The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

The functional characterisation of the C-type lectin: Clecsf8

University of Cape Town

Lisa Maria Graham

Department of Clinical Laboratory Sciences
Division of Immunology
Faculty of Health Sciences

A thesis submitted for the degree of Doctor of Philosophy at the University of Cape Town, June 2011

This thesis is dedicated to my parents

University of Cape Town

Table of Contents

Declaration (vi)
Abstract (vii)
Acknowledgements (viii)
Abbreviations (ix)

Chapter 1: Introduction

1.1 Innate immunity and leukocyte receptors 1
1.2 C-type lectins and lectin-like receptors 2
1.3 Activation and Inhibitory receptors 4
1.4 Selected functions of C-type lectins 8
1.5 The Dectin-2 family of C-type lectins: an overview 10
1.5.1 Blood dendritic cell antigen 2 (BDCA-2) 11
1.5.2 Dendritic Cell Immunoactivating Receptor (DCAR) 16
1.5.3 Dendritic cell Immunoreceptor (DCIR) 16
1.5.4 Dendritic Cell-associated C-type lectin-2 (Dectin-2) 20
1.5.5 Macrophage inducible C-type lectin (Mincle) 26
1.5.6 Clecsf8 33
1.6 Concluding remarks and aims of this thesis 34

Chapter 2: Materials and Methods

2.1 General reagents 36
2.2 Commonly used solutions 36
2.3 Cells 38
2.3.1 Cell lines 38
2.3.2 General cell culture 39
2.3.3 Primary cells 40
2.3.3.1 Isolation of human peripheral blood leukocytes (PBLs) 40
2.3.3.2 Isolation and culture of human monocytes and *in vitro* derived macrophages/DCs 40
2.3.3.3 Purification of human peripheral blood neutrophils 41
2.3.3.4 Cytokine and TLR agonist stimulation of cells 42
2.3.4 Generation of transduced cell lines 42
2.3.4.1 Retrovirus production 42
2.3.4.2 Retroviral transduction of cell lines 43
2.3.4.3 Transient transfection using Lipofectamine 43
2.4 General nucleic acid manipulation techniques 44
2.4.1 Polymerase chain reaction (PCR) 44
2.4.2 Agarose gel electrophoresis 44
2.4.3 Purification of DNA from agarose gels 45
2.4.4 TOPO cloning 45
2.4.5 Plasmid digestion 45
2.4.6 Ligation of digested vectors and insert DNA 46

University of Cape Town

2.4.7 Transformation of competent cells 46
2.4.8 Colony PCR 47
2.4.9 Production and isolation of plasmid DNA 47
2.4.10 Sequencing 48
2.5 Nucleic acid manipulation specific to this thesis 48
2.5.1 Generation of mClecsf8-HA, hClecsf8-HA, Mincle-HA, Dectin-2-HA and Dectin-1-HA 48
2.5.2 Generation of chimeric constructs 49
2.5.3 Other constructs 51
2.5.4 Generation of soluble FcClecsf8 fusion protein 51
2.6 Flow cytometry 52
2.7 Phagocytosis assays 53
2.7.1 Phagocytosis of zymosan particles by transduced RAW264.7 cells 53
2.7.2 FITC labelling and antibody coupling of Pan mouse IgG Dynabeads 54
2.7.3 Binding of anti-hClecsf8 coated beads by transduced RAW264.7 cells 55
2.7.4 Phagocytosis of anti-hClecsf8 coated beads by human neutrophils 55
2.8 Zymosan binding and cytokine production assays 56
2.9 Respiratory burst assays 57
2.9.1 Respiratory burst assay in RAW264.7 cells 57
2.9.2 Respiratory burst assay in neutrophils 57
2.10 Protein analyses 58
2.10.1 Cell lysates and SDS-PAGE 58
2.10.2 Western blotting 59
2.10.3 Immunoprecipitations 60
2.11 Ligand screening 61
2.11.1 Carbohydrate microarray screening 61
2.11.2 Endogenous ligand screening 61
2.12 Mice 62
2.12.1 DNA extraction and genotyping 62
2.12.2 Infection with *Candida Albicans* 63
2.12.3 Infection with *Listeria monocytogenes* 63
2.12.4 Infection with *Staphylococcus aureus* 64
2.12.5 Infection with *Nippostrongylus brasiliensis* 64
2.12.6 Sterile peritonitis 65
2.12.7 Peritoneal inflammation in response to necrotic EL4 cells 65
2.12.8 Neutrophil infiltration induced by thymocyte death 65
2.13 Statistics 66

Chapter 3: Characterisation of Clecsf8 expression and regulation

3.1 Introduction 67
3.2 Results 69
3.2.1 Distribution and regulation of Clecsf8 in human peripheral blood 69
3.2.2 Clecsf8 does not associate with known adaptors for expression at the cell surface 77

University of Cape Town

3.2.3 Intracellular retention of Clecsf8 requires the CRD 82
3.2.4 The intracellular retention of Clecsf8 is unique in requiring the CRD 84
3.3 Discussion 87

Chapter 4: Functional characterisation of Clecsf8

4.1 Introduction 91
4.2 Results 93
4.2.1 Clecsf8 ligand screening 93
4.2.1.1 Carbohydrate microarray analyses 93
4.2.1.2 Endogenous ligand screening 94
4.2.2 Clecsf8 functional assays 97
4.2.2.1 Clecsf8 mediates phagocytosis 100
4.2.2.2 Clecsf8 induces the production of TNFα 103
4.2.2.3 Clecsf8 can mediate the respiratory burst 105
4.2.2.4 Clecsf8 can signal via Syk kinase 107
4.3 Discussion 111

Chapter 5: *In vivo* characterisation of Clecsf8

5.1 Introduction 119
5.2 Results 121
5.2.1 Genotyping 121
5.2.2 The role of Clecsf8 in the inflammatory response 123
5.2.2.1 Thioglycollate induced inflammation 123
5.2.2.2 Necrosis induced inflammation 124
5.2.3 The role of Clecsf8 in infection 126
5.2.3.1 Fungal infections 128
5.2.3.2 Helminth infections 128
5.2.3.3 Bacterial infections 131
5.3 Discussion 133

Chapter 6: General summary and future directions

6.1 Review of the aims of this project 137
6.2 Summary of key findings 138
6.3 Future directions 139
6.3.1 Generate anti-mClecsf8 monoclonal antibody 139
6.3.2 Ligand screening and physiological function 141
6.3.3 Adaptor molecule identification 143
6.4 Conclusion 145

References 147

Appendix I: Publications 158

University of Cape Town

Declaration

I, Lisa Graham, 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.

I empower the University of Cape Town to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

University of Cape Town

____

Lisa Graham

June 2011

Abstract

Characterisation of the C-type lectin: Clecsf8

Lisa Graham

Division of Immunology, Faculty of Health Sciences

Submitted for the degree of Doctor of Philosophy, June 2011

Characterisation of the receptors belonging to the "Dectin-2 cluster" of C-type lectins has revealed exciting new insights into the functions of these molecules in both pathogen recognition and maintenance of homeostasis. Clecsf8 is a poorly characterised member of the "Dectin-2 cluster" and was originally thought to be expressed exclusively by macrophages. In this study it was demonstrated that Clecsf8 is primarily expressed by peripheral blood neutrophils and monocytes. The expression of this receptor is lost upon differentiation of monocytes into macrophages or dendritic cells, suggesting a role for the receptor in innate immunity. Clecsf8 can additionally be slightly up-regulated upon exposure to certain pro-inflammatory cytokines and microbial components. Like the other members of the Dectin-2 family, which require association of their transmembrane domains with signalling adaptors for surface expression, Clecsf8 is retained intracellularly when expressed in non-myeloid cells. However, it was demonstrated that Clecsf8 does not associate with any known signalling adaptor molecules, including DAP10, DAP12, or the FcRγ chain, and it was found that the carbohydrate recognition domain of Clecsf8 was responsible for its intracellular retention. Although Clecsf8 does not contain a signalling motif in its cytoplasmic domain, it was found that this receptor is capable of inducing signalling via Syk kinase in myeloid cells and that it can induce phagocytosis, pro-inflammatory cytokine production and the respiratory burst. This work additionally describes the search for a natural ligand for Clecsf8, which remains unidentified. Furthermore, the study characterised Clecsf8 deficient mice in an attempt to find a physiological role for the receptor and found that Clecsf8 deficient mice did not have defects in their ability to resist infection with fungi, bacteria or nematodes. Additionally, no effect of Clecsf8 deficiency was observed in models of sterile peritonitis or response to necrotic cell death. These data therefore indicate that Clecsf8 functions as an activation receptor on myeloid cells through an association with a novel adaptor molecule.

University of Cape Town

Acknowledgements

Firstly I would like to thank my supervisor Professor Gordon Brown, for his guidance, support, encouragement and patience. I feel extremely privileged to have been given the opportunity to work with and learn from him.

I would also like to thank Janet Willment, Vicky Tsoni and Kevin Dennehy for the time and expertise they so generously shared with me. Their support and guidance was invaluable to me. Cristal Huysamen, Ann Kerrigan and Matti Kimberg made my experience in the lab positive and vibrant. I am very grateful to them for their love, friendship, help, support and hours of mopping up my tears. I want to thank all the other members of the Brown lab: Georgia, Ines, Elwira, Chris, Gloria, Naren, Vandana and Janice, who all helped me along the way and provided a great working environment.

I would especially like to thank all my friends for their endless support and advice throughout this project, especially Ilze and Claire. Your encouragement and belief in me made this possible. Finally I am sincerely grateful to my fantastic parents who provided me with this wonderful opportunity. I owe everything I have achieved to them.

University of Cape Town

Abbreviations used in this thesis

Aa	Amino acid
AA	Arachidonic acid
Ab	Antibody
APC	Allophycocyanin
APC	Antigen presenting cell
BCR	B cell receptor
BSA	Bovine serum albumin
BMDC	Bone-marrow-derived DC
CEA	Carcinoma embryonic antigen
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CFU	Colony forming unit
CRD	Carbohydrate recognition domain
CTLD	C-type lectin-like domain
DAMP	Damage-associated molecular pattern
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
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
HA	Hemagglutinin
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
kDa	Kilodalton
LB	Luria Bertani
LPS	Lipopolysaccharide
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
moDC	Monocyte-derived DC
MS	Mass spectrometry
MUC1	Mucin 1
NK	Natural killer

University of Cape Town

NKC	Natural killer gene complex
NLR	Nod-like receptors
NOD	Nucleotide-oligomerisation domain
ns	Not significant
PAMP	Pathogen-associated molecular pattern
PBL	Peripheral blood leukocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid DC
PE	Phycoerythrin
PRR	Pattern recognition receptor
RIG-1	Retinoic acid-inducible gene 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Standard deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEA	Soluble egg antigen
SEM	Standard error of the mean
SSC	Side scatter
SH2 domain	Src homology 2 domain
SH3 domain	Src homology 3 domain
TAA	Tumour associated antigen
TCR	T cell receptor
TDB	Trehalose 6,6'-dibehenate
TDM	Trehalose 6,6'-dimycolate
TGF	Transforming growth factor
TH_1	T-helper 1
TH_2	T-helper 2
TH_{17}	T-helper 17
Thr	Threonine
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
Trp	Tryptophan
UV	Ultraviolet

University of Cape Town

Protein abbreviations / common names

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

BCL10	B-cell 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
CCR	C-C chemokine receptor
CEACAM3	Carcinoembryonic antigen-related cell adhesion molecule 3
CXCL2	Chemokine (C-X-C motif) ligand 2 (CXCL2)
DAP10	DNAX-activating protein of 10kDa
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
Dectin-2	Dendritic cell-associated C-type lectin-2
DNGR-1	DC, NK lectin group receptor-1
ERK	Extracellular regulated mitogen activated protein kinase
FcRγ	Fc receptor gamma chain
G-CSF	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
ICAM-2	Inter-Cellular Adhesion Molecule 2
LOX-1	Lectin-like oxidised low-density lipoprotein receptor-1
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAPK	Mitogen-activated protein kinase
MBL	Mannose binding lectin
MGL	Macrophage galactose type C-type lectin
MICL	Myeloid inhibitory C-type lectin-like receptor
Mincle	Macrophage inducible C-type lectin
MR	Mannose receptor
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear Factor Kappa B
NFAT	Nuclear factor of activated T-cells
NKG2D	Natural-killer group 2, member D
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
SAP130	Spliceosome-associated protein 130
SHIP	Src homology 2 domain-containing inositol 5-phosphatase
SHP-1	Src homology 2 domain-containing phosphatase 1
SHP-2	Src homology 2 domain-containing phosphatase 1
SLP-65	SH2 domain containing leukocyte protein of 65kDa
Syk	Spleen tyrosine kinase
TRAIL	TNF-related apoptosis-inducing ligand

University of Cape Town

Chapter 1

Introduction

1.1 Innate immunity and leukocyte receptors

The innate immune system offers a rapid first line of defence against invading pathogens and includes physical barriers such as skin, mucous membranes and secretions, as well as complex responses carried out by specific leukocytes and their receptors. Innate immune recognition relies on a broad range of leukocyte receptors which are functionally diverse and play essential roles in host defence and homeostasis. Cellular receptors are divided into families and superfamilies based on their function or the presence of defined structural domains in their amino acid sequence. Members of a family that are structurally similar have usually evolved from a common ancestor but the presence of structurally similar domains does not necessarily signify common functions. Indeed, proteins have often come to possess similar domains as a result of shuffling, adding or deleting domains [1]. The immunoglobulin superfamily (IgSF) is one of the largest structurally defined groups [1]. The members of this group all contain the Ig domain but can be diverse in terms of sequence structure and functions, such as those of antibody receptors, MHC proteins, antibodies and lymphocyte cell-surface proteins [1].

The ligands for cellular receptors vary greatly, ranging from endogenous ligands to specific molecular structures found in microbes called pathogen associated molecular

University of Cape Town

patterns (PAMPs). PAMPs are generally not found in the host, enabling discrimination between self and non-self, and are usually essential for the microbe's survival, limiting the possibility of escape via mutation [2]. Pattern recognition receptors (PRRs) are germ-line encoded receptors which specifically recognise PAMPs and provide the host with the ability to recognise a wide range of pathogens quickly, without the delay of the lengthy somatic mutations which occur in adaptive immunity [2]. Recognition can take place either directly via receptors expressed on the cell surface, in intracellular vesicles or in the cytoplasm, or indirectly whereby soluble receptors coat or opsonise the pathogen, allowing recognition via opsonic receptors. Toll-like receptors (TLRs) were the first group of signalling PRRs to be described. TLRs are present on the cell surface or within endosomes and have been shown to detect Gram-positive and Gram-negative bacteria, mycobacteria, RNA and DNA viruses, fungi and protozoans [3]. Other families of innate receptors include the NOD-like receptors (NLRs), which detect bacteria, the RIG-1-like receptors which detect viruses and the C-type lectins, which are the main focus of this study and will be discussed in detail [4].

University of Cape Town

1.2 C-type lectins and lectin-like receptors

C-type lectins are a large family of receptors which all contain at least one common fold known as the carbohydrate recognition domain (CRD), which in general contains six conserved cysteine residues that form three intra-chain disulfide bonds, stabilizing the fold [5-7]. This domain was more recently renamed the C-type lectin-like domain (CTLD) [6], but for clarity I will use CRD in this thesis. The C-type lectin superfamily

has been divided into 17 families based on the overall domain architecture or phylogenetic relationships (Figure 1.1) [6, 8, 9]. They can either be produced as soluble or transmembrane proteins, for example Mannose binding lectin (MBL), which is produced by hepatocytes and secreted into the serum, or DC-SIGN, which is expressed on the surface of DCs, macrophages and endothelium [10, 11]. Transmembrane receptors can have their amino-terminus directed either into or out of the cytoplasm and are termed, type II and type I respectively [12].

Historically, due to the presence of at least one CRD, these proteins were originally all thought to bind carbohydrates but have since been shown to have diverse ligands, including proteins, lipids and even inorganic compounds such as $CaCO_3$ and ice [13-17]. C-type lectins can therefore be broadly divided into two categories: Classical C-type lectins contain conserved residues in their CRDs which are responsible for forming Ca^{2+} binding sites and also generally contain conserved motifs which typically bind carbohydrate ligands, such as the EPN amino acid triplet which binds mannose-type carbohydrates or the QPD triplet which binds galactose-type carbohydrates [6]. Non-classical C-type lectins or lectin-*like* receptors generally do not contain these residues and are more likely to, but do not necessarily, bind non-carbohydrate ligands, such as those encoded by the Natural killer gene complex (NKC) for example, which control cellular activation by recognition of MHC class I molecules [8, 18]. Although the presence of these residues has previously been used to predict whether a C-type lectin of unknown function was likely to bind a sugar, it is important to note there are a growing number of examples of C-type lectins that do not contain the conserved motifs but do recognise

University of Cape Town

carbohydrates, such as Dectin-1 which recognises β-glucan [19]. C-type lectins have been shown to have diverse functions in both immunity and homeostasis; however the natural ligands and physiological functions of numerous receptors remain undefined. These functions are generally mediated by C-type lectin signalling via specific motifs which will be briefly discussed below.

1.3 Activation and Inhibitory receptors

The functional balance between cellular activation and inhibition can be controlled by receptors that signal via consensus signalling motifs (Figure 1.2). Inhibitory receptors generally possess an immunoreceptor tyrosine-based inhibitory motif (ITIM), while activation receptors contain an immunoreceptor tyrosine-based activation motif (ITAM). ITAMs were identified as common sequences in the signalling domains associated with the T cell receptor (TCR), B cell receptor (BCR) and FcεRI [20]. Several C-type lectins have been shown to signal through ITAMs and ITIMs to control cellular outcomes. This balance between cellular activation or inhibition controlled by C-type lectins is well described for NK cell function in response to virally infected and transformed cells [21, 22]. In particular, C-type lectins on NK cells recognise ubiquitously expressed MHC class I molecules. In the absence of MHC class I expression, due to infection or transformation, NK cells are released from the inhibitory effects of the interaction and kill the target cell [23]. More recently, the identification of C-type lectins on myeloid cells has raised the possibility that they may also serve functions in cellular activation and inhibition. Some C-type lectins have intrinsic signalling sequences in their cytoplasmic

University of Cape Town

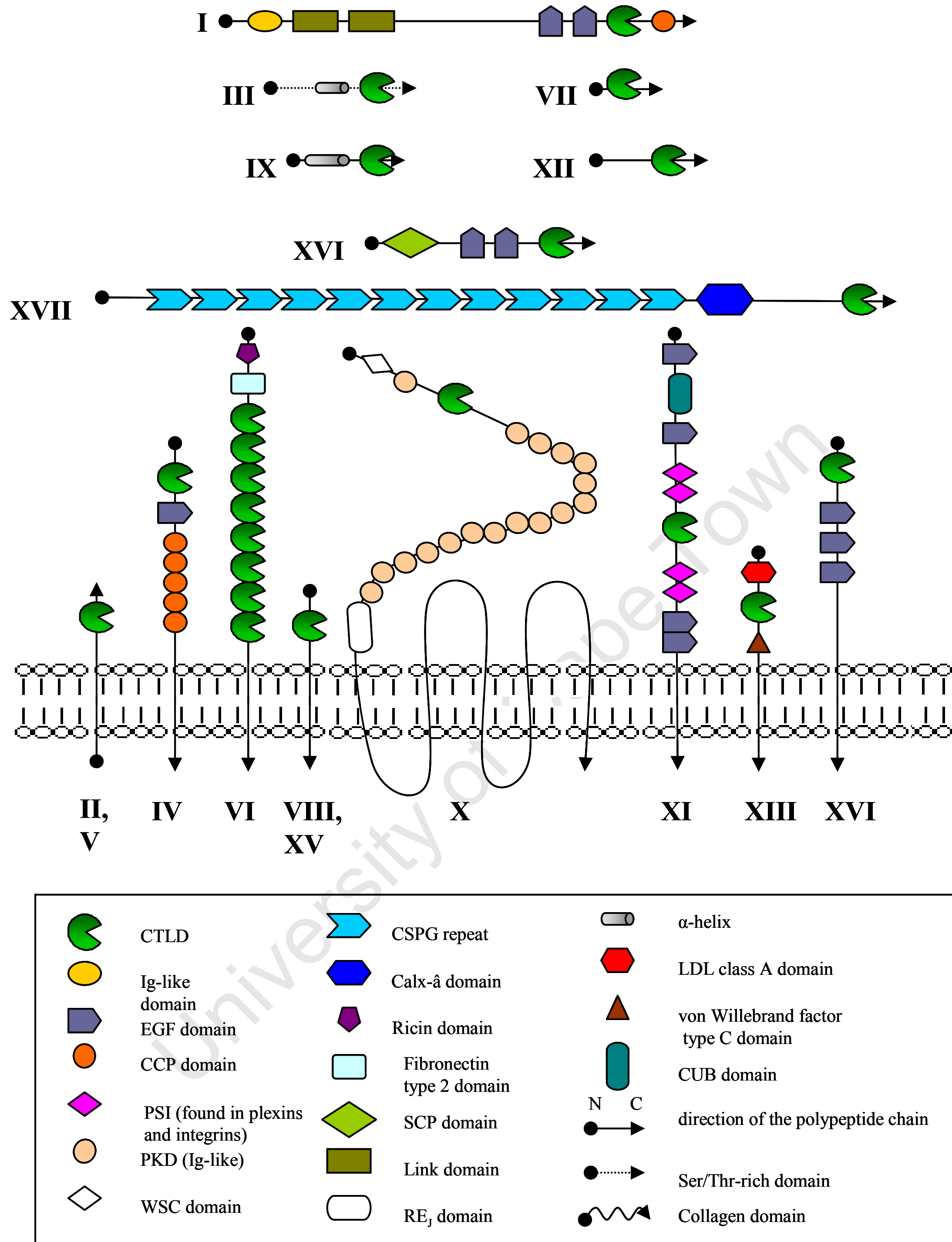


Figure 1.1 **Domain architecture of vertebrate C-type lectin domains from different groups.** Group numbers are indicated next to the domain charts. I- lecticans, II- the ASGR group, III- collectins, IV- selectins, V- NK receptors, VI- the macrophage mannose receptor group, VII- REG proteins, VIII- the chondrolectin group, IX- tetranectin group, X- polycystin 1, XI- attractin, XII- EMBP, XIII- DGCR2, XIV- thrombomodulin group, XV- Bimlec, XVI- SEEC, XVII- CBCP. Adapted from Zelensky and Gready [9].

domains (such as Dectin-1), while several others associate with ITAM-containing adaptor molecules (such as DAP10, DAP12 or FcRγ-chain) for signalling [24, 25]. These adaptors have minimal extracellular domains and no ligand binding capability and usually associate with ligand-binding receptors through complimentary charged residues in their transmembrane or cytoplasmic domains.

An ITAM's loose consensus sequence is YXXL/I(X_{6-8})YXXL/I (where X represents any amino acid) [20]. The signals produced by ITAM-containing receptors result in activation of cellular responses, including cellular adhesion and migration, proliferation and differentiation, as well as phagocytosis, degranulation and cytokine production, for example. Signalling through an ITAM results in tyrosine phosphorylation by Src family kinase which leads to recruitment and activation of Src homology 2 (SH2) domain-containing proteins, such as Syk kinase [26]. In myeloid cells, ITAM based signalling via Syk leads to caspase-recruitment domain (CARD) 9 aggregation with the adaptor protein B cell lymphoma 10 (BCL10) and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) [27]. This CARD9-BCL10-MALT1 pathway relays receptor proximal events to transcription factors, such as nuclear factor (NF)-κB, which controls expression of pro-inflammatory genes in immune cells [27, 28].

The consensus sequence for an ITIM is I/V/L/SXYXXL/V. ITIM-bearing receptors also become phosphorylated by Src family kinases, providing a docking site for SH2-containing phosphatases which dephosphorylate the tyrosines of activation kinases,

University of Cape Town

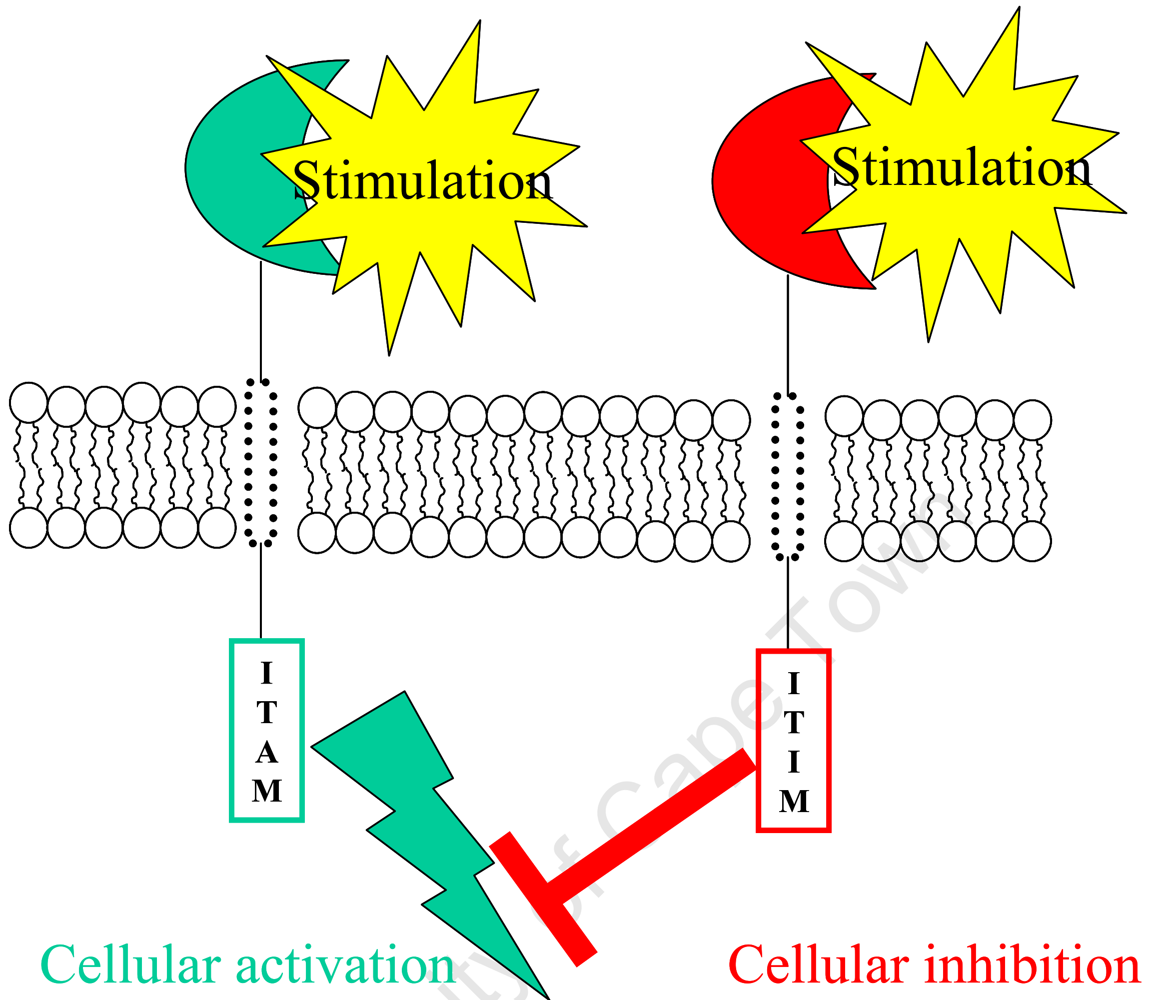


Figure 1.2 **Paired immunoreceptors maintain the balance between cellular activation and inhibition.** Schematic representation of an activation receptor which contains an ITAM and an inhibitory receptor which contains an ITIM. Stimulation of the receptors results in cellular activation or inhibition, respectively.

