphate) for 1 min at 37 °C before addition of lysis buffer (1% NP40, 25 mM Tris pH 8, 10 mM EDTA, 140 mM NaCl, 5 mM NaF, 1 mM NaVO4, 5 mM iodoacetamide) containing protease inhibitors (Roche). Lysate supernatants were incubated for 2 h with 30 μL streptavidin-agarose beads (Fluka) precoated with 25 μM biotinylated Dectin-1 phosphopeptide to precipitate Syk [9] or 30 μL protein G-Sepharose beads (Amersham, UK) precoated with 5 μg SLP-76 mbA. Beads were washed with lysis buffer, boiled in 50 μL SDS-PAGE sample buffer, and samples were Western-blotted and probed with phosphoantibodies mAb 4G10 (Becton Dickinson) and antibodies to Syk or SLP-76 (Santa Cruz Biotechnology, CA). For phospho-Syk staining, 129Sv thioglycollate-elicited macrophages were stimulated with 10 μg/mL particulate β-glucan for 5 min, fixed with paraformaldehyde, blocked and permethylated with Triton X-100. Cells were stained with anti-phosphoSyk (CellSignalling) followed by donkey anti-rabbit Cy3 (Jackson Immunoresearch) and analyzed by fluorescent microscopy.

IKK degradation and NFκB nuclear localization

RAW 264.7 cells expressing Dectin-1 placed at 2 × 103 cells/mL were stimulated with 10 μg/mL particulate β-glucan and 10 ng/mL Pam3CSK4 for 2 h, or the indicated times, before lysis and Western blotting of whole cell lysates with antibodies to IκB or actin (CellSignaling, MA) as a loading control. Nuclear lysates were prepared after 20 h stimulation as described [32], followed by Western blotting with antibodies to NFκB c-Rel or USF-2 (Santa Cruz Biotechnology) as a loading control. Band intensities were quantified using NIHImage software.

NFκB nuclear translocation

C57BL/6 thioglycollate-elicited macrophages plated at 2 × 105 cells/mL were stimulated with 10 ng/mL Pam3CSK4 for 90 min followed by 10 μg/mL particulate β-glucan for 60 min, fixed with paraformaldehyde, blocked and permethylated with Triton X-100. Cells were stained with Hoechst and NFκB c-Rel antibody, followed by anti-rabbit IgG-Cy3 (Jackson laboratories, USA) and analyzed by fluorescent microscopy. Three independent fields containing >100 cells were counted for each stimulation type, and data were analyzed using the Student's t-test.

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