University of Cape Town

thereby down-modulating cellular activation [29]. Recently however, it has become apparent that this dichotomy is not always clear cut, as some ITAMs have been shown to mediate cellular inhibition while ITIMs induced activation [30, 31]. As mentioned, C-type lectin signalling via these motifs triggers a range of cellular responses, a number of which will be discussed below.

1.4 Selected functions of C-type lectins

C-type lectins elicit a vast array of cellular responses necessary for their role in immunity to infections as well as maintenance of homeostasis. These responses include cytokine production, adhesion and migration of cells, leukotriene production, recognition of cellular stress or transformation, phagocytosis and induction of the respiratory burst, to name a few. In this section I will briefly introduce some of the functions of C-type lectins which are relevant to this thesis.

University of Cape Town

Phagocytosis is an active process involving the ingestion of large particles in an actin-dependent manner and is involved in many biological processes, including pathogen elimination and clearance of apoptotic cells [32]. A number of myeloid C-type lectins have been shown to function as phagocytic receptors for both endogenous and exogenous ligands, including Clec-2 and Dectin-1 for example [33-35]. These receptors often contain specific internalisation motifs in their cytoplasmic domains, such as triads of acidic amino acids and tyrosine based motifs [36]. Interestingly, signalling via Syk kinase is required for Dectin-1 dependent phagocytosis in dendritic cells and NIH3T3 cells, but not in macrophages, indicating the existence of independent signalling pathways in

different cell types [34, 37]. The process of phagocytosis begins with a receptor recognising a ligand on the surface of a particle. This recognition could either be direct, mediated by cell-based receptors or indirect, whereby soluble receptors (such as surfactants) coat the particle for recognition by opsonic receptors. The phagocyte then undergoes actin polymerization at the site of recognition and the particle is ingested by way of an actin-based mechanism into a phagosome. The phagosome then matures by a series of fusion and fission events with the endocytic pathway, generally culminating in the formation of a phagolysosome and degradation of the particle. Importantly, the ability of C-type lectins to mediate phagocytosis has implication in both pathogen clearance and antigen presentation to T cells.

Upon pathogen ingestion, phagocytes undergo dramatic physiological and biochemical changes that require the rapid consumption of oxygen and result in the production of superoxide anions. This process is known as the respiratory burst, the function of which is to generate microbicidal agents essential for killing a number of microorganisms. Signalling via the C-type lectin Dectin-1 for example, has been shown to induce the respiratory burst in macrophages, neutrophils, DCs and mast cells, in response to stimulation with zymosan and *Candida albicans* [38-42]. The importance of this antimicrobial function is made clear in patients with chronic granulomatous disease, a genetic disease characterized by the inability of patients to produce normal amounts of superoxides. These patients are considered to be immunocompromised as they are generally much more susceptible to infections with both bacteria and fungi [43, 44].

University of Cape Town

As mentioned, C-type lectins are able to phagocytose apoptotic cells and maintain homeostatic cell turnover. Apoptosis is generally non-immunogenic, while non-physiological cell death or necrosis, which can come about by infection or other pathological processes, can be seen as a potential threat to an organism and may result in immunogenic responses [45]. Recently, a number of C-type lectins have been shown to recognise preformed endogenous adjuvants called damage-associated molecular patterns (DAMPs), which are released during necrosis [46, 47]. This recognition may result in activation of local APCs which possibly explains how adaptive immune responses can be stimulated in the absence of infectious agents [48].

Signalling via C-type lectins clearly elicit a variety of functions beyond the scope this discussion, but of specific importance to this thesis are the functions carried out by the Dectin-2 family of C-type lectins.

University of Cape Town

1.5 The Dectin-2 family of C-type lectins: an overview

The Dectin-2 cluster is comprised of Dectin-2, DCIR, DCAR, BDCA-2, Mincle and Clecsf8, which are members of the Group II C-type lectin family. The genes belonging to this family are clustered in the telomeric region of the NK-gene cluster on human chromosome 12 and mouse chromosome 6 [9]. They are all encoded by six exons and share a common structure, consisting of a single extracellular CRD, a stalk region of varying length, a transmembrane region, and a cytoplasmic domain which is generally short (with the exception of DCIR; Figure 1.3). The receptors demonstrate type II

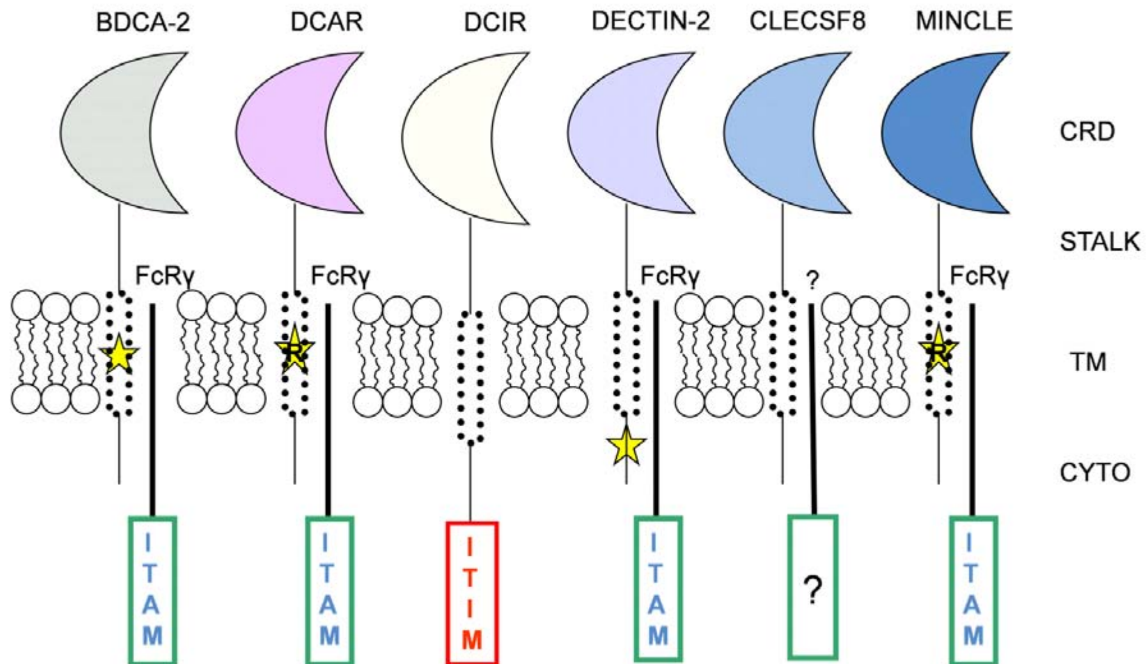
transmembrane topology and are classical C-type lectins, and indeed Dectin-2 and Mincle have been shown to bind sugar ligands, but there are also non-carbohydrate ligands such as SAP130, an endogenous protein recognized by Mincle. Members of this family generally lack consensus signalling motifs in their cytoplasmic domains; however the presence of a positively charged residue in the transmembrane or cytoplasmic regions of most receptors aids the association with the ITAM-containing FcRγ chain adaptor molecule. DCIR is the only member of this family to contain an integral ITIM signalling motif [49].

University of Cape Town

The Dectin-2 cluster has revealed exciting new insights into C-type lectin functions, although the physiological roles of many members remain poorly defined. The functions of this cluster of receptors include roles as PRRs for fungal, mycobacterial and viral pathogens, as well as necrotic cell-death. Facilitation of antigen cross-presentation reveals a role in development of adaptive immune responses. Additional functions include the control of the development of autoimmunity and maintenance of homeostasis. I will discuss each member of the family individually, describing expression, ligand recognition, signalling and known physiological functions.

1.5.1 Blood dendritic cell antigen 2 (BDCA-2)

BDCA-2 is most well known as a specific marker for human plasmacytoid DCs (pDCs). BDCA-2 was originally identified by a monoclonal antibody panel raised against $CD4^{+}$ blood DCs, and found to be exclusively expressed on $CD11c^{-}CD123^{high}$ pDCs with expression being lost upon maturation with IL-3 [50]. BDCA-2 transcript was weakly



University of

Figure 1.3 **Cartoon representation of the Dectin-2 family of C-type lectins.** Members of the Dectin-2 family of C-type lectins are type II proteins with a single C-terminal extracellular carbohydrate recognition domain (CRD), a stalk region, a transmembrane region (TM), and a cytoplasmic domain (CYTO). DCIR contains an immunoreceptor tyrosine-based signalling motif (ITIM) in its cytoplasmic domain, while BDCA-2, DCAR, Dectin-2 and Mincle associate with FcRγ chain which contains an ITAM. It is not yet known whether Clecsf8 associates with an adaptor molecule (?). The star represents the region responsible for association with the adaptor. R: arginine. From Graham et al. [53].

detected in tonsils, bone marrow, pancreas, testis, ovary, lymph nodes and peripheral blood leukocytes [51, 52]. In tonsils, BDCA-2 protein expression was restricted to $CD123^+$ pDCs found in the T cell-rich areas but not in the germinal centres [51]. Similarly, in immunohistochemical analysis of testis obtained from patients with testicular cancer, neoplastic and normal epithelium were negative for BDCA-2 staining while $CD123^+$ pDCs were found to associate with lymphoid aggregates in tumours [51]. This suggested that expression of BDCA-2 in tissues was linked to infiltrating pDCs and it is now widely accepted that BDCA-2 expression is in fact restricted to pDCs. At least five truncated BDCA-2 mRNA species have been detected, which if translated would give rise to variants lacking different domains, including three variants lacking the transmembrane domain, which may represent soluble forms of the receptor [51, 52]. To date, the murine homologue(s) of BDCA-2 remain unknown. BDCA-2 contains an EPN motif in the CRD but ligand(s) for this receptor have not yet been identified. Furthermore, the ligands may not be mannose-based, as BDCA-2 from cell lysates was shown not to recognise these carbohydrates [52, 54]. In the absence of a ligand, an anti-BDCA-2 monoclonal antibody was used to cross-link the receptor on pDCs and transfected U937 cells, resulting in src-family kinase dependent Ca^{2+} influx and intracellular protein phosphorylation [51, 55]. Immunoprecipitation and expression studies in transfected 293T and Jurkat cells and freshly isolated pDCs, revealed that BDCA-2 couples with FcRγ chain [55, 56]. Indeed, in Jurkat cells co-transfected with BDCA-2 and FcRγ chain, stimulation of BDCA-2 induced intracellular protein phosphorylation and Ca^{2+} influx which was not possible when BDCA-2 was expressed alone [51, 55]. The association with FcRγ chain has been shown to take place through the

University of Cape Town

transmembrane domain and this interaction is unusual as BDCA-2 does not contain a positively charged residue in this domain [56].

Signalling through BDCA-2 is dependent on the ITAM motif in the FcRγ chain and downstream pathways involve Syk, Src homology 2 domain-containing leukocyte protein of 65kDa (Slp65), Vav1, phospholipase C-gamma (PLCγ2) and ERK1/2 [55, 56]. This cascade in primary human pDCs resembles signalling via the B cell receptor in B cells. However, BDCA-2 signalling does not lead to activation of the NF-κB pathway. In fact, cross-linking of BDCA-2 on pDCs actually decreased activation of the NF-κB pathway following stimulation with TNFα [56]. Interestingly, amongst the proteins phosphorylated after BDCA-2 triggering, Röck et al. identified those involved in cytoskeletal rearrangement, including actin, tubulin and clatherin heavy chain [56]. This, and the fact that BDCA-2 contains an EEE (late endosomal sorting) motif in its cytoplasmic tail, may indicate targeting of the receptor to late endosomal compartments. Accordingly, when antibody was used to cross-link BDCA-2 on the surface of pDCs, BDCA-2-antibody complexes were internalised and efficiently presented to T cells, indicating a possible role for BDCA-2 in antigen capture and presentation [50, 51, 57]. However, when pDCs were targeted with antigen via CD36, a known antigen-uptake scavenger receptor, cross-linking of BDCA-2 diminished the ability of the pDCs to restimulate antigen-specific memory T cells, demonstrating an inhibitory effect of BDCA-2 signalling [57].

University of Cape Town

pDCs are characterised by the release of large amounts of type I interferon, especially in response to viral infection. However, following BDCA-2 cross-linking on pDCs, the cells' ability to produce IFNα, IFNβ and IL6 was found to be suppressed in response to CpG-DNA, but the production of other cytokines, including IL12p40, IL12p70, IL4 and IL10, remained unaffected [51, 55, 56]. Additionally, when BDCA-2 was cross-linked on CpG-DNA-stimulated pDCs, these pDCs had decreased ability to stimulate $CD4^+$ T cells to proliferate and produce IFNγ [58]. These stimulated $CD4^+$ T cells also showed enhanced expression of CCR5 and decreased expression of CCR4, which are markers of TH_2 and TH_1 differentiation respectively [58]. These results indicate that BDCA-2 may function in skewing the immune system away from a pro-inflammatory TH_1 response, by decreasing IFN production, towards a TH_2 response, characterised by increased $CCR5^+$ T cells. As pDCs have been shown to play a role in the pathogenesis of systemic lupus erythematosis, by being the main producers of increased levels of IFN-α/β associated with this autoimmune disease, BDCA-2 has been proposed to be a therapeutic target for treatment of this disease [59].

University of Cape Town

In addition to IFN I production, pDCs have been shown to release TNF-related apoptosis-inducing ligand (TRAIL), a soluble member of the TNF family of cytokines capable of inducing apoptosis in cells expressing the TRAIL death receptors. This cytokine was released upon autocrine IFN I production and in response to CpG-DNA stimulation and could induce apoptosis of TRAIL-sensitive Jurkat cells [60]. Interestingly, BDCA-2 ligation on these activated pDCs was able to abolish both TRAIL secretion and cytotoxic activity, indicating another negative regulatory role of the receptor [60].

1.5.2 Dendritic Cell Immunoactivating Receptor (DCAR)

There has been only one publication on DCAR, a molecule identified by a cDNA homology search with the DCIR CRD [49]. DCAR transcripts were strongly detected in lung, spleen and bone-marrow DCs, weakly in skin and lymph node and not at all in bone-marrow NK cells [49]. The cytoplasmic domain of DCAR is characteristically short and lacks a defined signalling motif but the receptor was found to associate with the FcRγ chain, partly by association with an arginine residue in the transmembrane region of DCAR. In A20 cells co-transfected with the FcRγ chain, cross-linking a chimeric receptor consisting of the extracellular region of FcγRIIB coupled to the transmembrane and intracellular domains of DCAR, resulted in tyrosine phosphorylation of intracellular proteins and Ca^{2+} mobilisation. When the tyrosine of the FcRγ chain was mutated to a phenylalanine, the Ca^{2+} mobilisation was no longer observed, demonstrating that signalling from DCAR takes place via the ITAM motif of the adaptor. The FcRγ chain is also likely to be required for surface expression of DCAR, as it enhanced receptor expression in transduced in 293T cells. Two isoforms of DCAR have been identified, one of which lacks the stalk region, but the ligands and biological functions of these isoforms still remain undefined.

1.5.3 Dendritic cell Immunoreceptor (DCIR)

DCIR was identified by screening a nucleotide database for molecules homologous to the Group II C-type lectin hepatic asialoglycoprotein receptors, which also contain a single CRD at the C-terminal [61]. DCIR mRNA was found to be highly expressed in human

University of Cape Town

peripheral blood leukocytes, and at lower levels in lymph node, spleen, bone marrow and thymus, while mouse DCIR was found to be expressed at highest levels in spleen and lymph node, although peripheral blood was not examined [61, 62]. A closer look at protein expression on cell types, revealed that DCIR was expressed on circulating APCs such as $CD14^+$ monocytes, $CD19^+$ B cells, macrophages, neutrophils as well as myeloid and pDCs, but not on $CD3^+$ T cells nor on $CD56^+$ or $CD16^+$NK cells [61-65]. DCIR was also expressed on epidermal Langerhans cells and dermal $CD14^-CD1a^+$ and $CD14^+CD1a^-$ DCs [63]. In vitro, DCIR expression was found to be higher in $CD14^+$ than $CD1a^+$ derived DCs, while monocyte-derived DCs (moDCs) had high levels of expression throughout differentiation, which was down-regulated upon maturation with LPS or CD40 ligand [61]. Additionally, DCIR expression on neutrophils was down-regulated by TNFα, IL1α and LPS stimulation but anti-inflammatory stimuli including IL4, IL10 and IL13 did not affect expression, suggesting that DCIR may be down-regulated during inflammation [65]. Interestingly, GM-CSF, IL3, IL4 and IL13 stimulation of neutrophils resulted in accumulation of a short form of DCIR mRNA, which encodes a putative non-functional protein which may act as an antagonist to the full length receptor [65, 66].

University of Cape Town

DCIR is distinct from other members of this receptor family in that it possesses a longer cytoplasmic tail which contains a consensus signalling motif and is the first reported DC-expressed ITIM-bearing C-type lectin [61]. The presence of this ITIM motif, and down-regulation of DCIR in pro-inflammatory settings, suggests a regulatory role for this receptor. The function of the intracellular domain of DCIR has been studied using B cells transformed with a chimeric receptor comprising the extracellular domain of FcγRIIB

coupled to the intracellular domain of DCIR [62]. Stimulation of the B cell receptor in these cells resulted in Ca^{2+} mobilization and intracellular protein tyrosine phosphorylation and these activities could be inhibited by co-ligation with the chimeric receptor [62]. This inhibition was completely lost when the tyrosine of the DCIR ITIM motif was mutated to a phenylalanine [62]. Moreover, immunoprecipitations using a phosphorylated peptide covering the DCIR ITIM demonstrated association with the inhibitory phosphates, Src homology 2-containing tyrosine phosphatase 1 (SHP-1) and SHP-2 [67]. Accordingly, in pDCs, which are generally considered to induce TH_1 polarisation in response to viral stimuli, stimulation with TLR9 ligand resulted in the production of large amounts of IFNα, which could be inhibited by cross-linking of DCIR with anti-DCIR antibodies, similarly to the effect of cross-linking BDCA-2 [64]. Cross-linking of DCIR on immature moDCs stimulated with other TLR ligands including LPS (TLR4), polyIC (TLR3), zymosan (TLR2 and 4) and R848 (TLR8), resulted only in the inhibition of the TLR8-mediated responses, demonstrating some selectivity in DCIR function [68].

University of Cape Town

Cross-linking DCIR in pDCs and moDCs also resulted in internalization of the receptor into intracellular vesicles in a clatherin-dependent manner [64, 68]. Furthermore, once internalized, DCIR was able to deliver antigen into the antigen-presentation pathway, resulting in efficient T cell proliferation [64]. Indeed, a recent study has shown that delivery of influenza matrix protein through DCIR on all human DC subsets results in expansion of specific memory $CD8^+$ T cells and that DCIR targeting could induce primary $CD8^+$ T cell responses to self and viral antigens [63]. Additionally, DCIR-

mediated cross-presentation and cross-priming could be enhanced by activating DCs with TLR 7/8 agonists which resulted in increased production of IFNγ and TNFα and IL4, IL5 and IL13 [63]. It was therefore suggested that targeting specific antigens through DCIR to DCs could be used to induce cytotoxic T cell responses which could aid in treatment and prevention of diseases.

DCIR also plays a role in the capture and transmission of HIV-1 by DCs [69]. Decreasing DCIR surface expression on human immature moDCs with small-interfering RNA, or blocking the receptor with antibodies, significantly reduced transfer of HIV-1 virions to autologous $CD4^+$ T cells [69]. Additionally, Raji-CD4 cells transiently expressing DCIR were shown to have both increased binding to HIV-1 and enhanced virus production, following infection, compared to DCIR-negative cells. Hypothetically, DCIR interaction with HIV could allow the virus to gain access to non-degradative endosomal organelles and lead to fusion of viral and endosomal membranes, allowing productive infection of the cells [69]. To study association with HIV-1, a DCIR mutant which lacked the stalk region was created. Although the receptor was expressed on the surface of Raji-CD4 cells, it did not increase attachment or replication of virus particles, indicating a crucial role for the stalk region of DCIR in HIV-1 interaction, perhaps by extending the CRD from the cell surface and making it available for attachment [69].

In addition to acting as a PRR for HIV, DCIR has been shown to play a role in controlling autoimmune disease [70]. Aged DCIR deficient mice were found to spontaneously develop joint abnormalities, have elevated levels of autoantibodies and

University of Cape Town

show higher levels of $CD11c^+$ DCs and a proportional expansion of T cell populations in their lymph nodes [70]. Additionally, in response to collagen-induced arthritis, young DCIR deficient mice were found to develop a more severe disease than their wild type littermates, indicating a protective role for DCIR [70]. DCIR deficient mice also had elevated levels of IL4, IL10, IL17 and IL23 after type II collagen immunization, consistent with a negative regulatory role played by the receptor [70]. Additionally, bone marrow cells from DCIR deficient mice differentiated into DCs and proliferated more efficiently than wild type cells, due to an increased responsiveness to GM-CSF. DCIR therefore appears to be crucial in maintaining appropriate DC numbers to prevent development of autoimmunity in mice [70]. When studying DCIR expression in patients with rheumatoid arthritis, the receptor was found to be abundantly expressed in synovial biopsies but was not found in those of healthy controls [71]. In these biopsies, the receptor was expressed on numerous cell types and surprisingly also on $CD56^+$ NK cells and $CD4^+$ and $CD8^+$ T cells [71]. $DCIR^+$ T cells in the synovial fluid were activated, as well as much more abundant, than those found in peripheral blood [71]. The function of DCIR on T cells in this disease setting is as of yet undefined. Overall, these observations suggest DCIR has an essential role in maintaining homeostasis of the immune system by controlling DC expansion and the development of autoimmune disease.

1.5.4 Dendritic Cell-associated C-type lectin-2 (Dectin-2)

Dectin-2 is the most well characterised member of this receptor family and was identified as an over-expressed transcript in a myeloid leukemia mouse model, macrophages, neutrophils and pleuripotent myeloid precursors [72]. However, Ariizumi et al. reported

University of Cape Town

Dectin-2 as a transcript which was highly expressed by the Langerhans-cell-like skin-derived cell-line compared to other cell-lines, including macrophages and fibroblasts [73]. The Dectin-2 promoter was also defined as a Langerhans cell specifc regulatory element [74] but Northern blot analyses revealed that the highest levels of transcript were present in the spleen and thymus [73]. Although originally proposed to be Langerhans cell specific, the results of Dectin-2 expression based on RNA analyses were therefore inconclusive. The use of a Dectin-2 specific monoclonal antibody however, demonstrated that the receptor is expressed predominantly in tissue macrophages, some DCs and at a low level on Langerhans cells and peripheral blood monocytes, where expression levels could be transiently increased upon induction of inflammation [75].

Dectin-2 was predicted to have mannose binding activity due to the presence of an EPN motif in the CRD [72]. Use of a soluble form of the CRD of Dectin-2 as a probe, revealed that the receptor could recognise zymosan and numerous pathogens including *Candida albicans, Saccharomyces cerevisiae, Mycobacterium tuberculosis, Microsporum audounii, Trichophyton rubrum Paracoccoides brasiliensis, Histoplasma capsulatum* and capsule-deficient *Cryptococcus neoformans.* Although the level of binding to these pathogens differed greatly, binding could always be inhibited by chelation of Ca^{2+} or competition with mannose [25, 76]. Additionally, a glycan microarray showed that the receptor had specificity for high-mannose structures [76]. Indeed, in BMDCs Dectin-2 has been shown to recognise α-mannans from *C. albicans* and to be responsible for production of cytokines such as IL12, IL6, TNF and IL10 [77].

University of Cape Town

Interestingly, Dectin-2 may also have an endogenous ligand on $CD4^{+}CD25^{+}$ T cells and interaction of the two molecules may mediate UV-induced immunosuppression [78]. More specifically, injection of a soluble form of Dectin-2 *in vivo* inhibited UV-induced suppression of contact hypersensitivity, supposedly by inhibiting the interaction of endogenous Dectin-2 with its putative ligand on regulatory T cells [78]. Indeed, UV-B irradiation was shown to increase Dectin-2 expression in Langerhans cells of the skin at both mRNA and protein levels [79]. It is possible that Dectin-2 recognises an endogenous ligand that is not a carbohydrate, perhaps via an alternative binding site to that which recognises fungi, as has been reported for other C-type lectins, such as Dectin-1 [80].

University of Cape Town

Delivering antigens directly to DCs has the potential to increase the efficacy of vaccination and Dectin-2 has been proposed to act as a target for this delivery [81]. Carter et al. showed that even though Dectin-2 is expressed at low levels on DCs, anti-Dectin-2 monoclonal antibodies conjugated to ovalbumin were capable of targeting this model antigen to the cells and increasing presentation to $CD8^{+}$ T cells [81]. Induction of a $CD8^{+}$ T cell response could even be achieved with an antigen dose which was too low to induce a response when administered alone [81].

As mentioned, Dectin-2 has been identified as a PRR for fungi. The receptor exhibits preferential recognition of hyphal over conidial forms [25]. Binding of *C.albicans* hyphae by RAW cells transduced with Dectin-2 or cross-linking the receptor on these cells, resulted in tyrosine phosphorylation of intracellular proteins, as well as internalization of the receptor into endosomes, activation of transcription factor NF-κB and production of

TNFα and IL1 receptor antagonist, indicating that the receptor is able to transduce intracellular signals [25]. Additionally, in macrophages stimulated with *C. albicans*, Dectin-2 has been implicated in regulating cytosolic phospholypase A_2 activation, a regulatory enzyme that releases arachidonic acid for the production of prostaglandins and leukotrienes [82]. Dectin-2 has the characteristic short cytoplasmic domain which lacks known signalling motifs and associates with FcRγ chain to transduce these signals [25, 83]. Interestingly, it was not the positively charged arginine in the transmembrane region which was responsible for association with FcRγ chain, as has been shown for other receptors, but rather a short region of the cytoplasmic domain proximal to the transmembrane region [25]. Indeed, Dectin-2 has also been shown to associate with FcRγ chain in DCs and cross-linking the endogenous receptor resulted in phosphorylation of Syk, ERK, p38 and JNK MAPKs and production of IL2, IL10 and TNF [83]. Similarly, blocking or knocking down Dectin-2 expression on BMDCs resulted in decreased IL2, IL10 and TNF production in response to *C. albicans* stimulation, which could be almost completely lost when Dectin-2 was blocked on Dectin-1 deficient BMDCs [83]. These results indicated that signalling via Dectin-1 and Dectin-2 together could account for the Syk-CARD9 dependent activation seen by DCs in response to fungi [83]. In bone marrow-derived macrophages however, it has been shown that Dectin-2 but not Dectin-1 is responsible for hyphal *C. albicans* induced NF-κB activation and knocking down Dectin-2 in these cells resulted in inhibition of IL12, IL10 and TNFα production [84]. Additionally, it was shown that in response to fungal stimulation, signalling via Dectin-2 was responsible for formation of the CARD9-BCL10 complex required for NF-κB activation [84]. *In vivo*, Dectin-2 deficient mice have been shown to have increased

University of Cape Town

susceptibility to *C. albicans* infection, with higher kidney fungal burden and lower survival than wild type mice [77]. Dectin-2 has also been shown to direct the TH_{17} response to *Candida albicans* [102, 108]. Robinson et al. reported that splenocytes from mice treated with anti-Dectin-2 mAb and infected with *C. albicans*, showed decreased IL17 production after fungal re-stimulation [83]. Accordingly, Saijo et al. showed that TH_{17} cell differentiation was reduced when $CD4^{+}$ T cells were cultured with *C. albicans*-stimulated BMDC supernatants from Dectin-2 deficient mice [77].

Dectin-2 has additionally been shown to play a role in response to allergens [85]. Dectin-2 on BMDCs was able to bind to extracts from house dust mite (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*) and *Aspergillus fumigatus* in a mannose-dependent manner [85]. Stimulation of mast cells co-expressing Dectin-2 and FcRγ chain with these extracts resulted in production of cysteinyl leukotrienes, pro-inflammatory lipid mediators which are not produced by untransfected cells [85]. Additionally, in primary BMDCs, signalling by Dectin-2 to produce cysteinyl leukotriene in response to the extracts was dependent on Syk kinase and FcRγ chain, and lentiviral knockdown of the receptor significantly reduced this activity [85]. Indeed, more recently it has been shown that Dectin-2 is essential for the development of a TH_2 response to house dust mite, including eosinophilic and neutrophilic pulmonary inflammation and TH_2 cytokine production in the lungs and re-stimulated lymph nodes of mice, revealing an interesting link between C-type lectin and TH_2 immunity [86].

University of Cape Town

Furthermore, Dectin-2 has recently been identified as the first Syk-coupled C-type lectin to be associated with helminth infections [87]. Dectin-2 was identified as a receptor for soluble egg antigen (SEA) from *Schistosoma mansoni* on BMDCs [87]. Stimulation of BMDCs with SEA was shown to activated the Nlrp3 inflammasome to induce IL-1β production by BMDCs, in a manner dependent on elevated ROS levels and signalling via Syk kinase [87].

Most of the work studying Dectin-2 has been performed in the mouse, however two reports describe identification and characterization of human Dectin-2 (hDectin-2) [88, 89]. In tissues, hDectin-2 transcripts were detected in lung, spleen, lymph node, leukocytes, bone marrow and tonsils, but unlike mouse, Dectin-2 was not expressed in the human thymus. In peripheral blood cells, hDectin-2 transcripts were shown to be preferentially expressed in plasmacytoid, rather than myeloid DCs and constitutively expressed in $CD14^+$ monocytes and B cells, and could be induced in $CD4^+$ T cells upon activation with Con A [88, 89]. Indeed, similar to mouse Dectin-2, hDectin-2 appears to be up-regulated in inflammatory settings, as gene expression in $CD14^+$ monocytes could be up-regulated by treatment with GM-CSF, TGF-β1 and TNFα and down-regulated with the addition of IL4 and IL10 [88]. In $CD8^+$ T cells however, transcripts were detectable in inactive cells but decreased upon activation with phytohemagglutinin [88]. hDectin-2 was also expressed on Langerhans cells, however, while this expression was up-regulated upon UV-B radiation in mice, it was down-regulated in human cells [88]. These contradicting findings may be due to the fact that $CD14^+$ monocytes were used as a surrogate model for epidermal Langerhans cells in the human experiment [79, 88].

University of Cape Town

Interestingly a truncated isoform of hDectin-2 has been identified which lacks part of the intracellular domain and most of the transmembrane domain of the receptor [88]. Speculatively, the lack of transmembrane region could encode a secreted protein which may act as an antagonist to full-length Dectin-2 [88]. Alternatively, this truncated version may act in a similar manner to human Dectin-1 isoform E, which also lacks a transmembrane region. This isoform was retained intracellularly where it interacted with Ran-binding protein, a molecule which is presumed to act as a scaffold protein to coordinate signals from cell surface receptors with intracellular signalling pathways [90].

1.5.5 Macrophage inducible C-type lectin (Mincle)

The Mincle gene was originally identified as a transcriptional target of nuclear factor (NF-) IL6 in peritoneal macrophages [91]. This transcription factor has low transcriptional activity unless activated by inflammatory stimuli and accordingly, Mincle gene expression can be induced in peritoneal macrophages upon stimulation with LPS, IFNγ, IL6 or TNFα [91]. Mincle transcript was also detected in RAW macrophages and could be up-regulated upon LPS stimulation, as well as LPS stimulated M1 myeloblastic leukaemia cells, but the receptor was not expressed in other cell lines including myeloma, mature B cells, NK cells, EL4 thymoma or NIH3T3 fibroblasts [91].

Like Dectin-2, Mincle was able to recognise fungi and induce inflammatory signals. Initially, microarray analysis of Mincle expression suggested that the gene was up-regulated in bone-marrow derived macrophages exposed to *Candida albicans* yeast and a

University of Cape Town

soluble Mincle protein was subsequently found to bind to *C. albicans* and *Sacccharomyces cerevisiae* in an ELISA-based assay [92]. In this study, both human (in transduced cells) and mouse (in wild type cells) Mincle were examined and it was found that although Mincle was not a phagocytic receptor for *C.albicans*, it was able to mediate inflammatory responses to the yeast. In transfected RAW cells, Mincle recognition of *C. albicans* induced the production of TNFα, which could be partially inhibited by blocking with an anti-Mincle antibody and bone-marrow derived macrophages from Mincle knockout mice produced less TNFα than wild type cells [92]. This study also used Mincle knockout mice to study systemic *C. albicans* infection and found that Mincle knockout mice had significantly higher fungal burdens in the kidneys than wild type mice, demonstrating that the receptor was involved in pathogen clearance [92]. Interestingly, mice deficient in the NF-IL6 transcription factor, which controls Mincle expression, are also susceptible to infection with *Candida* [93]. However, the susceptibility of knockout animals to a lethal dose of fungus has not been investigated.

University of Cape Town

Most recently however, another group were unable to demonstrate the recognition of *Candida* by Mincle. Using a non-myeloid cell-based NFAT-GFP reporter system to screen 50 different fungal species, including *Candida* spp. and *S. cerevisiae*, Mincle was found to specifically recognise *Malassezia* species [94]. It is however important to note that the *Candida* spp. screened in this system were not the same strains used in the study described above. As Mincle contains an EPN motif, it was postulated that it specifically recognises mannose on the fungal surface and accordingly, mutation of the EPN into QPD resulted in loss of recognition [94]. Additionally, a carbohydrate microarray showed

soluble Mincle bound a multivalent form of α-mannose in a Ca^{2+} dependent manner [94]. Upon stimulation with *Malassezia*, bone-marrow derived macrophages from wild type mice showed increased Mincle expression and production of MIP2, TNFα, KC and IL10, which was significantly reduced in Mincle knockout cells [94]. This indicates a role for Mincle in eliciting an immune response to *Malassezia* by macrophages. Additionally, intraperitoneal injection of *Malassezia* resulted in impaired IL6 and TNFα production as well as neutrophil infiltration in the Mincle deficient mice, compared to their wild type littermates [94].

Similar to BDCA-2, DCAR and Dectin-2, Mincle has been shown to associate with FcRγ chain in transduced HEK293T cells and mouse peritoneal macrophages [95]. This interaction was found to take place via the positively charged arginine in the transmembrane region of Mincle and was crucial for signalling through the receptor [95]. Indeed, cross-linking of Mincle on thioglycollate elicited peritoneal macrophages with an anti-Mincle antibody resulted in the production of TNFα, MIP2, KC and IL6, and this activity was lost in FcRγ deficient cells [95]. This signalling was shown to follow the Syk and caspase recruitment domain protein (CARD9) pathway and to be independent of MyD88 signalling [95]. While FcRγ chain was not essential for cell surface expression of Mincle, LPS stimulation was found to induce less Mincle expression on the surface of FcRγ deficient macrophages than wild type cells, indicating that the adaptor was involved, at least partly, in surface expression of the receptor [95].

University of Cape Town

Most recently, Mincle has been identified as a receptor for mycobacterial cord factor (trehalose 6,6'-dimycolate, TDM) and its synthetic analogue trehalose 6,6'-dibehenate (TDB) [96, 97]. Cord factor is the most abundant lipid found in the mycobacterial cell wall and has been shown to play essential roles in the pathogenesis of tuberculosis [98]. Cord factor has been shown to induce FcRγ, Syk and CARD9 dependent activation in macrophages and indeed, Mincle was identified as the relevant receptor, by means of bone marrow-derived macrophage microarray mining for C-type lectins which associate with FcRγ and CARD9, as well as an NFAT-driven GFP reporter cell system [96, 97, 99]. *In vitro*, TDM failed to induce the production of inflammatory cytokines or nitric oxide in Mincle deficient macrophages compared to wild type cells [96, 97]. Additionally, *in vivo* administration of TDM resulted in decreased IL6 and TNF sera levels as well as no granuloma formation in the lungs of Mincle deficient mice in contrast to their littermate controls [96]. TDM and TDB have previously been identified as adjuvants for induction of a protective T cell response by recombinant proteins from numerous pathogens, including *M. tuberculosis* [100, 101]. Mincle deficient mice however, failed to generate the adjuvant effect of TDB to drive TH_1 and TH_{17} immune responses to a subunit vaccine consisting of the *M. tuberculosis* fusion protein Ag85B-ESAT-6 [97]. These results therefore identified Mincle as a PRR that controls the generation of immunity to mycobacterial infection.

In addition to exogenous ligands, Yamasaki et al. have shown that Mincle can mediate inflammatory responses to necrotic cells [95]. In a search for an endogenous ligand using

University of Cape Town

the NFAT-GFP reporter cell system, the authors discovered that cellular activation occurred following prolonged cultured and noticed that this activity correlated with the presence of dead cells [95]. Mutation of the EPN motif in the CRD did not abrogate cellular activation in response to dead cells, nor was binding by a soluble Mincle protein inhibited in the absence of Ca^{2+}, suggesting that Mincle was recognising a non-carbohydrate endogenous ligand [95]. Immunoprecipitation from dead-cell lysates identified spliceosome-associated protein 130 (SAP130), a soluble protein that is localised in the nucleus of living cells and released during cellular necrosis. Stimulation of macrophages or Mincle expressing T cell hybridomas with purified SAP130 resulted in production of MIP2 and IL2 respectively, showing that SAP130 is a functional endogenous ligand for Mincle and that recognition of this protein could act as a signal for excessive cell death [95]. To study the function of Mincle in response to necrotic cell death *in vivo*, Yamasaki et al. inhibited Mincle function with blocking antibodies and found decreased neutrophil recruitment and cytokine production in response to cell death [95]. This model therefore proposes that Mincle recognises SAP130 released by necrotic cells, which results in cytokine production by macrophages and subsequent neutrophil infiltration into the damaged tissue (Figure 1.4). Speculatively, this infiltration could be either beneficial or detrimental to the host. The neutrophils may aid in clearance of apoptotic cells and promote repair or they may induce acute inflammation with a pathological outcome, such as in autoimmune diseases. Indeed, the Mincle gene complex has been reported to be associated with rheumatoid arthritis [102, 103]. Further studies in Mincle deficient mice are needed to understand the role of the receptor in autoimmunity.

University of Cape Town

Recently, Mincle mRNA expression in macrophages has been shown to be up-regulated during interactions with adipocytes [104]. Specifically, this up-regulation was found to be induced by saturated fatty acids released from adipocytes during macrophage induced adipocyte lipolysis and was induced in pro-inflammatory rather than anti-inflammatory macrophages [104]. Additionally, Mincle mRNA expression was increased along with macrophage markers in the adipose tissue of obese mice and humans [104]. Although the physiological relevance of these findings have not been revealed, it is possible that Mincle may play a role in obesity-induced tissue inflammation.

University of Cape Town

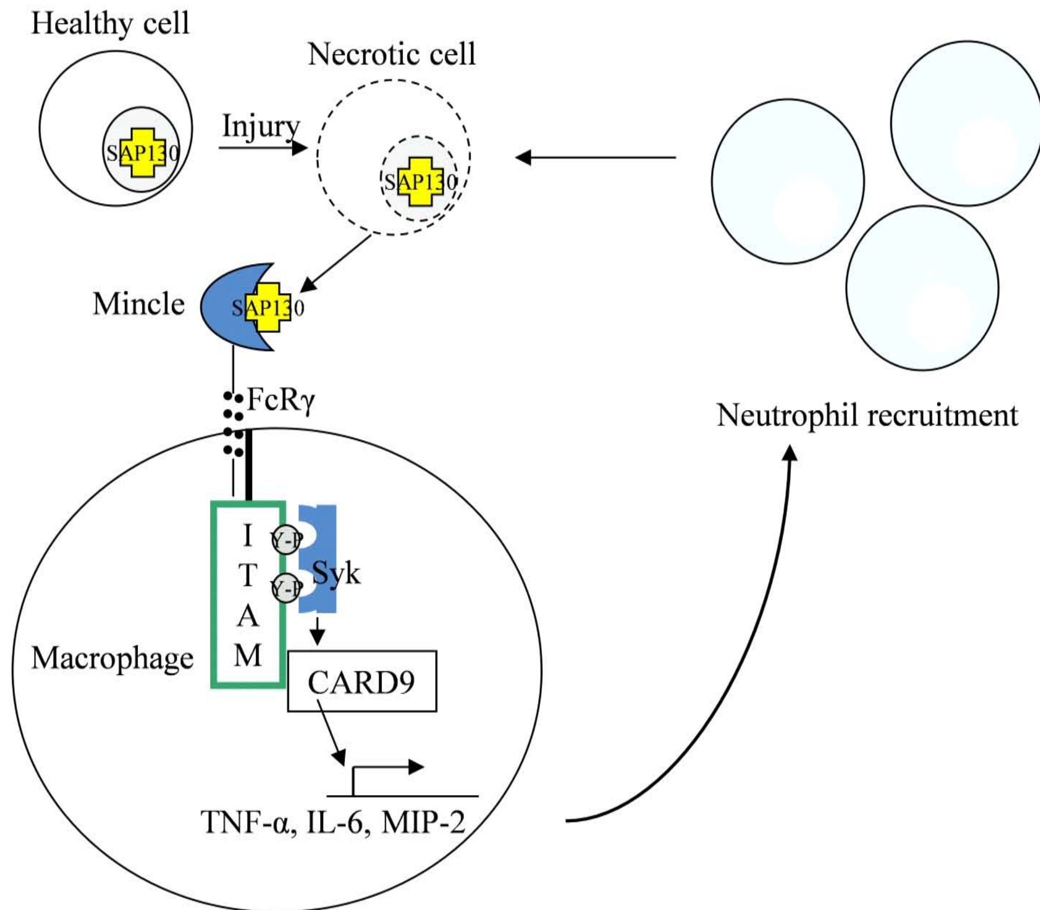


Figure 1.4 **Mincle is a detector of necrotic cell death.** Necrotic cell death caused by injury, such as radiation, results in release of preformed nuclear proteins such as SAP130. SAP130 is recognised by Mincle expressed on the surface of macrophages, which signals via FcRγ chain, in a Syk and CARD9 dependant pathway. This induces the production of pro-inflammatory cytokines such as MIP2, IL6 and TNFα, resulting in recruitment of neutrophils to the site of necrosis. From Graham et al. [53].

University

1.5.6 Clecsf8

Clecsf8 is a poorly characterised member of the Dectin-2 cluster of receptors and is the focus of this thesis. The receptor was first identified through a differential display PCR screen of numerous murine cell lines for macrophage-specific genes [105]. Northern blot analysis of mouse tissues revealed that Clecsf8 transcripts were predominantly expressed in resident peritoneal macrophages and at lower levels in bone marrow, spleen, lung and lymph nodes, however peripheral blood was not examined [105]. The Clecsf8 open reading frame encodes a 219 amino acid protein with typical Group-II characteristics, including a short cytoplasmic domain with no signalling motif, and a CRD with conserved Ca^{2+} association residues [105]. The CRD however, does not contain the conserved EPN or QPD amino acid triplets associated with carbohydrate recognition and the transmembrane region lacks charged residues normally involved in association with an adaptor molecule. Cross-linking of Clecsf8 on 293T cells transiently expressing the receptor resulted in internalisation of the molecule, indicating a potential role for the receptor in antigen uptake [106].

University of Cape Town

The human orthologue of mouse Clecsf8 was also found to be expressed in a monocyte/macrophage restricted manner, and interestingly, freshly isolated peripheral blood monocytes were found to have higher mRNA levels than cultured or buffy-coat isolated monocytes [106]. The expression of this receptor could be up-regulated on these cells by stimulation with IL6, IL10, TNFα or IFNγ, but was down-regulated with LPS [106]. Clecsf8 transcripts were detected in peritoneal macrophages from a patient suffering from *Pseudomonas aeruginosa* induced peritonitis as well as in synovial fluid

macrophages isolated from a patient with rheumatoid arthritis, but not from a patient with gout [106]. In gout, macrophages have been implicated in the generation of anti-inflammatory rather than pro-inflammatory cytokines [107]. Although no ligand or biological function has as yet been described for Clecsf8, the receptor has been shown to be up-regulated at the transcript level in a number of disease settings, including TNFα over-expressing myocarditis and *M. tuberculosis* infection [108, 109]. Overall these preliminary indications suggest that Clecsf8 could be up-regulated in pro-inflammatory settings, as has been described for Mincle and Dectin-2.

Very little is known about the true function of the receptor but a Clecsf8 deficient mouse has been generated, offering an exciting reagent for future studies. This mouse has been generated and phenotypically characterised by the Consortium for Functional Glycomics. Clecsf8 deficient mice suggested slightly impaired T cell and B cell proliferation with stimulants that cross-linked the antigen receptors, as well as a possible increase in mature re-circulating B cells found in the bone marrow, but none of these findings were significantly different to wild type mice (www.functionalglycomics.org/glycomics/publicdata/phenotyping.jsp).

University of Cape Town

1.6 Concluding remarks and aims of this thesis

The Dectin-2 family are multifunctional receptors, and have been shown to be important in both homeostasis and immunity. Many can act as PRRs and they recognise a range of pathogens, including fungi, viruses and mycobacteria. Many also recognise endogenous

ligands, highlighting the importance of this family in homeostasis. Indeed, the Dectin-2 locus has been identified as a susceptibility region associated with autoimmune disorders, including multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis and type I diabetes [110]. The diverse repertoire of ligands and functions of these receptors reveals an exciting area of research.

Clecsf8 is one of the least characterised members of the Dectin-2 cluster of C-type lectins and the main aim of my project was to functionally characterise the receptor. To this end my objectives were to:

- Characterise human Clecsf8 expression in peripheral blood
- Characterise human and murine Clecsf8 localisation in transfected cells
- Determine whether Clecsf8 associates with an adaptor molecule
- Identify ligands of Clecsf8
- Determine whether Clecsf8 could induce cellular responses such as cytokine production, phagocytosis and the respiratory burst
- Define the signalling capacity of Clecsf8
- Characterise the role of Clecsf8 *in vivo* using the Clecsf8 knockout mice

University of Cape Town

Chapter 2

Materials and Methods

2.1 General reagents

The majority of chemicals of analytical grade were purchased from Gibco, Invitrogen, Sigma Aldrich and Merck, unless stated otherwise. Distilled water was produced using a milliQ water purification system.

2.2 Commonly used solutions

- **Cell lysis buffer for lysates and immunoprecipitations:** dH_20, 10mM Tris pH8, 150mM NaCl, 10mM EDTA, 1% (v/v) Igepal CA-630 and 10mM NaN_3. 10mM NaF, 1mM Na_3VO_4 and 1 complete EDTA-fee protease inhibitor cocktail tablet (Roche) were added immediately prior to use.
- **ELISA stop solution:** 2M H_2SO_4
- **FACS block:** PBS, 5% (v/v) heat inactivated serum, 0.5% (w/v) BSA, 2mM NaN_3
- **FACS wash:** PBS, 0.5% (w/v) BSA, 2mM NaN_3
- **Formaldehyde fix:** PBS, 2% (v/v) formaldehyde

University of Cape Town

- **Gey's solution :** Directly prior to use, combine 20% of solution A, 5% of solution B, 5% of solution C and 70% dH_2O and filter sterilise.

  Gey's solution A: 3.5% (w/v) NH_4Cl, 0.185% (w/v) KCL, 0.15% (w/v) $NA_2HPO_4.12H_2O$, 0.5% (w/v) glucose

  Gey's solution B: 0.42% (w/v) $MgCl_2.6H_2O$, 0.34% (w/v) $CaCl_2$, 0.14% (w/v) $MgSO_4.7H_2O$

  Gey's solution C: 2.25% (w/v) $NaHCO_3$

- **Lidocaine/EDTA:** PBS, 4mg/ml Lidocaine hydrochloride, 10mM EDTA pH8, filter sterilised
- **Lysis buffer for genotyping:** 50mM Tris-HCl pH8, 100mM EDTA pH8, 100mM NaCl, 1% SDS in dH_2O and 0.5mg/ml Proteinase K added directly prior to use.
- **Paraformaldehyde solution:** dH_2O, 4% (v/v) paraformaldehyde, 250 mM HEPES
- **Pervanadate solution:** PBS, 25mM Na_3VO_4, 0.05M H_2O_2, prepared fresh for each use.
- **PBS-Tween wash buffer:** PBS, 0.1% Tween-20
- **Ponceau stain:** dH_2O, 0.2% (w/v) ponceau S, 5% (v/v) acetic acid
- **SDS-PAGE running buffer:** dH_2O, 25mM Tris, 90mM glycine, 0.1% (w/v) SDS
- **SDS-PAGE loading dye (2X):** 0.25M Tris pH6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.001% (w/v) bromophenol blue. Reducing loading dye contains 10% (v/v) β-mercaptoethanol , non-reducing does not contain β-mercaptoethanol

University of Cape Town

- **SOC medium:** dH_2O, 0.5% (w/v) yeast extract, 2% (w/v) tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$. 20mM Glucose added after autoclaving solution. Final solution filter sterilised
- **Staining buffer (for sample preparation for confocal microscopy):** PBS, 0.25% (w/v) saponin, 1% (w/v) BSA
- **Thioglycollate solution:** Boil 40.5g Brewer's Thioglycollate (DIFCO laboratories) in 1 litre of dH_2O until dissolved, autoclave for 15min and then leave for 1 month at room temperature. Thereafter store aliquots at -20°C.
- **Tris/EDTA (TE):** 10mM Tris pH8, 10mM EDTA pH8
- **Trypsin/EDTA:** PBS, 0.05% (v/v) trypsin solution, 5mM EDTA, filter sterilised
- **Western blot transfer buffer:** dH_2O, 25mM Tris, 90mM glycine, 20% (v/v) methanol

2.3 Cells

2.3.1 Cell lines

All cell lines in this study were obtained from the IIDMM cell bank at UCT and were cultured according to ATCC recommendations. A summary of the cell lines used and their growth conditions are listed in Table 2.1

University of Cape Town

2.3.2 General cell culture

Cells were grown in the appropriate media supplemented with 10% heat-inactivated foetal calf serum (FCS, Gibco), 100units/ml penicillin, 0.1mg/ml streptomycin and 2mM L-glutamine (all from Cambrex). FCS was heat inactivated at 56°C for 30min prior to use. Cells were cultured on tissue culture plastic in a 5% CO_2 humidified incubator at 37°C and passaged every 3 to 4 days. Adherent cells were detached with either trypsin/EDTA or lidocaine/EDTA for 5min at room temperature. For long term storage of cell lines, cells were resuspended in FCS containing 10% DMSO (v/v) and stored in liquid nitrogen.

Table 2.1 Summary of cell lines

Cell line	**Description**	**Media used**	**ATCC number**
NIH3T3	Adherent murine embryonic fibroblasts	DMEM	CRL-1658
A20	Non-adherent murine B lymphoma cells	DMEM	TIB-208
RAW264.7	Adherent murine macrophages	RPMI	TIB-71
293T	Adherent derivative of the human embryonic kidney 293 cell line	DMEM	CRL-71
Plat-E	Adherent ecotropic retroviral packaging cell line	RPMI or DMEM containing 10µg/ml blasticidin and 1µg/ml puromycin	N/A

University of Cape Town

2.3.3 Primary cells

2.3.3.1 Isolation of human peripheral blood leukocytes (PBLs).

For flow cytometric analyses of human Clecsf8 expression, blood was collected from healthy volunteers in EDTA coated BD vacutainers to prevent coagulation and 10ml Gey's solution was added to 2ml blood to lyse erythrocytes. After 3-5min on ice the diluted blood was underlaid with 2ml FCS and centrifuged for 10min at 300*g* without acceleration or brake [111]. The supernatant was discarded and the process repeated if red blood cells were still present. The final pellet was resuspended in cold FACS wash and the cells were counted using Trypan blue.

2.3.3.2 Isolation and culture of human monocytes and *in vitro* derived macrophages/DCs.

Peripheral blood monocytes were obtained and differentiated as previously described [111]. Essentially, 25ml of blood (obtained from the Western Province Transfusion Service, Cape Town, South Africa) was diluted 1:1 in room temperature PBS and 35ml was layered over 15ml Ficoll-Hypaque solution (Amersham). After centrifugation at 900*g* for 20min at room temperature, with no brake, 10ml serum was removed from the top layer, heat inactivated at 56°C for 30min, centrifuged for 10min at 1250*g* and 0.22µm filtered. The layer of mononuclear cells was also isolated from the gradient and clumps were disrupted by vigorous pipetting and washed with ice cold PBS by centrifugation at 500*g* for 7min at 4°C, with low brake. Washing was repeated 3-4 times with PBS and once with RPMI with centrifugation at 250*g* for 5min at 4°C with full brake. The pellet was resuspended in X-VIVO supplemented with 1% of the isolated heat-inactivated

University of Cape Town

autologous serum and spread evenly on 15cm Petri dishes pre-coated with 2% sterile gelatin (Sigma). After 90min incubation at 37°C, the monocytes had adhered to the gelatin and non-adherent mononuclear cells (mainly lymphocytes) were gently washed off with RPMI. Fresh X-VIVO with serum was added to the dishes containing the remaining monocytes and these were cultured over night at 37°C. Monocytes (day 1) were then harvested and some were analysed for Clecsf8 expression. For differentiation into macrophages, monocytes were cultured in X-VIVO supplemented with 1% autologous serum for 4 and 7 days. For generation of monocyte-derived DCs, day 1 monocytes were cultured with RPMI supplemented with 10% FCS, 25ng/ml IL4 (R&D systems) and 50ng/ml GM-CSF (R&D systems) and analysed on day 4. To induce maturation of DCs, cells were given fresh medium with IL4 and GM-CSF for two days and then treated with media containing 1µg/ml *Salmonella typhimurium* LPS and analysed on day 8.

2.3.3.3 Purification of human peripheral blood neutrophils.

To isolate neutrophils from peripheral blood for use in functional assays, blood was collected in an EDTA coated BD vacutainer to prevent coagulation. Leukocytes were then separated by centrifugation over a two-layer (62% and 75%) Percoll Plus (GE Healthcare) gradient at 20°C for 25min (10min at 200*g* and 15min at 400*g*, without interruption). The neutrophils were collected from the interface between the two Percoll solutions and washed with PBS containing 5mM glucose and 0.2% BSA, to prevent clumping. After centrifugation for 7min at 4°C, contaminating erythrocytes were lysed by hypotonic treatment for 30s with 0.2% NaCl, flowed by restoration of isotonicity with

University of Cape Town

1.6% NaCl. After centrifugation, neutrophils were counted using Trypan blue. To isolate autologous serum, blood was collected without EDTA and allowed to clot at room temperature and centrifuged at 3000*g* for 5min at 21°C.

2.3.3.4 Cytokine and TLR agonist stimulation of cells

To test the effect of cytokines and TLR agonists on human Clecsf8 surface expression, PBLs were plated at 5x10^{5} cells/well in 24-well plates and stimulated for 6h with IL4 (20ng/ml), IL6 (80ng/ml), IL10 (20ng/ml), TNFα (10ng/ml), IFNγ (10ng/ml), Pam3CSK4 (TLR1/2; 100ng/ml), LPS (TLR4; 100ng/ml), flagellin (TLR5; 20ng/ml) and FSL-1 (TLR2/6; 20ng/ml) (All TLR agonists were from Invitrogen and all cytokines from R&D systems). Cells were then analysed by flow cytometry.

2.3.4 Generation of transduced cell lines

2.3.4.1 Retrovirus production

Retroviral vectors (either pFBneo or pMXs-IP) containing cloned genes of interest were introduced into Plat-E cells which enabled production of infectious retroviruses [112]. These retroviruses could infect and stably transmit the cloned gene of interest into the chromosome of target cells. To produce retroviral particles, recently passaged Plat-E cells were plated at 2x10^{6} cells/well in 6-well plates in 2ml DMEM on day 1. The following day, the media was replace with 2ml DMEM and the cells were transfected with 1µg DNA using Fugene6 (Roche), according to the manufacturers' instructions and incubated at 37°C overnight. On day 3, the cells were moved into a 5% CO_2 filled airtight container

University of Cape Town

and incubated overnight at 32°C. The following day, supernatants were harvested and polybrene (Sigma) was added at 5µg/ml to reduce electrostatic repulsive forces between virus and target cells. Supernatants were filtered through 0.45µM filter and used immediately to transduce various cell lines or stored at -80°C for future use.

2.3.4.2 Retroviral transduction of cell lines

In parallel with retrovirus production, on day 3, NIH3T3 cells were plated at 1x10^{5} cells/well and RAW264.7 cells were plated at 5x10^{5} cells/well in 6-well plates. On day 4 RAW264.7 cells were treated with 0.2µg/ml tunicamycin (Sigma) for at least 6h, which inhibits N-glycosylation and enhances transduction efficiency. Cells were then washed and viral supernatant was added. For transduction of A20 cells, on day 4, cells were plated at 1x10^{5} in 6-well plates and viral supernatant was added. Plates were centrifuged at 2500rpm at 25°C for 90min to bring viral particles into contact with cells. After incubation overnight at 37°C, viral supernatants were replaced with fresh media and the following day the relevant selection media was added (800µg/ml G418 for pFBneo or 2µg/ml puromycin for pMXs-IP, both from Invivogen). Cells were monitored daily and detached and transferred to flasks once colonies became clearly visible.

University of Cape Town

2.3.4.3 Transient transfection using Lipofectamine

The FcClecsf8 fusion plasmid (described in 2.5.4) was transfected into HEK293T cells using Lipofectamine (Invitrogen) according to the manufacturers' protocol. Cells were cultured to 60% confluency in T175 flasks and transfections were performed in OPTI-MEM (Gibco).

2.4 General nucleic acid manipulation techniques

2.4.1 Polymerase chain reaction (PCR)

General PCR reactions contained 2x PCR Master Mix (Fermentas) containing 0.05u/µl *Taq* DNA polymerase, reaction buffer, 4mM $MgCl_2$ and 0.4mM each of dATP, dCTP, dGTP and dTTP. Added to this was 0.2µM of appropriate forward and reverse primers and approximately 100ng template DNA in a 20-50µl reaction in PCR grade H_2O (Sigma). Reactions were incubated using a Gradient PTC-200 Peltier Thermal Cycler (MJ Research) and the cycling parameters were dependent on primers and template size. However, standard parameters involved denaturation at 94°C for 2min, followed by 30-35 cycles of denaturation, annealing and extension. Annealing took place at 5°C below the lowest primer melting point and extension took place for approximately 1 minute per 1Kb of template. After cycling, the reaction was incubated for a further 4-10min at 72°C to ensure all PCR products were full length. For high fidelity PCR used to amplify cDNA for cloning, the Advantage 2 PCR kit (Clontech) was used, according to manufacturers' instructions. All reactions were analysed by agarose gel electrophoresis.

University of Cape Town

2.4.2 Agarose gel electrophoresis

0.8% (w/v) agarose gels were prepared by dissolving electrophoresis grade agarose (Whitehead Scientific) in 0.5X TBE (Sigma). Ethidium bromide (Sigma) was added to the dissolved agarose to a final concentration of 0.2µg/ml to allow visualisation of the

DNA. DNA loading gel was added to the samples which were run alongside DNA molecular weight markers (Fermentas) to allow for identification of fragment sizes. Gels were run at a constant voltage of 90-100V and gels were analysed using a long wavelength (366nm) UV transilluminator (UV Wirsam Scientific).

2.4.3 Purification of DNA from agarose gels

DNA fragments were excised from agarose gels and the DNA was purified using the Wizard SV Gel and PCR Clean-Up System (Promega) according to manufacturers' instructions.

2.4.4 TOPO cloning

TOPO TA Cloning (Invitrogen) was carried out for direct insertion of PCR products into the pCR2.1-TOPO vector, according to the manufacturers' protocol. TOPO reactions were used to transform chemically competent *Escherichia coli*.

2.4.5 Plasmid digestion

Digestion of plasmid vectors with or without DNA inserts was performed using appropriate restriction enzymes according to manufacturers' guidelines. In general, 25-50ng of DNA was digested for 2h (or overnight) at 37°C in the appropriate buffer and then enzymes were heat inactivated at recommended temperatures. When necessary, cut

University of Cape Town

vectors were dephosphorylated by incubation with 2U of shrimp alkaline phosphatase (Fermentas) to prevent religation of empty vectors. The reaction was incubated at 37°C for 60min and heat inactivated at 65°C. Digestions were confirmed by agarose gel electrophoresis and digested DNA was excised and gel purified, as described earlier.

2.4.6 Ligation of digested vectors and insert DNA

Ligations were carried out using 1U T4 DNA ligase (Fermentas) in a reaction volume of 20µl containing 20-100ng linearised vector DNA and 1:1 to 5:1 ratio of insert:vector DNA, in the recommended buffer. Controls with no insert and/or no vector DNA were included to observe false positive background, self-ligating vector and the presence of contaminating vector. Ligations were performed overnight at 16°C and 2µl of the reaction was used to transform chemically competent *E. coli*.

University of Cape Town

2.4.7 Transformation of competent cells

One Shot TOP10 Chemically Competent *E. coli* (Invitrogen) were stored at -80°C and thawed on ice for use in transformations. 2µl DNA was added to 12-30µl competent cells, mixed gently and incubated on ice for 5-30min. Cells were heat shocked for 30s at 42°C and transferred back to ice. 250µl room temperature SOC medium was added and the reaction was incubated at 37°C for 1h on a horizontal shaking incubator. 200µl from each reaction was plated onto pre-warmed LB agar plates containing 100µg/ml ampicillin (Sigma) and incubated overnight at 37°C. All vectors in this study contained an

ampicillin resistance cassette and only bacteria successfully transformed with a ligated vector would grow under selection.

2.4.8 Colony PCR

Transformants were screened by PCR to confirm that the insert was present in the desired orientation. Single colonies were sampled from the ampicillin agar plates using a pipette tip and transferred onto a replica plate and numbered for identification. Each colony was also transferred directly to a tube containing a PCR reaction mixture and the necessary primers, which would only yield a product if the insert was in the desired orientation. PCR and agarose gel analyses were performed as previously described.

2.4.9 Production and isolation of plasmid DNA

Transformants which contained the desired insert were selected from the replica plate and inoculated into 10ml LB broth containing 100µg/ml ampicillin. Cultures were grown at 37°C with shaking overnight. Plasmid DNA was isolated from the culture using the Wizard Plus SV Miniprep DNA Purification System (Promega), according to manufacturers' instructions. DNA samples were analysed for concentration and purity using a NanoDrop ND-1000 Spectrophotometer prior to sequence analyses.

University of Cape Town

2.4.10 Sequencing

To confirm the fidelity of all constructs, purified plasmid DNA was sequenced at the Central Analytical Facility at the University of Stellenbosch, South Africa. Sequences were analysed using DNAMAN software.

2.5 Nucleic acid manipulation specific to this thesis

2.5.1 Generation of mClecsf8-HA, hClecsf8-HA, Mincle-HA, Dectin-2-HA and Dectin-1-HA

The complete mClecsf8, hClecsf8, Mincle and Dectin-2 open reading frames were isolated by PCR from the sources and using the primers listed in Table 2.2. The PCR products were purified and inserted into TOPO vector as described (section 2.4.4) and the fidelity of the sequences was confirmed. The gene of interest was digested with the relevant restriction enzyme and ligated into the pFBneo retroviral vector resulting in the introduction of the influenza hemagglutinin (HA)-tag at the C-terminus of the receptor, essentially as previously described [113]. The HA-tag provides a convenient method to detect the expression of proteins by flow cytometry, fluorescence microscopy, Western blotting and immunoprecipitation. Full-length Dectin-1-HA and truncated Dectin-1-HA were available in the laboratory and were generated as previously described [24].

University of Cape Town

2.5.2 Generation of chimeric constructs

C-terminally HA-tagged chimeric constructs in pFB-neo or pMXs-IP were generated using overlapping extension PCR such that the final constructs translated as: $\text{D1}^{\text{CTLD}}/\text{SF8}^{\text{TM-cyto}}$ $({}^{\text{SF8-76}}\text{TGGTW}^{\text{SF8-80}}/{}^{\text{D1-110}}\text{QTTGG}^{\text{D1-73}})$; $\text{SF8}^{\text{CTLD}}/\text{D1}^{\text{TM-cyto}}$ $({}^{\text{D1-112}}\text{TGGFSQ}^{\text{D1-117}}/{}^{\text{SF8-74}}\text{GATGG}^{\text{SF8-78}})$; Janus $({}^{\text{SF8-215}}\text{WKPSK}^{\text{SF8-219}}/{}^{\text{D1-69}}\text{FWRHN}^{\text{D1-73}})$; $\text{D1}^{\text{CTLD}}/\text{Min}^{\text{TM-cyto}}$ $({}^{\text{MIN-80}}\text{CPLNWK}^{\text{MIN-85}}/{}^{\text{D1-113}}\text{GGFSQS}^{\text{D1-118}})$; $\text{Min}^{\text{CTLD}}/\text{D1}^{\text{TM-cyto}}$ $({}^{\text{D1-119}}\text{CLPNWI}^{\text{D1-124}}/{}^{\text{MIN-74}}\text{GSVKNC}^{\text{MIN-79}})$; $\text{D1}^{\text{CTLD}}/\text{D2}^{\text{TM-cyto}}$ $({}^{\text{D2-80}}\text{PNHWK}^{\text{D2-84}}/{}^{\text{D1-113}}\text{GGFSQSC}^{\text{D1-119}})$ and $\text{D2}^{\text{CTLD}}/\text{D1}^{\text{TM-cyto}}$ $({}^{\text{D1-119}}\text{CLPNWI}^{\text{D1-124}}/{}^{\text{D2-73}}\text{EKMWGC}^{\text{D2-78}})$. Primers and template DNA used are listed in Table 2.3. **$\text{D1}^{\text{CTLD}}/\text{SF8}^{\text{TM-cyto}}$, $\text{SF8}^{\text{CTLD}}/\text{D1}^{\text{TM-cyto}}$ and Janus were constructed by Gordon Brown. $\text{D1}^{\text{CTLD}}/\text{Min}^{\text{TM-cyto}}$, $\text{Min}^{\text{CTLD}}/\text{D1}^{\text{TM-cyto}}$, $\text{D1}^{\text{CTLD}}/\text{D2}^{\text{TM-cyto}}$ and $\text{D2}^{\text{CTLD}}/\text{D1}^{\text{TM-cyto}}$ were generated by Vandana Gupta and Georgia Schafer.**

University of Cape Town

Table 2.2 Primers and template DNA used to clone receptors

Receptor	Template	Forward Primer	Reverse Primer
mClecsf8	Biogel elicited macrophage cDNA	5' GGATCCACCATGTG GCTGGAAGAATCC 3'	5'GAATTCCTCGAGCTTC GAGGGCTTCCAATT 3'
hClecsf8	Bone marrow cDNA	5' GGATCCACCATGGGG CTAGAAAAACC 3'	5' TTCTCGAGGTTCAAT GTTGTTCCAGGTAT 3'
Mincle	RAW264.7 cDNA	5'AAAAGTCGACGGAAGAA AGGCAGGAAAAAGGAAG 3'	5'GTACTCGAGGTCCAG AGGACTTATTTCTGGCAT 3'
Dectin-2	Spleen cDNA	5'AAAGAATTCACCCCTGA CCTTCTGAACATACAC 3'	5'GTACTCGAGGTAAA TCTTCTTCATTTCACA 3'

Table 2.3 Primers and template DNA used to generate chimeras.

Chimera Name	Template DNA	Primer 1	Primer 2
D1^{CTLD}/SF8$^{TM-cyto}$	pFBneo Dectin-1	5' ACAGGAGGTTTTTCTCAG 3'	3' pFB neo
	pFBneo Clecsf8	5' Retro	5' CTGAGAAAAACCTCCTGTAG CTCC 3'
SF8^{CTLD}/ D1$^{TM-cyto}$	pFBneo Clecsf8	5' CAAACTACAGGAGGTAC TTGG 3'	3' pFB neo
	pFBneo Dectin-1	5' Retro	5' CCAAGTACCTCCTGTAGTTTG 3'
D1^{CTLD}/ Min$^{TM-cyto}$	pFBneo Dectin-1	5' GGTTCAGTCAAGAATTGC TGCCTTCCTAATTGGATC 3'	3' pFB neo
	pFBneo Mincle	5' Retro	5' GATCCAATTAGGAAGGCAGC ATTCTTGACTGAACC 3'
Min^{CTLD}/ D1$^{TM-cyto}$	pFBneo Mincle	5' GGAGGTTTTTCTCAGTCT TGTCCTTTGAACTGGAAA 3'	3' pFB neo
	pFBneo Dectin-1	5' Retro	5' TTTCCAGTTCAAAGGACA AGACTGAGAAAAACCTCC 3'
D1^{CTLD}/D2$^{TM-cyto}$	pFBneo Dectin-1	5'GAAAAAATGTGGGGATGC TGCCTTCCTAATTGGATC 3'	3' pFB neo
	pFBneo Dectin-2	5' Retro	5' GATCCAATTAGGAAGGCA GCATCCCCACATTTTTTC 3'
D2^{CTLD}/D1$^{TM-cyto}$	pFBneo Dectin-2	5' GGAGGTTTTTCTCAGTCTT GCCCAAATCACTGGAAG 3'	3' pFB neo
	pFBneo Dectin-1	5' Retro	5'CTTCCAGTGATTTGGGCA AGACTGAGAAAAACCTCC 3'
Janus	pFbneo Dectin-1	5' TGGAAGCCCTCGAAG TTTTGGCGACACAAT 3'	3' pFB neo
	pFBneo Clecsf8	5' Retro	5' ATTGTGTCGCCAAAACTTCGAG GGCTTCCA 3'

University of Cape Town

2.5.3 Other constructs

DNA constructs encoding DAP10, DAP12 and FcRγ in pMXs-IP were a kind gift from Phil Taylor (University of Cardiff) and NKG2D in pRV137G from Brian Rabinovich (University of Texas). NKG2D was amplified from the pRV137G vector by PCR using primers 5' GTCGACCCACCATGAGCAAATGCC 3' (forward) and 5' CTCGAGCACCGCCCTTTTCATGCAGAT 3' (reverse), TOPO cloned and inserted into pFBneo, essentially as described for the other receptors.

2.5.4 Generation of soluble FcClecsf8 fusion protein

A soluble fusion protein containing the extracellular CTLD and stalk region of Clecsf8 fused to the Fc portion of human IgG1 was **generated by Gordon Brown**, essentially as described for Dectin-1 [114]. The Clecsf8 CTLD was amplified by PCR with primers: 5' GGTACCATCATTACTTTTTACGC 3' (forward) and 5' GAATTCCTCGAGCTTCGAGGGCTTCCAATT 3' (reverse). To generate soluble protein the construct was transfected into HEK293T cells and transfected cells were selected with 200µg/ml zeocin (Invivogen). FcClecsf8 was harvested from conditioned supernatants by chromatography on protein A sepharose (GE Healthcare).

University of Cape Town

2.6 Flow cytometry

Flow cytometry was performed on live cells according to conventional protocols in the presence of 2mM NaN_3 and at 4^oC to limit membrane mobility. To inhibit non-specific binding, cells were blocked with FACS block containing 5% heat-inactivated rabbit serum (murine cells) or murine serum (human PBLs) and 50µg/ml human IgG (*in vitro* cultured human cells). For permeabilisation of cells prior to staining, cells were fixed with 1% formaldehyde at room temperate and permeabilised by the addition of 0.5% saponin (Sigma). Cells were stained in V-bottomed 96-well plates. Primary antibodies were generally used at 10µg/ml for an hour, cells were washed three times with FACS wash and secondary antibodies were generally added at 1/200 in FACS block for an hour. Stained cells were fixed with 1% formaldehyde prior to flow cytometry. The following antibodies were used: anti-HA (clone 16B12, Covance), anti-hClecsf8 (MAB2806; R&D systems), CD14-PE, CD16-FITC, CD3-FITC, CD4-FITC, CD8-FITC, CD86-PE, F4/80-PE, CD11b-FITC, 7/4-biotin (all from Serotec), Ly6G-PE, CD19-PE, CD56-PE, HLA-DR-FITC and DC-SIGN-FITC (all from BD Pharmingen); as well as irrelevant PE- or FITC-labelled or unlabelled mouse IgG_1 (BD Pharmingen), IgG_{2b} and IgG_3 (Serotec) control antibodies. The secondary antibodies used were goat anti-mouse PE and donkey anti-mouse APC (both from Jackson Immunoresearch) and streptavidin-APC (BD Pharmingen). Flow cytometric analyses using FcClecsf8, FcDectin-1 and FcClec9A was performed using the Fc proteins at 10µg/ml and PE-conjugated donkey anti-human IgG (Jackson Immunoresearch). Fold changes in surface staining were determined using the following equation: receptor surface expression after treatment (mean fluorescence of

University of Cape Town

receptor specific mAb – mean fluorescence of isotype control)/ receptor expression of untreated cells (mean fluorescence of receptor specific mAb – mean fluorescence of isotype control).

2.7 Phagocytosis assays

2.7.1 Phagocytosis of zymosan particles by transduced RAW264.7 cells

University of Cape Town

RAW264.7 cells transduced with Janus, Dectin-1 or truncated Dectin-1 (which lacks a cytoplasmic domain) were seeded at $5x10^5$ cells/well in 12-well plates the day prior to the assay. Some cells were treated with 5µM Cytochalasin D (Calbiochem) for 40min prior to and throughout the assay, to inhibit phagocytosis. After washing, FITC-zymosan (Molecular Probes) was added at a ratio of 10 particles/cell and allowed to settle for 1h on ice, to allow synchronised phagocytosis to occur. After washing three times with ice cold media to remove unbound particles, cells were incubated at 37^oC for 30min. Cells were then cooled to 4^oC and detached by incubation with Lidocaine/EDTA. Cells were transferred to a 96-well plate, blocked with FACS block for 30min and stained with a rabbit anti-zymosan antibody (Invitrogen) for 1h on ice. Cells were washed three times with FACS wash prior to staining with APC-conjugated goat anti-rabbit antibody (Molecular Probes) for 45min. Cells were washed and resuspended in 1% formaldehyde and analysed by flow cytometry. $FITC^+$ cells which had bound or internalised zymosan particles were gated and the percentage of phagocytosis was determined by comparing the APC^- (internal zymosan) and APC^+ (external zymosan) populations.

For fluorescent microscopy, transduced cells were seeded onto acid-treated glass coverslips at $3x10^4$ cells/well in 24-well plates the day prior to the experiment. FITC-zymosan particle binding on ice and phagocytosis at 37^oC were allowed to take place as described above. Cells were washed three times with cold media and fixed with 4% paraformaldehyde for 30min at room temperature. Cells were permeabilised with 0.25% saponin, 1% BSA in PBS for 30min at room temperature and actin was stained by adding 1µM TRITC-phalloidin (Sigma) for 1h, rocking gently in the dark. Cells were washed three times with PBS. Coverslips were inverted onto drops of Vectashield (VECTOR laboratories) mounting medium containing Hoechst nuclear dye on microscope slides. Samples were analysed by fluorescence microscopy on a Zeiss Axiovert 40.

University of Cape Town

2.7.2 FITC labelling and antibody coupling of Pan mouse IgG Dynabeads

Pan mouse IgG Dynabeads (Invitrogen) are uniform superparamagnetic polystyrene beads, 4.5µM in diameter, which are coated with monoclonal human anti-mouse IgG and therefore specifically recognise mouse antibodies. $8x10^8$ beads were washed and resuspended in 2ml bicarbonate buffer. FITC solution (Sigma) was prepared in DMSO at 1mg/ml and 90µl was added to the beads in 5µl aliquots and rotated overnight in the dark at 4^oC. The following day the beads were washed twice with PBS and resuspended in PBS with 10mM NaN_3. Flow cytometry was used to confirm FITC-labelling. Anti-hClecsf8 or mouse IgG2b isotype control antibodies were added to the beads at 0.5µg per

$1x10^6$ beads and allowed to rotate at 4^oC for 45min. Beads were washed and used to analyse Clecsf8 phagocytosis in neutrophils.

2.7.3 Binding of anti-hClecsf8 coated beads by transduced RAW264.7 cells

To determine the specificity of the antibody coated beads, RAW264.7 cells transduced with hClecsf8 were seeded at $1x10^5$ cells/well in 24-well plates, the day prior to the experiment. After blocking with FACS block for 30min on ice, anti-hClecsf8 or isotype coated beads were added at 2 beads/cell for 1h on ice. After washing to remove unbound beads, cells were lifted with lidocaine/EDTA and bead binding was analysed by flow cytometry.

2.7.4 Phagocytosis of anti-hClecsf8 coated beads by human neutrophils

To analyse the phagocytic ability of Clecsf8 on neutrophils, 24-well plates were coated with autologous serum for at least one hour at 37^oC. After isolation of neutrophils on a Percoll gradient (described in section 2.3.3.3), cells were plated at $5x10^5$ cells/well in the pre-coated 24-well plates and allowed to bind at 37^oC for 30min. Some cells were treated with 5µM Cytochalasin D or 50µM piceatannol (Sigma), to inhibit actin polymerisation and signalling via Syk kinase respectively, for 30min prior to and throughout the assay. Cells were rinsed gently with fresh media and FITC-labelled anti-hClecsf8 or isotype coated beads were added at 2 beads/cell and allowed to bind for 30min at 10^oC. Unbound beads were washed away and cells were placed at 37^oC for 30min to allow phagocytosis

University of Cape Town

to occur. Cells were placed on ice and external beads were stained with goat anti-mouse-PE for 30min in FACS block. Cells were lifted, washed three times and analysed by flow cytometry. Cells which had bound or internalised FITC-beads were gated and the percentage of cells which had phagocytosed beads was determined by comparing the PE^- and the PE^+ populations.

2.8 Zymosan binding and cytokine production assays

To determine the zymosan binding ability of RAW264.7 transfectants, cells were plated at $2x10^5$ cells/well in 24-well plates the day prior to the assay. Cells were washed three times with ice-cold RPMI and 100µg/ml glucan phosphate (a kind gift from David Williams, ETSU, TN) was added to block Dectin-1 where appropriate, for 20min on ice. FITC-zymosan was added at 25 particles/cell and allowed to bind on ice for 1h. Following extensive washing to remove unbound particles, cells were lysed with 3% (v/v) Triton X-100 and the amount of zymosan associated with the cells was measures by fluorometery using a Thermo Fluoroskan Ascent FL. To allow cytokine production to occur, cells were incubated at 37°C for 3h after washing away unbound zymosan. Supernatants were harvested and TNFα levels were measured by ELISA (BD Biosciences). Some cells were treated with piceatannol for 40min prior to the addition of zymosan particles, to inhibit signalling via Syk kinase. The synergistic activity between TLR4 and Clecsf8 was determined similarly, except transduced RAW264.7 cells were stimulated with either 100µg/ml particulate β-glucan or 10ng/ml LPS alone or in combination.

University of Cape Town

2.9 Respiratory burst assays

2.9.1 Respiratory burst assay in RAW264.7 cells

To examine reactive oxygen species (ROS) production, transduced RAW264.7 cell suspensions ($1x10^6$ cells/ml RPMI) were loaded with 20µM dihydrorhodamine 123 (Sigma). Cells were incubated in a 37^oC water bath for 30min before stimulation with 50µg/ml particulate β-glucan. One ml aliquots were removed at particular time points and added to 3ml ice-cold PBS with 1% BSA. Samples were centrifuged at 1000rpm for 5min and resuspended in FACS wash. Conversion of dihydrorhodamine 123 to rhodamine by H_2O_2 results in a bright fluorescent signal which was measured by flow cytometry. Cells loaded with dihydrorhodamine 123 but not stimulated with particulate β-glucan were included to assess background production of H_2O_2. Mean fluorescent intensity of untreated samples was subtracted from mean fluorescent intensity of treated samples.

2.9.2 Respiratory burst assay in neutrophils

Analysis of H_2O_2 production by human neutrophils in response to Clecsf8 stimulation was performed by cross-linking the receptor on 24-well poly-L-lysine plates (BD Biocoat). On the day prior to the experiment, plates were activated with 2.5% gluteraldehyde (Sigma) in PBS for 15min at room temperature, washed three times with dH_2O and once with PBS. Wells were then coated with 50µg/ml cross-linking Sheep anti-mouse IgG (Jackson Immunoresearch) for 30min at room temperature and free aldehyde

University of Cape Town

groups were quenched by the addition of 0.2M glycine in PBS for at least 2h at room temperature. Wells were washed three times with PBS and 10µg/ml anti-hClecsf8 or isotype antibody was added to the wells and allowed to bind overnight at 4°C. The following day, wells were washed extensively with PBS to remove unbound antibody before use. Freshly isolated neutrophils were loaded with 20µM dihydrorhodamine 123, added to the wells and incubated in a 37°C water bath for 30min. Cells loaded with dihydrorhodamine 123 but not cross-linked were included as a negative control and ROS production was measured as described above.

2.10 Protein analyses

2.10.1 Cell lysates and SDS-PAGE

To prepare cellular extracts, cells were counted and lysed with ice cold lysis buffer, at $1x10^{6}$ cells/20µl buffer, on ice for 10-30min. Lysates were centrifuged at full speed for 10min at 4°C to remove cellular debris. Supernatants were mixed with sample buffer and stored at -20°C. Protein separation on the basis of mass was carried out by SDS-PAGE. All samples were mixed with reducing or non-reducing sample buffer and boiled for 5min at 95°C prior to use. 1.5mm thick gels consisting of a 12-20% resolving polyacrylamide gel overlaid by a 10% stacking polyacrylamide gel were cast using a Mini-PROTEAN 3 System (Biorad). Gels were immersed in SDS-PAGE running buffer and 20µl samples were loaded into the wells alongside molecular weight markers. Gels

University of Cape Town

were run at a constant voltage of 100-200V for as long as required for suitable separation and were subsequently analysed by Western blotting.

2.10.2 Western blotting

Following SDS-PAGE, resolved proteins were transferred from acrylamide to Hybond-C Nitrocellulose or Hybond-P PVDF membranes (Amersham biosciences) in Western blot transfer buffer by applying a constant voltage of 100V for 1-2h with cooling. The uniformity of transfer of protein was examined by staining the membranes with Ponceau S (Merck) and destaining with water. Membranes were blocked for 1h at room temperature or overnight at $4^{o}C$ with either 0.5% BSA in PBS or 5% milk powder in PBS and stained with the relevant specific antibodies. After washing three times for 10min with PBS-Tween, membranes were probed with the relevant secondary antibodies for 45min at room temperature and washed to remove unbound antibody. Incubation times and antibody concentrations were dependent on the antibody in use. Blots were developed with SuperSignal West Chemiluminescent Substrate (Thermo Scientific), according to manufacturers' instructions. Specific antibodies used were anti-phosphotyrosine (clone 4G10), anti-Syk, anti-phospho-Syk (Cell Signalling Technology), anti-HA (clone 16B12, Covance), anti-DAP10 and DAP12 (kind gift from Toshiyuki Takai, Tohoku University), or anti-FcRγ (Millipore), followed by appropriate HRP-linked secondary antibodies (Jackson Immunoresearch).

University of Cape Town

2.10.3 Immunoprecipitations

Immunoprecipitations from transduced RAW264.7 were performed by coating 2×10^7 cells/time point with 10µg/ml anti-HA for 2h with rotation at 4°C. When necessary, cells were stimulated with pervanadate for 1 or 3min and lysed as previously described. Pervanadate is a protein tyrosine phosphatase inhibitor which stimulates recruitment of signalling molecules. Lysates were added to pan mouse IgG Dynabeads (Invitrogen) pre-blocked with PBS containing 0.5% BSA and rotated for 2h at 4°C. After extensive washing, immunoprecipitates were analysed by SDS-PAGE and Western blotting.

For immunoprecipitations to confirm transductions with the adaptor molecules and study association of the adaptors with the relevant receptor, A20 cell lysates were prepared as described and pre-cleared by rotation with Protein A Sepharose beads (GE Healthcare) for 30min at 4°C and the beads removed by centrifugation for 10min. Anti-FcRγ, anti-DAP10 or anti-DAP12 were added to the lysates and rotated for 2h at 4°C before addition of Protein A Sepharose beads for an additional hour. Beads were washed extensively with lysis buffer and immunoprecipitates were analysed by SDS-PAGE and Western blotting.

University of Cape Town

2.11 Ligand screening

2.11.1 Carbohydrate microarray screening

Carbohydrate microarray analysis was carried out in collaboration with Angelina S. Palma and Ten Feizi at The Glycosciences Laboratory, Imperial College London. Microarrays of 362 lipid-linked oligosaccharide probes, neoglycolipids and glycolipids were analysed by probing with FcClecsf8 and FcDectin-1, essentially as previously described [115]. The oligosaccharide probes were printed in duplicate on nitrocellulose glass slides at 2 and 7fmol/spot. A comprehensive list of probes can be found in Schallus et al., Molecular Biology of the Cell, 2008 [116]. FcClecsf8 and FcDectin-1 were tested at 20 and 5µg/ml respectively and binding of the probes was detected after 2h using biotinylated anti-IgG, followed by streptavidin-HRP.

2.11.2 Endogenous ligand screening

The organs used for endogenous ligand screening were isolated from sacrificed mice, disaggregated by maceration and strained through a 70µM nylon cell strainer to remove clumps. Cells were centrifuged at 1500rpm for 5min and pellets were resuspended in FACS block and probed with the Fc fusion proteins as described in section 2.6.

University of Cape Town

2.12 Mice

C57BL/6 or Clecsf8$^{-/-}$ mice were obtained from the specific-pathogen-free facilities of the University of Cape Town. The Clecsf8$^{-/-}$ mice were generated on a C57BL/6 background by the Consortium for Functional Glycomics (CFG). Full details of the generation of these animals and their phenotypic analysis can be obtained from the following CFG websites:

https://www.functionalglycomics.org/static/consortium/resources/DataCoreFmcl.shtml

https://www.functionalglycomics.org/glycomics/publicdata/phenotyping.jsp

All animal experimentation conformed to institutional guidelines for animal care and welfare. All experiments utilized at least 6 mice per group.

University of Cape Town

2.12.1 DNA extraction and genotyping

DNA was extracted from 1cm mouse tail clippings by incubating the clipping with 750µl lysis buffer at 55°C overnight. 250µl saturated NaCl was added, the mixture was mixed well and centrifuged for 10min at full speed. After transferring the supernatant to a new tube, DNA was precipitated by the addition of 0.6x volume of isopropanol. After centrifugation at full speed for 5min, the pellet was washed with 70% ethanol and allowed to air dry. DNA pellets were resuspended in dH_2O and used for genotyping by PCR using primers 5' CTGAAAAACTTATTGCTCATAATTACACAGTAT 3' (forward), 5' GGAGGCTTTGGGAGCACATG 3' (wild type reverse) and 5' GTATAATGTATGCTATACGAAGTTATCTCGAG 3' (knockout reverse).

2.12.2 Infection with *Candida Albicans*

C. albicans SC5314 was streaked onto on Sabouraud dextrose (SAB) agar for isolation of individual colonies. Colonies were inoculated into 5ml SAB broth and cultured in a shaking incubator for 24h culture at 30°C. Cultures were washed twice with PBS and diluted to the required concentration. Eight to ten week old female wild type or Clecsf8$^{-/-}$ mice were anaesthetized intraperitoneally and weighed before infection. Live *C. albicans* (1x10^{5} CFU) was administered intravenously in a final volume of 100µg/ml PBS. Mice were weighed and monitored daily and were sacrificed once they had lost 20% of their body weight or if they became moribund, as determined by an animal welfare scoring sheet. Experiments continued for a maximum of 21 days.

2.12.3 Infection with *Listeria monocytogenes*

L. monocytogenes strain EGD was inoculated into tryptic soy broth (TSB) and incubated in a shaking incubator overnight at 37°C. The overnight culture was diluted 1/10 in TSB and allowed to grow with agitation at 37°C until an OD of 0.5 was reached. The culture was washed twice with PBS and diluted to the required concentration. Wild type and Clecsf8$^{-/-}$ female mice (9-10 weeks old) were infected intraperitoneally with 2x10^{6} CFU/mouse in 0.2ml PBS. Mice were weighed and monitored daily and were sacrificed once they had lost 20% of their body weight or if they became moribund, as determined by an animal welfare scoring sheet. Experiments continued for a maximum of 21 days.

University of Cape Town

2.12.4 Infection with *Staphylococcus aureus*

S. aureus was inoculated into 5ml Luria Bertani (LB) medium and incubated with shaking at 37°C overnight. 1 ml of culture was inoculated into 1litre LB medium and cultured for a further 6h before washing twice with PBS and storing at -80°C. The CFU concentration was determined by serial dilution on LB agar and stocks were diluted in PBS to the required concentration. Male wild type and Clecsf8$^{-/-}$ mice (10-11 weeks old) were infected intravenously with 2x10^{7} CFU *S. aureus* in 100µl PBS. Mice were weighed and monitored daily and were sacrificed once they had lost 20% of their body weight or if they became moribund, as determined by an animal welfare scoring sheet. Experiments continued for a maximum of 21 days.

2.12.5 Infection with *Nippostrongylus brasiliensis*

N. brasiliensis was maintained by passage through Wistar rats. Male and female wild type and Clecsf8$^{-/-}$ mice (7 weeks old) were infected subcutaneously with 750 *N. brasiliensis* L3 larvae in 0.65% NaCl and sacrificed 10 days post infection. Serum IgE levels were analysed by ELISA. Adult worm burden was analysed by removing the small intestine, slicing it open and placing it in a gauze bag submerged in 0.9% NaCl solution at 37°C. The worms were allowed to migrate out of the tissue, and were then counted to determine parasite load.

University of Cape Town

2.12.6 Sterile peritonitis

To induce sterile peritonitis, Clecsf8$^{-/-}$ and wild type mice were injected intraperitoneally with 1ml of 4% thioglycollate broth, 18 or 96h prior to peritoneal lavage. Inflammatory cells were collected by peritoneal lavage with ice-cold 5mM EDTA in PBS. Cells were counted and analysed by flow cytometry. Antibodies used were F4/80-PE (Serotec), Ly6G-PE, CD11b-FITC, 7/4-biotin and streptavidin-APC (BD).

2.12.7 Peritoneal inflammation in response to necrotic EL4 cells

University of Cape Town

EL4 cells were heat-shocked to induce necrosis as previously described [117]. Mice (11-12 weeks old) were injected intraperitoneally with $7x10^6$-$1x10^7$ necrotic EL4 cells in 0.15ml PBS and 16h after challenge the numbers of peritoneal cells and percentage of neutrophils were analysed by flow cytometry as described [117].

2.12.8 Neutrophil infiltration induced by thymocyte death

Thymocyte death was induced and neutrophil infiltration analysed as previously described [95, 118]. In particular, female mice (9-10 weeks old) were whole body irradiated with X-rays (4Gy). The thymus was removed 12h post irradiation and the percentage of thymic neutrophils was analysed by flow cytometry using CD11b-FITC and Ly6G-PE (BD Pharmingen).

2.13 Statistics

Comparisons between two groups were performed using the students *t*-test. Comparisons between multiple groups were performed using one way ANOVA, with Dunnett's post test. Infections were analysed using Kaplan-Meier survival curves and log rank tests. All experiments were performed in duplicate or triplicate, and independently at least twice. *; $p < 0.05$.

University of Cape Town

Chapter 3

Characterisation of Clecsf8 expression and regulation

University of Cape Town

3.1 Introduction

Determining the cellular distribution, regulation and physical characteristics of a protein could provide clues as to a C-type lectin's physiological role(s). Prior to studying Clecsf8 expression, it is useful to note the interesting expression characteristics of the Dectin-2 cluster of receptors, which are discussed in detail in Chapter 1. The cell type on which a receptor is expressed, for example, could hint at specific functions. Expression on the surface of macrophages could indicate a function in immune surveillance or pathogen recognition, as is seen for Mincle which recognises fungi and mycobacteria [92, 94, 96, 97, 119]. Similarly, expression on DCs could point towards a function in antigen capture and presentation to T cells. As mentioned in chapter 1, cross-linking BDCA-2 on pDCs resulted in internalisation and presentation of BDCA-2-antibody complexes to T cells [56]. Additionally, studying the regulation of cell surface expression could give some indication as to the role of a receptor in specific cells. For example, DCIR expression on neutrophils appears to be down-regulated in pro-inflammatory settings, which may be a strategy used by the cell to prevent the inhibitory effect of the receptor [65]. An

additional example of interesting regulation of C-type lectin expression is found with Dectin-2. This receptor is a known PRR for certain pathogens and has been shown to be up-regulated in pro-inflammatory settings, presumably as a way of enhancing signalling via this activation receptor under these conditions [75, 88].

Post-translational modification such as glycosylation or dimerisation of a receptor may have implications for expression and ligand binding. For example, lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) is a highly N-glycosylated C-type lectin but once this protein is deglycosylated, it is not efficiently transported to the surface of certain cell types [120]. Additionally, the deglycosylated form of LOX-1 which is expressed on the cell surface has decreased affinity for its ligand, oxidised low density lipoprotein [120]. Similarly, N-linked glycosylation of Dectin-1 has been shown to be essential for the recognition of fungal β-glucan and activation of NF-κB [121].

University of Cape Town

Many receptors are composed of separate ligand-binding and signal-transducing chains. This allows a certain signalling chain to be shared between diverse receptors with different ligand-binding specificities [122]. In myeloid cells, DAP10, DAP12 and FcRγ are abundantly expressed and have an acidic aspartic residue in their transmembrane domains which allows non-covalent association with ligand-binding receptors [123-125]. DAP12 and FcRγ both contain a single ITAM motif, which recruit Syk kinase in myeloid cells, while DAP10 contains a YINM motif which signals via phosphatidylinositol-3 kinase [21]. Therefore, whether a receptor associates with an adaptor molecule containing a signalling motif could also indicate a role in cellular activation as has been shown for

members of the Dectin-2 family [53]. Specifically, Mincle, Dectin-2 and DCAR association with FcRγ is essential for cellular activation via the receptors. Importantly, it has been shown that ITAM signalling under certain conditions can induce inhibitory responses [30, 126]. For example, DAP12 been shown to suppress the activation of macrophages or DCs stimulated via TLRs by inhibiting the production of pro-inflammatory cytokines [127, 128], indicating that signalling via an ITAM motif does not always result in cellular activation.

This chapter focuses on characterising the expression of Clecsf8. I looked at expression of hClecsf8 in peripheral blood cells as well as regulation of the receptor upon cellular differentiation and stimulation with a variety of cytokines and TLR ligands. I additionally analysed receptor localisation in transduced cell lines and examined possible association with adaptor molecules.

University of Cape Town

3.2 Results

3.2.1 Distribution and regulation of Clecsf8 in human peripheral blood

Arce et al. have previously characterised the distribution of hClecsf8 gene expression by Northern blot analysis and detected hClecsf8 expression in freshly isolated monocytes from peripheral blood [106]. The presence of a particular mRNA does not however necessarily translate to protein expression and I therefore wished to examine Clecsf8 expression on the surface of peripheral blood cells. To my advantage, an antibody to

human Clecsf8 was commercially available for use. To test the antibody and verify the specificity for human Clecsf8, I transfected RAW264.7 macrophages with HA-tagged full-length human or murine Clecsf8. I verified surface expression of the receptors by anti-HA flow cytometry (Figure 3.1). Staining with anti-hClecsf8 could detect hClecsf8 on the cell surface but not mClecsf8, indicating that the anti-hClecsf8 does not cross-react with the murine receptor (Figure 3.1). Although the anti-hClecsf8 antibody could be used to stain live cells for surface expression, I found that it was not functional under fixed and permeabilised conditions and could therefore not be used for intracellular staining.

I subsequently used the anti-hClecsf8 antibody to study the expression of hClecsf8 on peripheral blood leukocytes by flow cytometry. Cells were initially separated into granulocytes, monocytes and lymphocytes based and size and granularity and then further characterised using cell-specific markers. In the granulocyte population which were gated by high side scatter (SSC), hClecsf8 expression was detected on $CD16^+$ neutrophils but not on $CD16^-$ eosinophils (Figure 3.2). Monocytes, gated as forward scatter (FSC) high and SSC low, were also hClecsf8 positive. Monocytes were divided into two distinct subsets, namely "recruited" ($CD14^{low}CD16^+$) and "inflammatory" ($CD14^+CD16^-$) cells [129] but hClecsf8 was only detected on the $CD14^+CD16^-$ population. Lymphocytes were gated as FSC low and SSC low and were hClecsf8 negative. Indeed, using specific markers to subdivide the lymphocytes revealed no hClecsf8 expression on $CD4^+$ or $CD8^+$ T cells, $CD19^+$ B cells or $CD56^+$ NK cells (Figure 3.2).

University of Cape Town

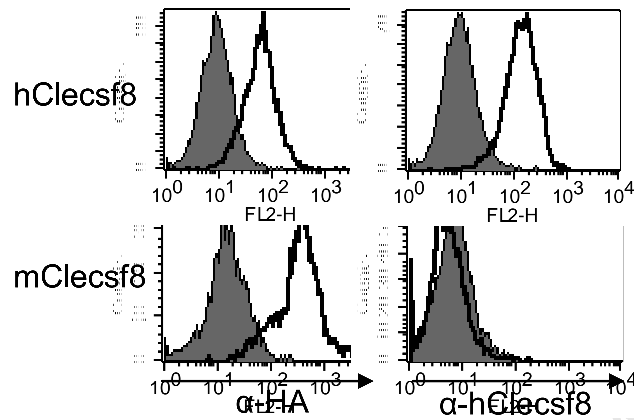


Figure 3.1 **Anti-hClecsf8 specifically recognises human Clecsf8 and does not cross-react with murine Clecsf8.** Flow cytometric analyses of RAW264.7 cells expressing HA-tagged human Clecsf8 or murine Clecsf8 and stained with anti-HA or anti-hClecsf8. Grey histograms represent vector only control cells and open black histograms represent receptor expression.

University of Cape Town

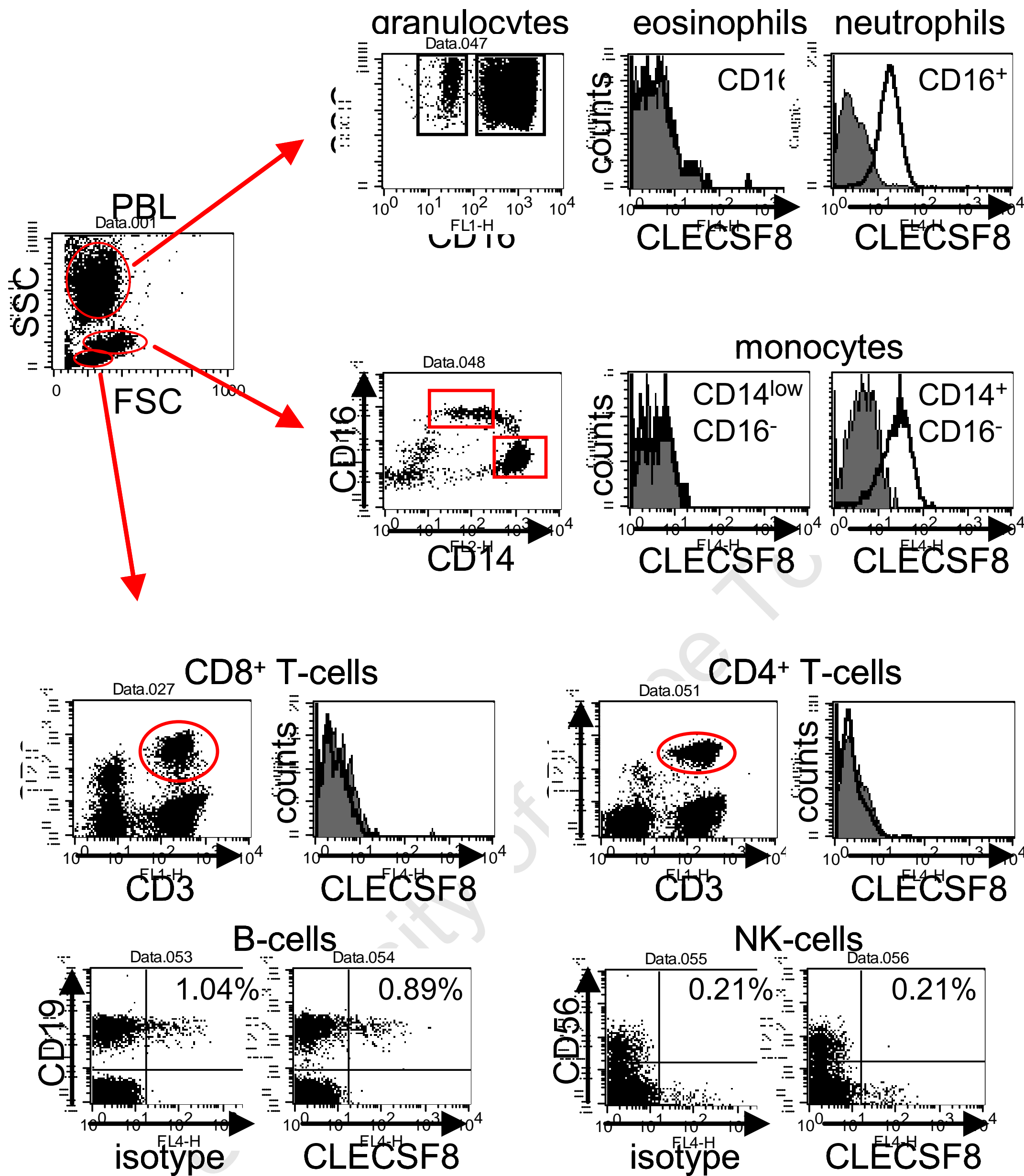


Figure 3.2 **Expression of hClecsf8 on peripheral blood leukocytes.** Flow cytometric analysis of peripheral blood showing the identification and gating of granulocytes, monocytes and lymphocytes. The major cell populations were further differentiated by specific cell markers and stained for hClecsf8 expression. The grey histograms represent the isotype controls and the black open histograms represent anti-hClecsf8 staining. These data are representative of five independent donors.

As I had demonstrated that hClecsf8 is expressed on peripheral blood monocytes, which are cells that are recruited from the blood into tissues where they differentiate into either macrophages or DCs, I next examined expression of Clecsf8 in mature cells. The process of cellular differentiation and maturation into macrophages and DCs could be mimicked by *in vitro* culture of monocytes over several days. Peripheral blood monocytes were isolated and cultured as described and examined at different time points for hClecsf8 expression along with specific markers of differentiation. At day 1, as in peripheral blood, monocytes were positive for hClecsf8 expression as well as CD14, CD86 and HLA-DR. As expected, DC markers such as DC-SIGN were not present on the monocytes [111]. However upon differentiation into macrophages, the expression of hClecsf8 was completely lost by day 2 of culture (data not shown) and could subsequently not be detected on day 4 or day 7 (Figure 3.3). These macrophages were characterised by a decrease in CD14 and increase in HLA-DR expression.

University of Cape Town

I next wished to look at expression of hClecsf8 on immature and mature DCs. To induce a DC-like phenotype, monocytes were cultured with GM-CSF and IL4. This resulted in a decrease in CD14 and increase in DC-SIGN expression on day 4 immature DCs. No hClecsf8 expression was detected on these cells (Figure 3.3). Cells were cultured with LPS to induce activation/maturation of the DCs, characterised by up-regulation of the co-stimulatory molecule CD86. Maturation of the DCs did not however induce hClecsf8 expression (Figure 3.3). As mentioned, due to the limitations of the antibody, I could not investigate whether this decrease in Clecsf8 expression was due to internalisation of the receptor.

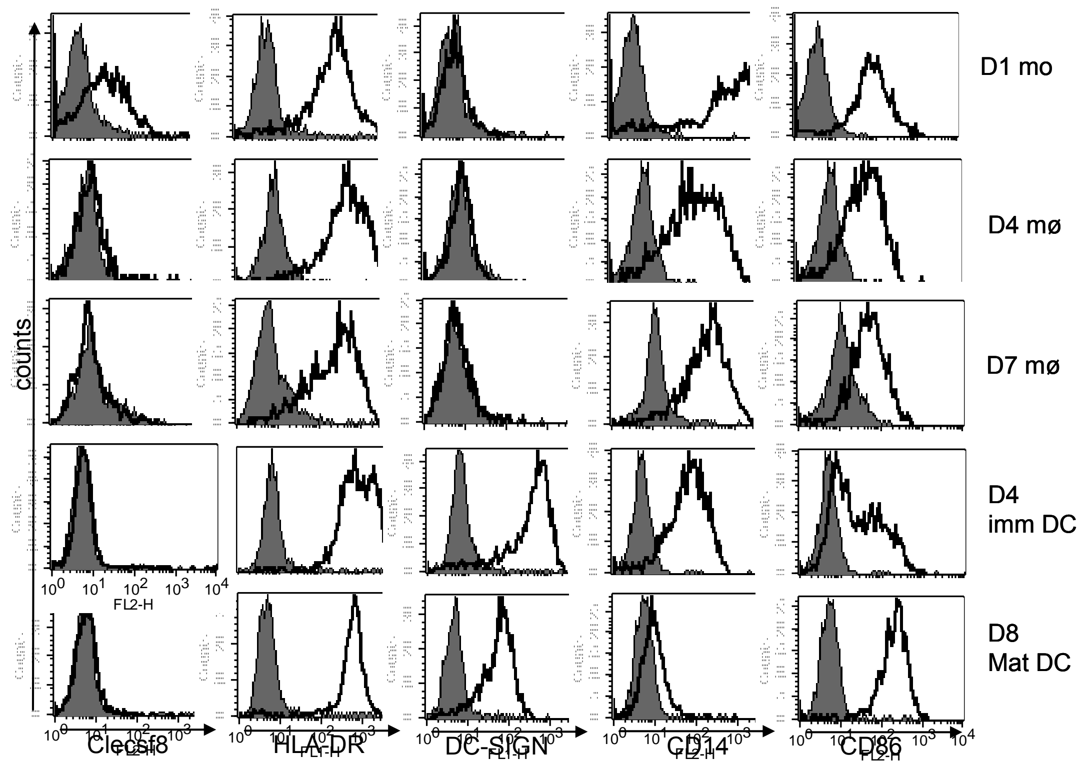


Figure 3.3 **Expression of hClecsf8 on monocytes is lost upon differentiation into monocyte-derived macrophages and DCs.** Analysis of hClecsf8 expression on monocyte-derived macrophages and DC. HLA-DR, CD14, DC-SIGN and CD86 were included as markers of macrophage and DC maturation, as described in the text. The filled grey histograms represent the isotype controls and the black histograms represent specific antibody staining, as indicated. The data are representative of at least five independent donors.

The expression of numerous C-type lectins, including Dectin-1, Clec2 and MICL, have previously been shown to be influenced by certain cytokines or TLR ligands [33, 130, 131]. Indeed, this is true for members of the Dectin-2 family. For example, the activation receptor, Dectin-2 has been shown to be up-regulated in inflammatory settings on $CD14^+$ monocytes stimulated with GM-CSF, TGF-β1 and TNFα and down-regulated with the addition of IL4 and IL10 [88]. Additionally, the inhibitory receptor DCIR expression on neutrophils has been shown to be down-regulated in inflammatory settings [65]. As mentioned, Arce et al. have previously shown that treatment of monocytes with IL6, TNFα, IL10 and IFNγ could up-regulate hClecsf8 mRNA [106]. I therefore wished to determine whether these and other cytokines or TLR agonists could influence hClecsf8 expression at the protein level. As hClecsf8 expression on monocytes was down-regulated as early as one day after culture and Arce et al. showed gene regulation after six hours of stimulation, I stimulated freshly isolated monocytes and neutrophils from peripheral blood for six hours with cytokines or TLR ligands, and examined expression by flow cytometry. Specifically, cells were treated with the anti-inflammatory cytokines IL4 and IL10, pro-inflammatory cytokines IL6, TNFα, and IFNγ, as well as the TLR agonists PAM_3CSK_4 (TLR1/2), LPS (TLR4), flagellin (TLR5) and FSL-1 (TLR2/6). Although I found a significant increase of hClescf8 expression on monocytes treated with LPS, and on neutrophils treated with TNFα, IFNγ, PAM_3CSK_4 and LPS, the overall effect of these agents on receptor expression were relatively modest (< 2-fold) (Figure 3.4). Notably, none of the cytokines or TLR agonists significantly down-regulated hClecsf8 expression.

University of Cape Town

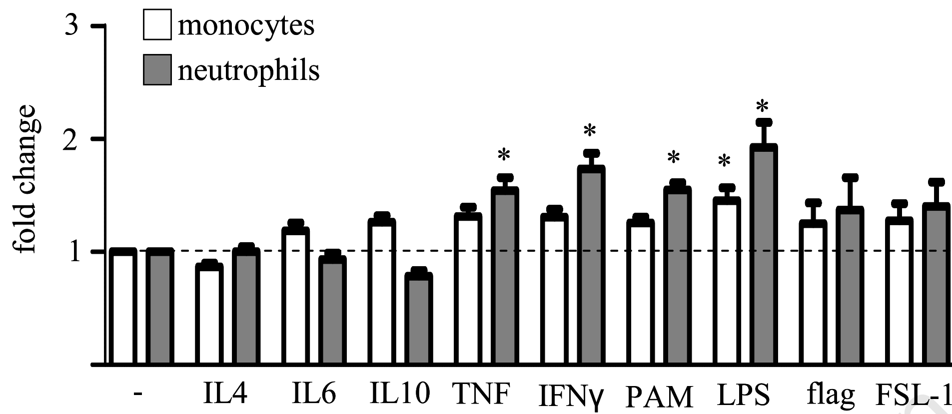


Figure 3.4 **The influence of cytokine and TLR agonist on hClecsf8 expression.** Freshly isolated peripheral blood monocytes (open bars) and neutrophils (grey bars) were treated with various cytokines and TLR agonists and hClecsf8 expression was analysed by flow cytometry. The data were normalised to untreated control value and shown as the mean ± SEM of pooled data from at least five independent donors. *, $p < 0.05$ compared to unstimulated controls. PAM, PAM_3CSK_4; flag, flagellin.

University of Cape Town

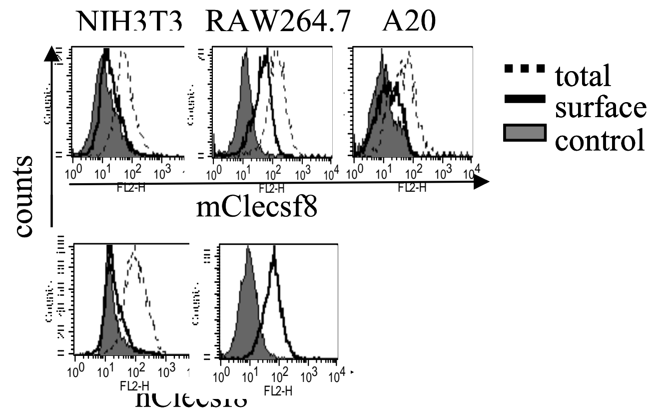
These data therefore indicate that Clecsf8 is expressed on a population of circulating monocytes and neutrophils and that the expression of this receptor is down-regulated following differentiation of monocytes into macrophages or DCs, and can be slightly up-regulated upon exposure to certain pro-inflammatory cytokines and microbial components.

3.2.2 Clecsf8 does not associate with known adaptors for expression at the cell surface.

Most members of the Dectin-2 cluster of receptors possess short cytoplasmic tails and have been shown to associate with FcRγ signalling adaptor for transport to the cell surface [53]. I therefore next wished to examine the cellular localisation of human and murine Clecsf8 in different cell types. To this end, I generated a variety of transduced murine cells expressing HA-tagged hClecsf8 and mClecsf8, in RAW264.7 macrophages, A20 B cells or NIH3T3 fibroblasts and examined expression by staining with an anti-HA antibody. FACS analysis of live cells showed hClecsf8 expression on the surface of the RAW264.7 macrophages but not on NIH3T3 fibroblasts (Figure 3.5A). Both cell lines were positive for hClecsf8 when stained intracellularly, suggesting hClecsf8 retention in NIH3T3 fibroblasts but not macrophages (Figure 3.5A) Similarly, mClecsf8 was expressed on the surface of RAW264.7 macrophages and retained intracellularly in A20 B cells and NIH3T3 fibroblasts (Figure 3.5A). Notably, even though Clecsf8 exhibited surface expression in RAW264.7 cells, significant quantities were also detected intracellularly, as this is an over-expression system. This result was further verified by fluorescent microscopy, whereby mClecsf8 was only detected on the surface of live

University of Cape Town

A



B

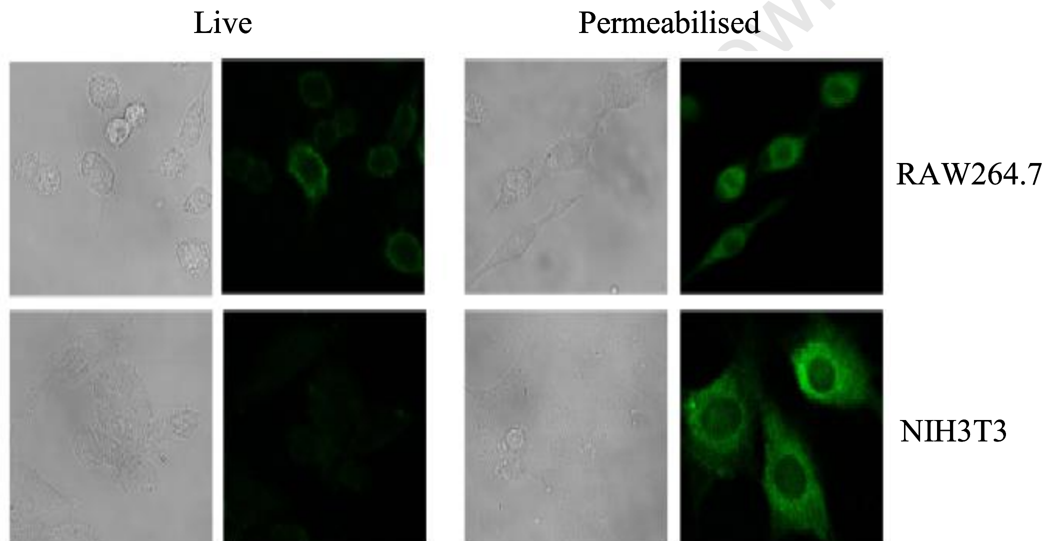


Figure 3.5 **Clecsf8 is expressed on the surface of RAW264.7 macrophages but retained intracellularly in NIH3T3 fibroblasts and A20 B cells.** (A) Anti-HA flow cytometric analyses of NIH3T3, RAW264.7 and A20 B cells transduced with HA-tagged mClecsf8 and hClecsf8. Filled grey histograms represent staining of vector control cells. Solid black histograms represent surface staining of live cells and dashed histograms represent total staining of fixed and permeabilised cells. (B) mClecsf8 expression was assessed in transduced RAW264.7 and NIH3T3 cells by fluorescence microscopy after staining with FITC-labelled anti-HA.

RAW264.7 cells but not on NIH3T3 cells (Figure 3.5B). After permeabilisation, both cell lines stained positive for mClecsf8 (Figure 3.5B). These results show that mClecsf8 is expressed on the surface of myeloid cells but retained intracellularly in fibroblasts and B cells. This suggests that like other members of the Dectin-2 family, Clecsf8 may associate with a myeloid expressed adaptor molecule for expression on the cell surface.

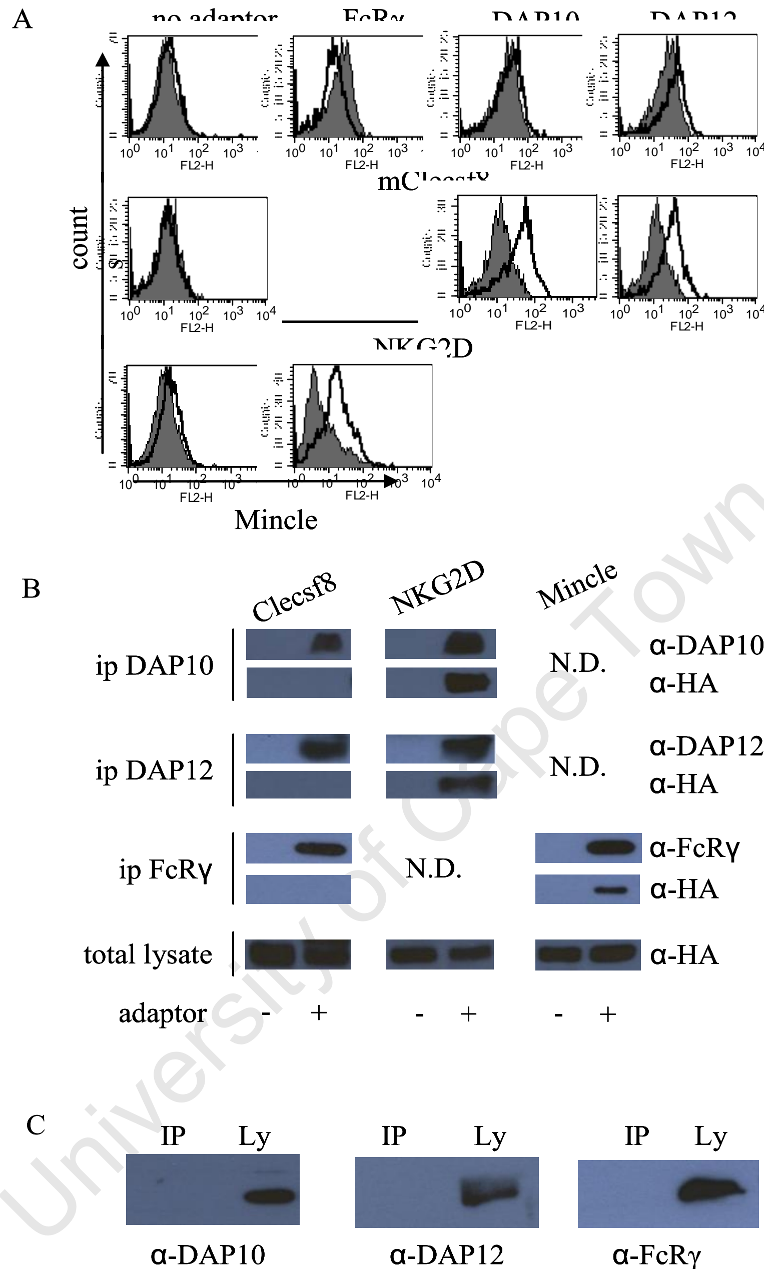
As mentioned, most members of the Dectin-2 family, have been shown to require association with an adaptor molecule for cell surface expression. Indeed, FcRγ chain enhanced DCAR expression in transduced 293T cells [49] as well as Mincle expression in macrophages [95]. As I had shown that Clecsf8 is expressed on the surface of myeloid cells while retained intracellularly in non-myeloid cells, I wished to determine whether the receptor needs association with an adaptor molecule for surface expression. I therefore co-transfected A20 cells expressing mClecsf8 with DAP10, DAP12 or FcRγ chain and examined expression by flow cytometry. As positive controls, I co-transfected A20 cells expressing NKG2D with DAP10 or DAP12 [132] and A20 cells expressing Mincle with FcRγ chain [95]. NKG2D is a C-type lectin-like receptor which has been shown to associate with DAP10 and DAP12 to stabilize cell surface expression and initiate signalling [133]. As previously mentioned, Mincle is a member of the Dectin-2 cluster of receptors which associates with FcRγ chain for intracellular signalling [95]. NKG2D and Mincle were both retained intracellularly in A20 cells unless co-expressed with the relevant adaptor molecule (Figure 3.6A). None of the adaptor molecules induced surface expression of mClecsf8 (Figure 3.6A). To verify that A20 cells were transfected with the relevant adaptors and receptors and to confirm association of receptors with

University of Cape Town

adaptor molecules, I performed immunoprecipitations from transfected cells. Immunoprecipitations were performed with anti-DAP10, anti-DAP12 or anti-FcRγ from A20 cells transfected with receptor only or co-transfected with receptor and adaptor molecule (Figure 3.6B). Immunoprecipitates were analysed by Western blot for the presence of each adaptor molecule and association of the relevant receptors were confirmed by anti-HA staining. Whereas Clecsf8 was not detected with any of the immunoprecipitates, NKG2D was immunprecipitated with DAP10 and DAP12 and Mincle was immmunoprecipitated with FcRγ (Figure 3.6B). Western blot analyses of total cell lysates with anti-HA, confirmed the presence of the receptors.

If Clecsf8 associates with an adaptor molecule for surface expression, as we hypothesize, we would assume that the adaptor is present in RAW264.7 cells as Clecsf8 is expressed on the cell surface. To further confirm that Clecsf8 does not associate with DAP10, DAP12 or FcRγ chain, I immunoprecipitated Clecsf8 from transduced RAW264.7 cells and probed the precipitates with anti-DAP10, anti-DAP12 and anti-FcRγ (Figure 3.6C). As a positive control I confirmed the presence of each adaptor in RAW264.7 cell lysates. As expected, from the flow cytometric data, none of the adaptors were associated with mClecsf8 upon immunoprecipitation. I therefore show conclusively, that Clecsf8 does not associate with DAP10, DAP12 or FcRγ chain, in transduced A20 and RAW264.7 cells. Taken together these results indicate that Clecsf8 is only expressed on the surface of myeloid cells, which is likely to be due to the need for association with an adaptor molecule.

University of Cape Town



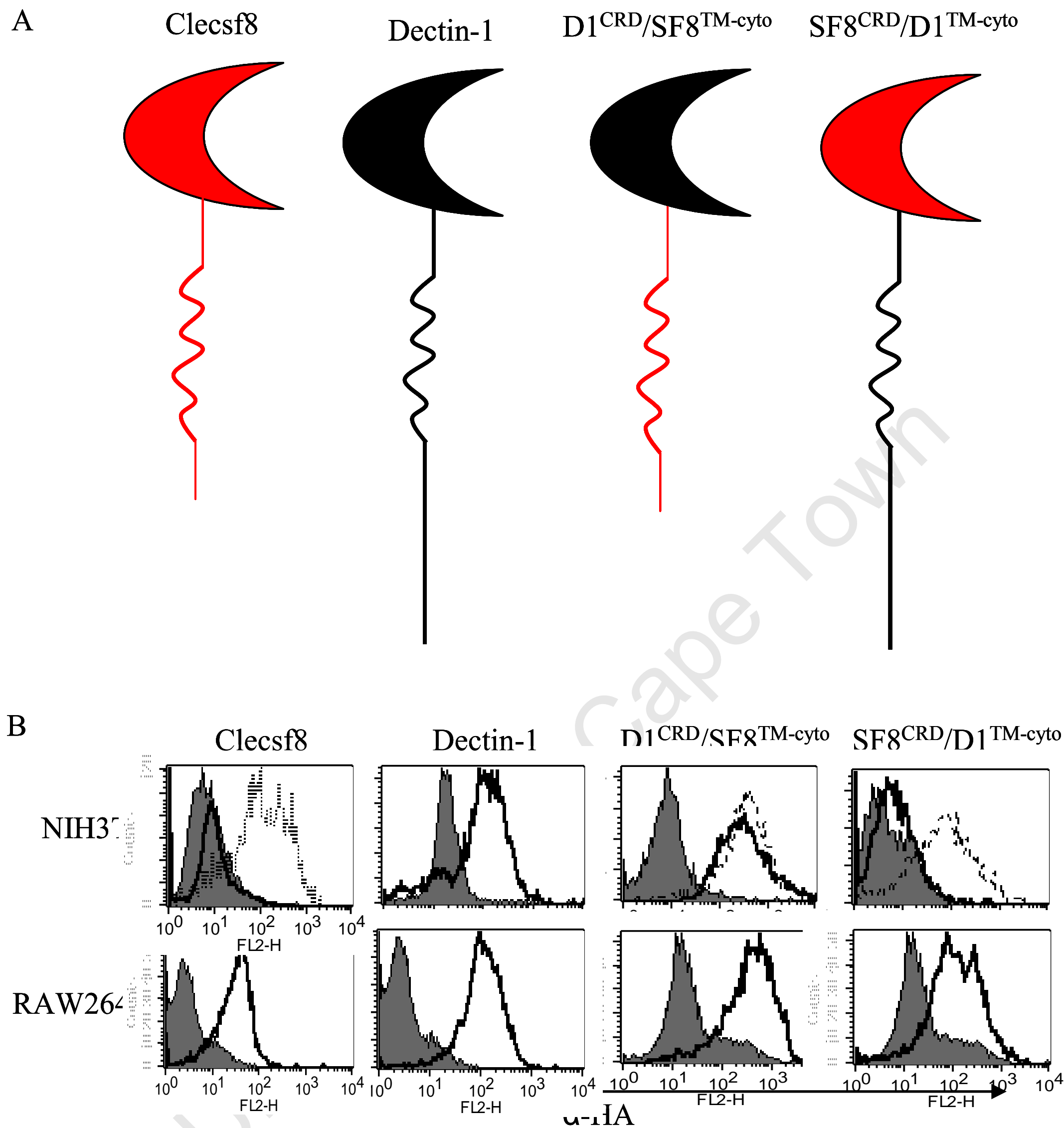
University of Cape Town

Figure 3.6 **Clecsf8 does not associate with FcRγ, DAP10 or DAP12.** (A) Flow cytometric analyses of A20 B cells transduced with HA-tagged mClecsf8, Mincle or NKG2D with or without FcRγ, DAP10 or DAP12. Grey filled histograms represent vector control cells and solid black histograms represent surface expression (B) Western blot analyses of anti-FcRγ, anti-DAP10 or anti-DAP12 immunoprecipitations from the transduced A20 B cells, showing presence of the adaptor molecules and the C-type lectin receptors (probed with anti-HA), as indicated. N.D. Not determined (C) Western blot analyses of anti-HA immunoprecipitations (IP) and whole cell lysates (Ly) from RAW264.7 cells transduced with mClecsf8, probed for the presence of the adaptors, as indicated.

3.2.3 Intracellular retention of Clecsf8 requires the CRD.

Other members of the Dectin-2 family have been shown to associate with signalling adaptors via association with the receptors' transmembrane or cytoplasmic domains, generally via charged amino acid residues [53]. As Clecsf8 appears to lack the appropriate charged residues and does not associate with DAP10, DAP12 or FcRγ, I wished to determine which domain of the receptor was responsible for its intracellular retention. To explore this I generated chimeric receptors, whereby I replaced various domains of mClecsf8 with that of an unrelated C-type lectin, Dectin-1 (Figure 3.7A). I chose to use Dectin-1 for these experiments, as this receptor does not associate with nor require an adaptor for expression at the cell surface [19]. Furthermore, the specificity of the Dectin-1 CRD for β-glucans has been well documented [19], allowing chimeric receptors to be used in future functional studies. I generated two chimeras; the first comprising of the CRD of Dectin-1 coupled to the transmembrane and intracellular regions of mClecsf8 (termed $D1^{CRD}/SF8^{TM\text{-}cyto}$), and the second consisted of the CRD of mClecsf8 coupled to the transmembrane and intracellular regions of Dectin-1 (termed $SF8^{CRD}/D1^{TM\text{-}cyto}$). I then examined the expression of these chimeras in transduced NIH3T3 and RAW264.7 cells. I found that the chimera $SF8^{CTLD}/D1^{TM\text{-}cyto}$, containing the CRD of mClecsf8, displayed the same expression profile as full-length mClecsf8 (Figure 3.7B). In contrast, the chimera $D1^{CRD}/SF8^{TM\text{-}cyto}$ was expressed on the surface of both cell types. As expected [19], full-length Dectin-1 was expressed on the surface of both NIH3T3 and RAW264.7 cells. These findings demonstrate that the CRD of mClecsf8 is responsible for its intracellular retention in NIH3T3 cells.

University of Cape Town



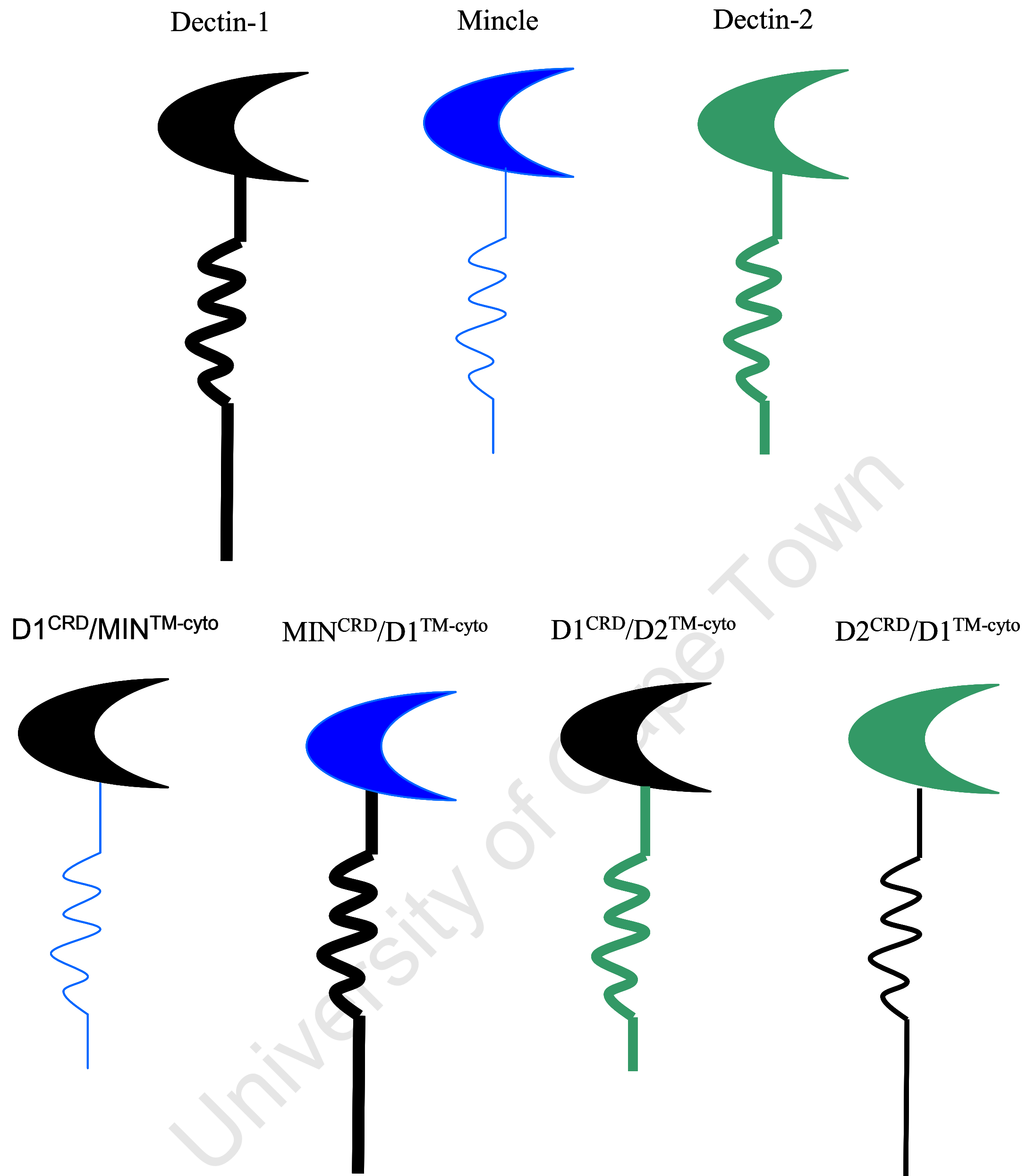
Cape Town

Figure 3.7 **The CRD of Clecsf8 is responsible for intracellular retention in NIH3T3 cells. (**A) Cartoon representations of Clecsf8, Dectin-1 and the chimeras $D1^{CRD}/SF8^{TM\text{-}cyto}$ and $SF8^{CRD}/D1^{TM\text{-}cyto}$. (B) Flow cytometric analyses of Clecsf8, Dectin-1 and the chimeras expressed in NIH3T3 and RAW264.7 cells and stained with anti-HA. Grey histograms represent vector control cells. Solid black histograms represent staining of live cells and dotted black histograms represent staining of fixed and permeabilised cells.

3.2.4 The intracellular retention of Clecsf8 is unique in requiring the CRD

Interestingly, intracellular retention of C-type lectins has not previously been shown to depend on the CRD of a receptor. I therefore wished to determine whether the other members of the Clecsf8 family i.e. Mincle and Dectin-2 would display the same dependency and cellular localisation. **This question was addressed in collaboration with Vandana Gupta and Georgia Schafer.** We generated HA-tagged full-length Mincle and Dectin-2, as well as chimeras (Figure 3.8) consisting of the CRD of Dectin-1 coupled to the body of Mincle ($D1^{CRD}/MIN^{TM\text{-}cyto}$), the CRD of Mincle coupled to the body of Dectin-1 ($MIN^{CRD}/D1^{TM\text{-}cyto}$), the CRD of Dectin-1 coupled to the body of Dectin-2 ($D1^{CRD}/D2^{TM\text{-}cyto}$) and the CRD of Dectin-2 coupled to the body of Dectin-1 ($D2^{CRD}/D1^{TM\text{-}cyto}$) and examined the expression profile of each of these receptors in transduced NIH3T3 and RAW264.7 cells. Full-length Mincle and Dectin-2 displayed the same localisation as full-length mClecsf8 and were expressed on the surface of RAW264.7 cells but retained intracellularly in NIH3T3 cells (Figure 3.9A). However, chimeras $D1^{CRD}/MIN^{TM\text{-}cyto}$ and $D1^{CRD}/D2^{TM\text{-}cyto}$, which contain the Dectin-1 CRD and body of Mincle and Dectin-2 respectively, were retained intracellularly in NIH3T3 cells, in contrast to Chimera $D1^{CRD}/SF8^{TM\text{-}cyto}$ (Figure 3.9B). Conversely, Chimeras $MIN^{CRD}/D1^{TM\text{-}cyto}$ and $D2^{CRD}/D1^{TM\text{-}cyto}$, which contained the CRDs of Mincle and Dectin-2 and the body of Dectin-1, were expressed on the surface of NIH3T3 cells (Figure 3.9B). All chimeras were expressed on the surface of RAW264.7 cells (Figure 3.9B). This indicated that, unlike Clecsf8 which is retained intracellularly due to the presence of the

University of Cape Town



University of Cape Town

Figure 3.8 **Cartoon representations of Dectin-1, Mincle, Dectin-2 and their chimeras.**

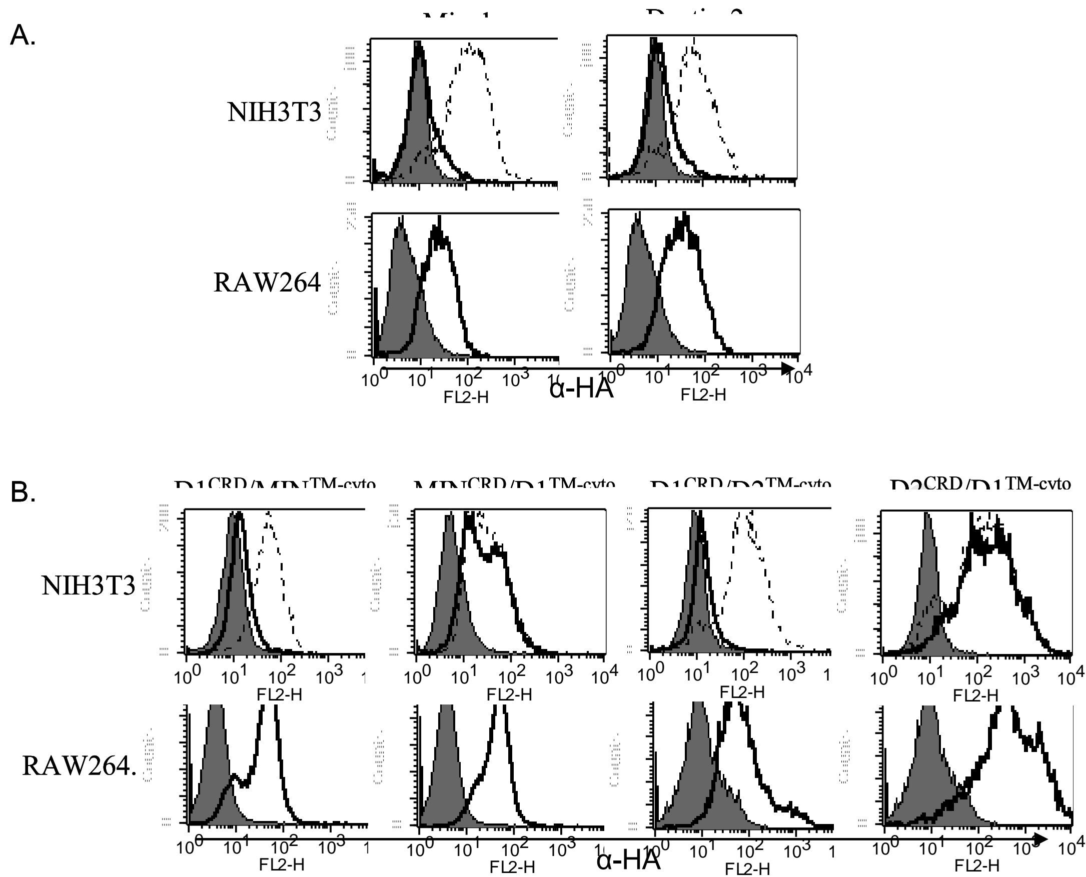


Figure 3.9 **The CRDs of Mincle and Dectin-2 are not responsible for their intracellular retention in NIH3T3 cells.** Flow cytometric analyses of (A) Mincle and Dectin-2 and (B) chimeras consisting of Mincle and Dectin-1 or Dectin-2 and Dectin-1, in NIH3T3 and RAW 264.7 cells stained with anti-HA. Grey histograms represent cells transfected with vector controls, solid black histograms represent surface staining of live cells and dashed black histograms represent total staining of fixed and permeabilised cells.

CRD, Mincle and Dectin-2 are retained intracellularly due to a portion of the transmembrane or intracellular regions of the receptors.

3.3 Discussion

Previous studies on Clecsf8 mRNA reported that both murine and human Clecsf8 were macrophage/monocyte restricted genes [105, 106]. Using a specific anti-hClecsf8 antibody however, I demonstrate that hClecsf8 is expressed on a population of monocytes as well as on neutrophils in peripheral blood (Figure 3.2). The population of monocytes which express hClecsf8 are $CD14^{+}CD16^{-}$ and have generally been classified as "inflammatory" monocytes which express the inflammatory chemokine receptors CCR1, CCR2, CXCR1 and CXCR2 [129]. Adoptive monocyte transfer experiments with the putative murine counterparts of these "inflammatory" monocytes characterised these cells as short-lived with a high homing potential to inflamed tissues [129]. "Inflammatory" monocytes are able to differentiate into macrophages and DCs [129] and I therefore examined hClecsf8 expression upon differentiation. hClecsf8 expression on monocytes was lost upon differentiation into macrophages or DCs *in vitro* (Figure 3.3) but could be marginally up-regulated upon stimulation with LPS (Figure 3.4). Similarly, Arce et al. previously reported that hClecsf8 mRNA was most prevalent in freshly isolated peripheral blood monocytes and expression was lost upon cell culture but could be up-regulated by IL6, TNFα, IFNγ and IL10 [106]. I additionally found that hClecsf8 expression on neutrophils could be up-regulated upon stimulation with TNFα, IFNγ, PAM_3CSK_4 and LPS (Figure 3.4). In comparison to some other members of the Dectin-2

University of Cape Town

family which are widely expressed on different cell types, Clecsf8 expression appears to be limited to short-lived circulating monocytes and neutrophils. Speculatively, expression of Clecsf8 on these specific leukocytes and up-regulation of the receptor in response to pro-inflammatory cytokines and LPS may suggest a role for the receptor in innate immune surveillance and perhaps pathogen recognition.

Having examined the expression of hClecsf8 in peripheral blood I next wished to examine whether Clecsf8 was associating with an adaptor molecule, as seen for other members of the Dectin-2 family. Notably, mClecsf8 and hClecsf8 were expressed on the surface RAW264.7 macrophages but retained intracellularly in non-myeloid cells (NIH3T3 fibroblasts and A20 B cells). Additionally, although the receptor was expressed on the surface of RAW264.7 cells, the cells also contained intracellular pools of Clecsf8 which may be due to artificial over-expression. Speculatively, the presence of the myeloid adaptor necessary to transport Clecsf8 to the cell surface may also be the limiting factor in surface expression of the receptor in these transduced cells. These results showed that Clecsf8 was expressed on the surface of myeloid cells, which is likely to occur via association with an adaptor molecule, and was retained intracellularly in fibroblasts and B cells. I next investigated whether Clecsf8 associates with an adaptor that is present in RAW264.7 but not in NIH3T3 or A20 cells. I therefore co-transfected A20 cells expressing mClecsf8 with DAP10, DAP12 or FcRγ chain but none of these adaptors were able to transport the receptor to the cell surface (Figure 3.6). As controls, I showed that the adaptors were functional as DAP10 and DAP12 were able to transport NKG2D and the FcRγ chain was able to transport Mincle to the cell surface.

University of Cape Town

Generally, receptors which associate with an adaptor molecule do so via a charged amino acid in their transmembrane or cytoplasmic domains [53]. Consistent with the fact that Clecsf8 does not associate with DAP10, DAP12 or the FcRγ chain, the receptor does not contain such a charged residue in these domains. Therefore, to determine which domain of Clecsf8 was responsible for intracellular retention in certain cell types we generated chimeras, whereby we replaced particular Clecsf8 domains with that of Dectin-1 (Figure 3.8). Unexpectedly, I found that surface expression of Clecsf8 involved the CRD and not the transmembrane or cytoplasmic domains. This finding is consistent with my observation that Clecsf8 did not associate with a typical adaptor protein, and suggested that the receptor may interact with a novel molecule. Although the nature of this adaptor remains unknown, it could possibly be another C-type lectin, as discussed in chapter 6.

In an effort to determine whether CRD involvement in surface expression is a trait shared by members of the Dectin-2 cluster, we performed similar expression analyses with chimeras of Dectin-2 and Dectin-1 as well as Mincle and Dectin-1. Unlike Clecsf8, Mincle and Dectin-2 expression was controlled by a portion of the transmembrane or intracellular domains. This is consistent with previous results showing that Mincle association with an adaptor occurs via the transmembrane domain and Dectin-2 association occurs via the cytoplasmic domain [25, 95]. Both receptors associate with FcRγ chain and this would explain why chimeras which contain the body of either Mincle or Dectin-2 are retained intracellularly in NIH3T3 fibroblasts, which do not contain

University of Cape Town

endogenous FcRγ chain. Clecsf8 therefore displays a novel expression profile, whereby the receptor CRD is responsible for intracellular retention in particular cell types.

Taken together these results show that Clecsf8 is a receptor expressed on circulating cells in the blood and on the surface of myeloid cells but retained intracellularly in fibroblasts and B cells, suggesting association with an adaptor molecule. However, the receptor does not associate with DAP10, DAP12 or FcRγ chain and unlike Mincle or Dectin-2, is retained intracellularly due to the presence of its CRD. These results suggest that Clecsf8 may associate with an as yet unidentified adaptor molecule for cell surface expression.

University of Cape Town

Chapter 4

The functional characterisation of Clecsf8

4.1 Introduction

University of Cape Town

Clecsf8 is a C-type lectin which I have shown to be expressed on neutrophils and monocytes in peripheral blood. This expression hints at a possible role in immunity and this chapter focuses on elucidating the function of the receptor. The functions of C-type lectins range from homeostatic roles to that of pathogen recognition and are described in detail in Chapter 1. Ultimately, the aim in characterising any molecule is to elucidate its function(s). For cell surface receptors, their functions can be determined by examining what the receptor interacts with and what occurs once receptor-ligand interactions take place. Extracellular interactions involve binding to ligand(s) which may be either endogenous or exogenous, or both. Ligand binding may induce intracellular signalling that involves recruitment of signalling molecules and the initiation of signalling cascades. These cascades could result in a range of cellular functions, including cytokine production, phagocytosis or induction of the respiratory burst.

The members of the Dectin-2 cluster in particular, act as pathogen recognition receptors as well as receptors for endogenous ligands (discussed in detail in Chapter 1). Analogous to these receptors, Clecsf8 could therefore function in similar manners. Additional clues

as to function(s) may be found by examining the receptor's primary amino acid sequence for the presence of conserved motifs. For example, the CRD may contain the conserved residues responsible for forming Ca^{2+} binding sites or the EPN or QPD amino acid triplets, which would indicate that the receptor is likely to bind mannose- or galactose-type carbohydrates respectively [134]. Similarly, the presence of a conserved cytoplasmic signalling motif such as an ITIM or ITAM, may suggest inhibitory or activation functions of the receptor and could be investigated by studying the receptor's signalling capacity as well its ability to induce certain cellular functions.

The aim of this chapter is to characterise the physiological function of Clecsf8. I commenced by trying to identify possible ligand(s) for Clecsf8, which may provide clues as to the receptor's function(s). Clecsf8 does not contain a cytoplasmic signalling motif but I hypothesised that it may associate with an as yet unidentified adaptor molecule, as explained in Chapter 3. It is possible that this adaptor may contain an ITAM motif through which Clecsf8 may be able to signal. I therefore investigated intracellular signalling via Clecsf8 as well as the possible functions which may be carried out by the receptor. In particular, based on functions held by other members of the receptor's family and due to Clecsf8s expression profile, I looked at functions performed by neutrophils and myeloid cells including phagocytosis, cytokine production and induction of the respiratory burst.

University of Cape Town

4.2 Results

4.2.1 Clecsf8 ligand screening

To screen for Clecsf8 ligands, I generated a soluble FcClecsf8 fusion protein, comprised of the CRD of mClecsf8 fused with human IgG1 Fc domain (Figure 4.1A). The FcClecsf8 construct was transfected into HEK 293T cells and the secreted protein was isolated from the culture supernatants. The Fc domain allowed for the isolation of highly purified protein by means of protein A affinity chromatography, with a molecular weight of approximately 49kDa, under reducing conditions (Figure 4.1B). This construct is similar to the FcDectin-1 fusion protein which I had previously characterised as a tool to detect the Dectin-1 ligand, β-glucan [114]. We were able to use this protein to screen for Clecsf8 ligands by means of flow cytometry and microarray analyses.

4.2.1.1 Carbohydrate microarray analyses

Clecsf8 retains the residues required for calcium coordination, but lacks the conserved triplet motifs that are normally associated with sugar recognition [105]. Despite this I speculated that it may bind a carbohydrate ligand, as is seen for Dectin-1 which also lacks these triplet motifs [135]. We therefore used the FcClecsf8 fusion protein to probe a neoglycolipid-based oligosaccharide microarray in collaboration with Ten Feizi and Angelina Palma at Imperial College London. This microarray included lipid-linked oligosaccharide probes from desired endogenous and exogenous sources and has previously been used to determine the precise carbohydrate specificity of Dectin-1 [115].

University of Cape Town

The probes used 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. [116]. FcClecsf8 was not able to recognise any of the probes included in the microarray (Figure 4.2). FcDectin-1 was included as a positive control and showed strong, selective binding to 11mer and 13mer β1-3 linked glucose sequences, as expected [115] (Figure 4.2).

4.2.1.2 Endogenous ligand screening

As numerous C-type lectins, including those from the Dectin-2 cluster, such as Mincle and DCIR, have been shown to have endogenous ligands, I wished to determine whether the same may be true for Clecsf8. I therefore used the FcClecsf8 protein as a probe to screen live and dead cells from a variety of murine tissues by means of flow cytometry. I probed kidney, spleen, liver, lung, and heart but did not detect a ligand for Clecsf8 in any of these tissues (Figure 4.3A). FcDectin-1 was included as a negative control, as it has previously been shown to not contain an endogenous ligand in the tissues probed [130]. FcClec9A was included as a positive control, as this receptor has previously been shown to recognise a ubiquitous preformed endogenous protein, which is released during necrotic cell death (C. Huysamen, unpublished data) [46].

University of Cape Town

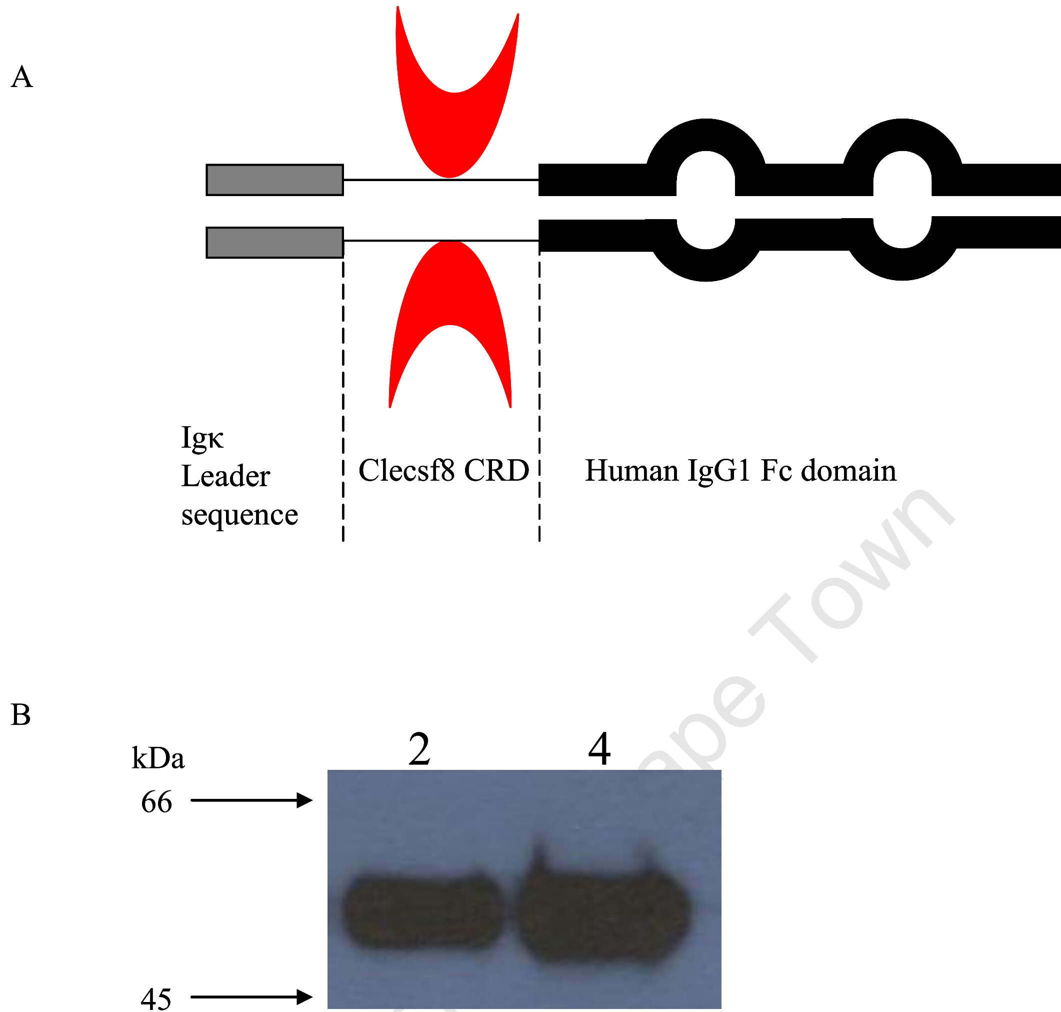


Figure 4.1 **Generation of the FcClecsf8 fusion protein.** (A) Cartoon representation of the FcClecsf8 fusion protein consisting of the Clecsf8 CRD coupled to the human IgG1 Fc domain. (B) Western blot showing 2 and 4µg of purified FcClecsf8 with a MW of 49kDa under reducing conditions.

University of Cape Town

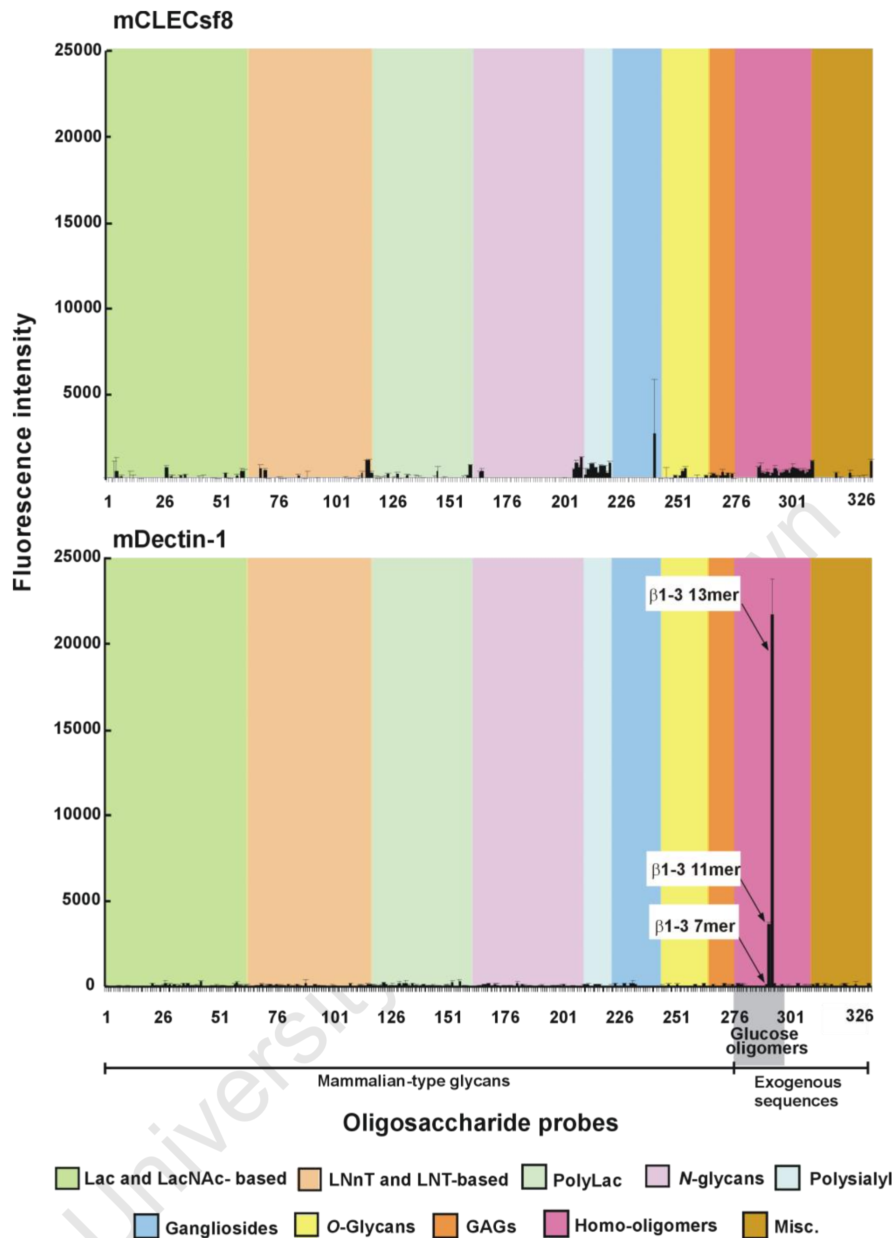


Figure 4.2 **Microarray analyses of FcClecsf8 and FcDectin-1**
Binding signals at 7 fmol/spot (with error bars) are shown for 326 oligosaccharide probes arrayed. None of the probes included in the microarrays elicited significant binding signals with Clecsf8 (the signal at position 234 has a large error bar and is not significant). This is in contrast to Dectin-1, which showed binding to β1-3-linked glucose sequences (11 and 13mers) derived from a glucan polysaccharide, in accord 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, polylactosamine; GAGs, glycosaminoglycans; and Misc., miscellaneous.

Interestingly, some C-type lectin receptors have also been shown to recognise other C-type lectins as their ligands. These receptor-ligand pairs are often genetically linked and encoded adjacently in opposite directions. For example, activation-induced C-type lectin (AICL) is a myeloid-specific receptor which recognises NKp80, an activating C-type lectin receptor expressed exclusively on human NK cells [136]. Interaction of the two receptors results in cross-talk between NK and myeloid cells, stimulating cytokine release from both cell types as well as NK cell mediated cytolysis of malignant myeloid cells [136]. More recently, the genetically coupled NKp65-KACL (keratinocyte-associated C-type lectin) receptor pair has also been identified and engagement similarly stimulates NK cell cytotoxicity and cytokine release [137]. I was therefore interested in whether a ligand for Clecsf8 could be encoded in the same gene cluster. Mincle and Dectin-2 are encoded adjacent to Clecsf8 and as there were RAW264.7 macrophages overexpressing HA-tagged versions of these receptors available to me, I probed the cells with the FcClecsf8 protein and looked at receptor association by flow cytometry. Cells were stained with anti-HA to confirm receptor expression (Figure 4.3B). FcClecsf8 did not specifically recognise Dectin-2 or Mincle expressing RAW264.7 cells, above vector control cells (Figure 4.3C). These results indicate that Clecsf8 does not recognise an endogenous ligand in the tissues screened, nor does it recognise Dectin-2 or Mincle.

University of Cape Town

4.2.2 Clecsf8 functional assays

In the absence of a known C-type lectin ligand, it is a well established approach to generate receptor chimeras to investigate receptor functions. Previous studies have

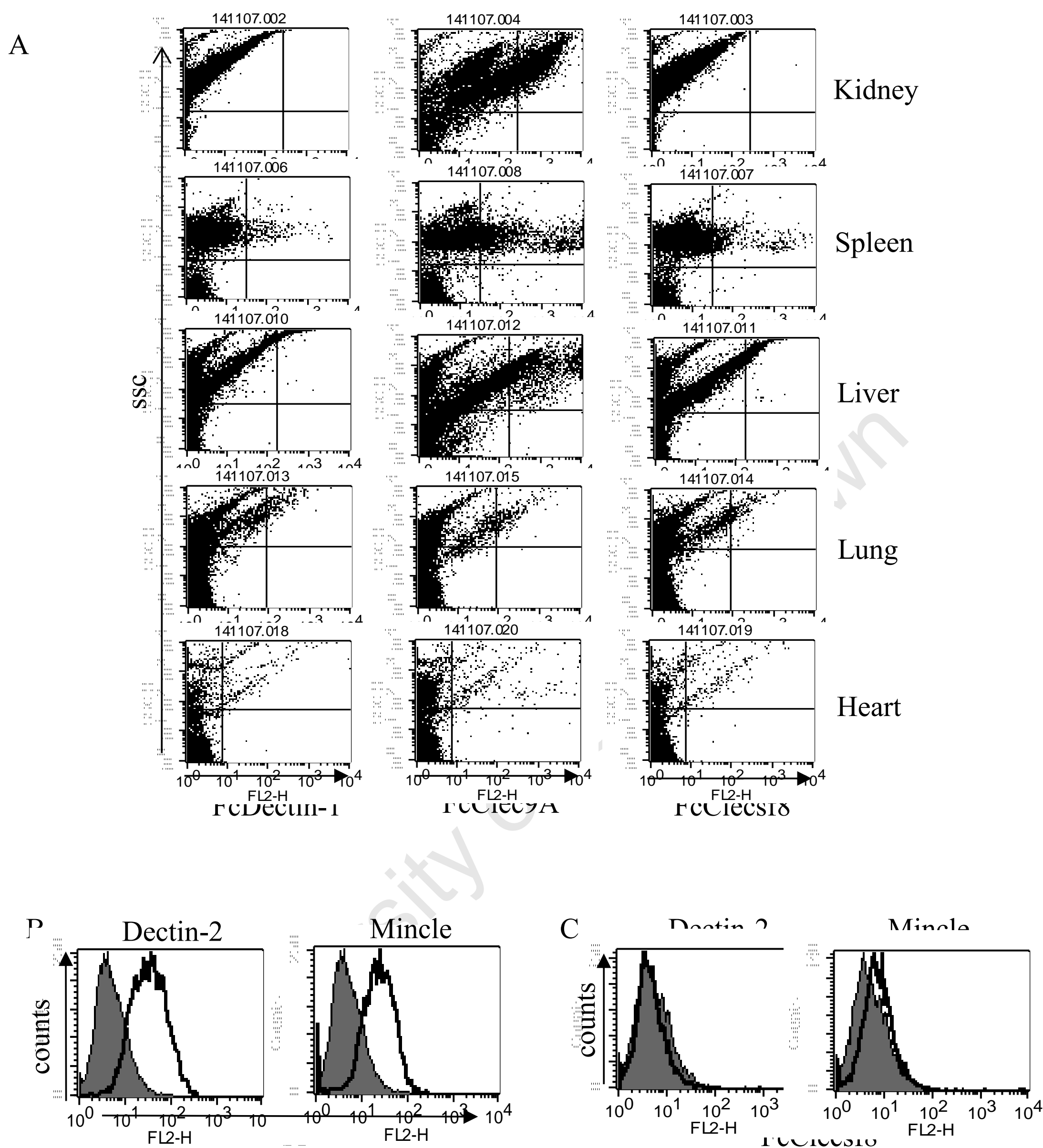


Figure 4.3 **Endogenous ligand screens.** (A) Flow cytometric analyses of non-fixed single cell suspensions isolated from various murine tissues, stained with FcDectin-1, FcClec9A and FcClecsf8. (B) Flow cytometric analyses of RAW264.7 cells expressing Dectin-2 or Mincle probed with anti-HA (open histograms). Cells transduced with vector only were included as a negative control (filled histograms). (C) RAW264.7 cells transduced with vector only (filled histograms) or Dectin-2 or Mincle (open histograms) were stained with FcClecsf8.

generated chimeras consisting of the CRD of Dectin-1 coupled to the cytoplasmic, transmembrane and stalk regions of receptors of interest, such as Clec9A, Clec2 and MICL [33, 113, 138]. The benefit of using such a chimera is that the CRD of Dectin-1 is able to recognise defined ligands, and thus triggering of the chimera using Dectin-1 ligands would induce intracellular signalling through the heterologous cytoplasmic domain [135]. As mentioned in Chapter 3, I generated such a chimera, called $D1^{CRD}/SF8^{TM\text{-}cyto}$ and noted that the chimera was expressed on the surface of both NIH3T3 cells and RAW264.7 cells (Figure 3.8). In NIH3T3 cells, full-length Clecsf8 is not transported to the cell surface, presumably due to the absence of the required adaptor molecule. Thus, as the chimera did not display the same cellular localisation as full-length Clecsf8, it would not suffice for functional analyses. Indeed, following the same hypothesis, as the chimera lacked the Clecsf8 CRD, it would not associate with the necessary adaptor molecules. Therefore, in an effort to mimic natural Clecsf8 localisation and association with the putative signalling adaptor, I generated a novel chimera, Janus, consisting of the CRD of Dectin-1 coupled to full-length mClecsf8 (Figure 4.4A) and expressed the chimera in NIH3T3 fibroblasts and RAW264.7 macrophages. I could demonstrate by means of flow cytometric analyses of live and fixed and permeabilised cells that Janus displayed the same expression profile as full-length Clecsf8 as it was expressed on the surface of RAW264.7 cells. Also, the presence of the Clecsf8 CRD caused intracellular retention in NIH3T3 cells (Figure 4.4B).

University of Cape Town

I next wished to determine whether Janus would be a functional receptor in binding the Dectin-1 ligand, zymosan. I therefore examined the ability of RAW264.7 cells transduced with HA-tagged Janus, Clecsf8 and Dectin-1 to bind zymosan. As expected, expression of Dectin-1 conferred the ability to bind zymosan and this binding could be inhibited by the addition of the soluble β-glucan, glucan phosphate [135]. Similar levels of zymosan binding were obtained by the Janus expressing cells, which could also be blocked by the addition of β-glucan (Figure 4.4C). Cells transfected with pFBneo vector only or Clecsf8 were unable to recognise zymosan or β-glucan (Figure 4.4C). This shows that zymosan and β-glucan are not ligands for Clecsf8. Taken together, I therefore generated a novel chimera with the same cellular localisation as full-length Clecsf8, which was able to bind zymosan and could therefore be used for functional analyses.

4.2.2.1 Clecsf8 mediates phagocytosis

As I identified Clecsf8 expression on monocytes and neutrophils which are known phagocytic cells, I wished to determine whether Clecsf8 could mediate phagocytosis, a function held by other C-type lectins [35]. I initially examined the phagocytic ability of murine Clecsf8 in transduced cells. Characterisation of receptor mediated phagocytosis is generally performed in non-myeloid cell lines, such as NIH3T3 cells to demonstrate whether the receptor under investigation can confer phagocytic activity in normally non-phagocytic cells. However, it has previously been shown that investigating the phagocytic ability of a receptor in RAW264.7 cells mimics results obtained in NIH3T3 cells [34]. Therefore, as Janus is not expressed on the surface of NIH3T3 cells, we examined the ability of RAW264.7 cells transduced with Janus to phagocytose bound

University of Cape Town

FITC-labelled zymosan particles. RAW264.7 cells expressing Dectin-1, which are able to phagocytose zymosan particles, were included as a positive control [34]. As a negative control, I included RAW264.7 cells expressing a truncated version of Dectin-1 which lacks the cytoplasmic domain. It has previously been shown that Dectin-1 lacking the cytoplasmic domain or with a mutation in the ITAM motif, is able to bind zymosan but cannot internalise the particles [34]. Indeed, RAW264.7 cells expressing Dectin-1 were able to phagocytose bound particles. Similarly, RAW264.7 cells expressing Janus were able to phagocytose the particles to comparable levels (Figure 4.5A). As expected, cells expressing truncated Dectin-1 were unable to induce particle uptake. Phagocytosis occurred in an actin-dependent manner as cells treated with cytochalasin D, a fungal toxin which inhibits actin polymerisation, were unable to internalise the particles (Figure 4.5A). These results were confirmed by fluorescence microscopy, which showed that cells expressing Janus and Dectin-1 had internalised zymosan particles, while cells expressing truncated Dectin-1 could bind the particles but were unable to mediate phagocytosis (Figure 4.5B).

University of Cape Town

As I demonstrated that Clecsf8 functions as a phagocytic receptor in transduced macrophages, I next wished to determine whether the human receptor could carry out this function in primary neutrophils. To this end, I made use of antibody-coated ~4.5µm FITC-labelled Dynabeads. These Dynabeads were either coated with anti-hClecsf8 or an isotype control antibody. To verify the specificity of these beads, I examined the ability of RAW264.7 cells transfected with hClecsf8 to bind to the beads. Transduced RAW264.7 cells showed increased binding of anti-hClecsf8 beads compared to isotype

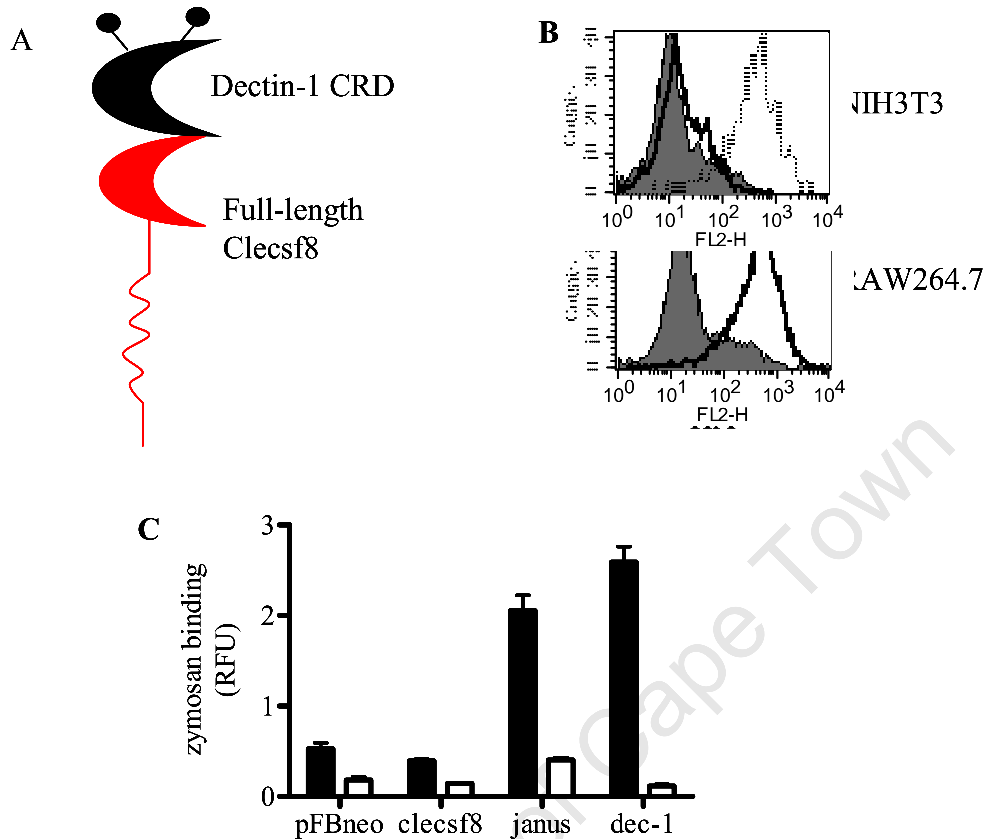


Figure 4.4 **Janus is a functional chimeric receptor.** (A) Cartoon representation of Janus, consisting of the CRD of Dectin-1 coupled to full-length Clecsf8. (B) Flow cytometric analyses of RAW264.7 and NIH3T3 cells expressing Janus stained with anti-HA. Grey filled histograms represent cells transduced with vector only. Solid black histograms represent staining of live cells and dashed black histograms represent staining of fixed and permeabilised cells. (C) Fluorometric quantification of zymosan binding by transduced RAW264.7 cells, in the presence (white bars) or absence (black bars) of glucan phosphate. RFU, relative fluorescence units.

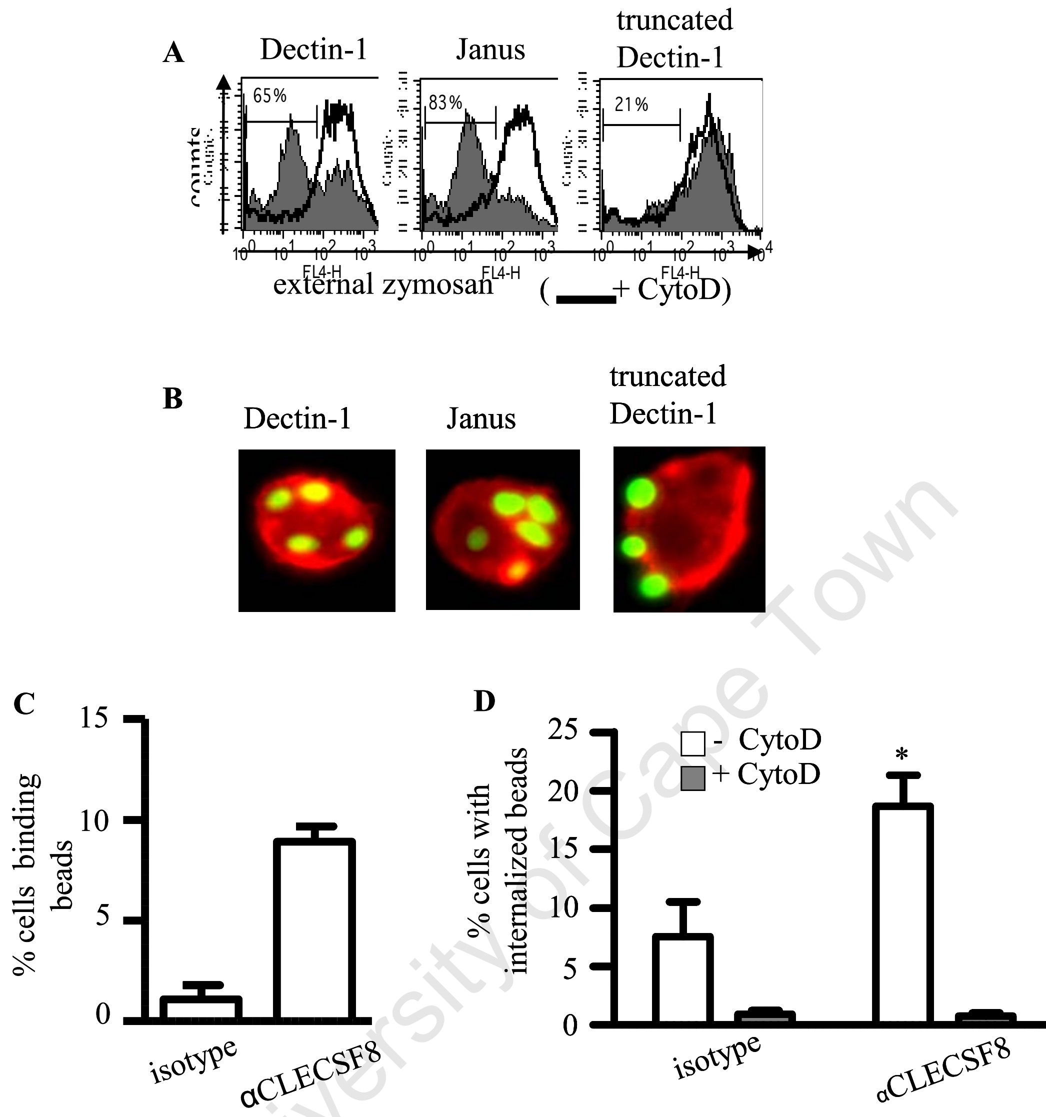
University of Cape Town

coated beads (Figure 4.5C). These results showed that the antibody linked to the beads could still specifically recognise hClecsf8. Furthermore, these beads were bound and internalised by freshly isolate neutrophils to a much greater extent than the isotype control beads. As I had observed for the RAW264.7 cells, internalisation of the beads occurred in an actin dependent manner, as it could be inhibited by the treatment of cytochalasin D (Figure 4.5D). These studies in macrophage cell lines and primary neutrophils demonstrate that Clecs8 is a phagocytic receptor.

University of Cape Town

4.2.2.2 Clecsf8 induces the production of TNFα

As I have shown that Clecsf8 can mediate phagocytosis, I next wished to determine whether stimulation of the receptor could result in cytokine production. To this end, I used RAW264.7 cells transduced with vector control, Janus and Dectin-1 and stimulated the cells with zymosan. Cells expressing Clecsf8 were not included in this experiment as I had previously shown that Clecsf8 is not able to recognise zymosan. In comparison to vector control cells, the expression of Janus was found to induce TNFα production following stimulation with zymosan (Figure 4.6A). The ability of soluble β-glucans to inhibit this cytokine production demonstrated the dependency on the Dectin-1 CRD for ligand recognition by the chimeric receptor. Cells expressing full length Dectin-1 were also able to induce TNFα, as expected [24], and served as a control in these experiments (Figure 4.6A). It has previously been shown that Dectin-1 lacking the cytoplasmic domain or with a mutated ITAM motif, was able to bind zymosan but could not induce cytokine production [24]. This suggests that cytokine production by Janus could occur by



University of Cape Town

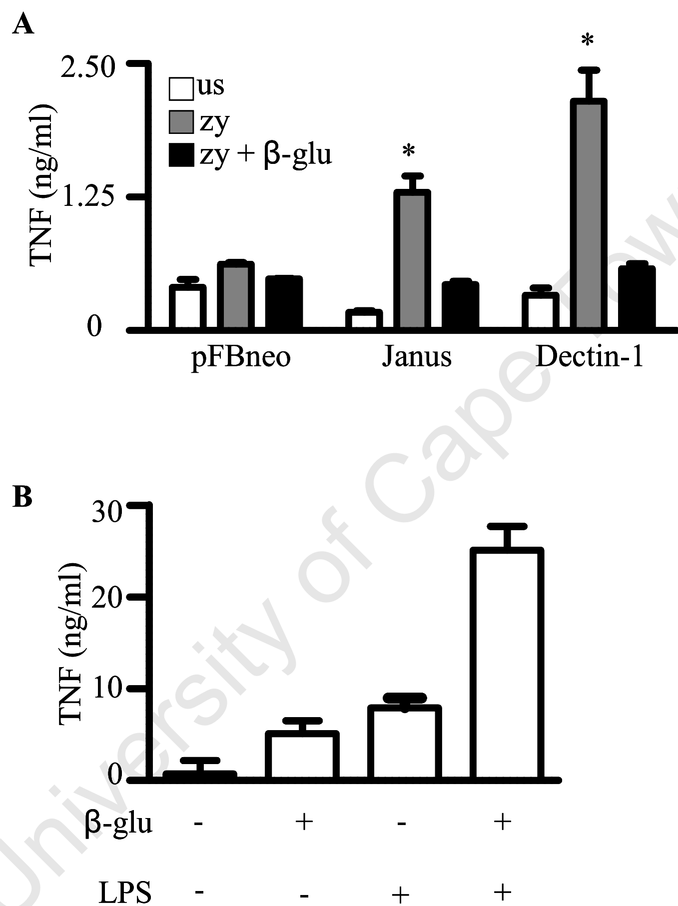
Figure 4.5 **Clecsf8 is a phagocytic receptor.** (A) FACS based phagocytosis assay showing the extent of zymosan internalisation (grey histograms) by RAW264.7 cells expressing Dectin-1, Janus or truncated Dectin-1. The bars indicate the percentage of cells with internalised zymosan. Cytochalasin D (CytoD; unfilled histograms) was included as a control to inhibit phagocytosis. (B) Fluorescence microscopy showing the interaction of TRITC-phallodin stained RAW264.7 cells expressing Dectin-1, Janus or truncated Dectin-1 with FITC-labelled zymosan (green particles). (C) Binding of anti-hClecsf8 or isotype-coated FITC-dynabeads to RAW264.7 cells, as indicated. (D) Binding and internalization of anti-hClecsf8 or isotype-coated FITC-dynabeads to peripheral blood neutrophils, in the presence (grey bars) or absence (white bars) of cytochalasin D (CytoD). Shown are the mean ± SD and the data are representative of at least three independent experiments. *, $p < 0.05$ versus isotype.

signalling via association with an intracellular adaptor molecule, which could possibly contain an ITAM motif. The production of pro-inflammatory cytokines in response to zymosan or fungi can take place in a collaborative manner between Dectin-1 and TLR2 in macrophages and DCs [38, 39]. Indeed, zymosan is a complex *Saccharomyces cerevisiae* cell wall preparation, which consists not only of β-glucans but also mannans, mannoproteins and chitin and is therefore recognised by a variety of receptors. To determine whether cytokine production via Clecsf8 in macrophages was also taking place in collaboration with TLRs, I stimulated Janus expressing RAW264.7 cells with pure receptor ligands. Stimulation with particulate β-glucan or LPS only, induced the production of low levels of TNFα (Figure 4.6B). However, together the stimuli were strongly synergistic (Figure 4.6B). These data show that stimulation of Clecsf8 can induce pro-inflammatory cytokine production and that the receptor may act synergistically with TLR4 to enhance this response.

University of Cape Town

4.2.2.3 Clecsf8 can mediate the respiratory burst

The respiratory burst is an important antimicrobial mechanism which can be activated in neutrophils, macrophages, DCs and mast cells by the C-type lectin Dectin-1 [39-42, 139]. As Clecsf8 is expressed on neutrophils, I wished to determine whether it may also be able to mediate induction of the respiratory burst. To this end, RAW264.7 macrophages transduced with pFBneo vector, Clecsf8, Janus and Dectin-1 were loaded with dihydrorhodamine (DHR) 123 and subsequently stimulated with zymosan. DHR is a nonfluorescent indicator of reactive oxygen species (ROS) production. It is localised in the mitochondria, is freely permeable, and emits a fluorescent signal upon oxidation by



University of Cape Town

Figure 4.6 **Clecsf8 can induce pro-inflammatory cytokine production.** (**A**) Induction of TNFα in RAW264.7 cells transfected with vector control, Janus or Dectin-1 following stimulation with zymosan (zy) in the presence (black bars) or absence (grey bars) of β-glucan (β-glu). Unstimulated (us) cells are included as a control. *, $p < 0.05$ compared to control cells. (**B**) TNFα production by RAW264.7 cells transfected with Janus, stimulated with 100μg/ml β-glucan or 10ng/ml LPS alone or in combination, as indicated. The data shown are mean ± SD and are representative of at least three independent experiments.

H_2O_2 and O_2^- [140]. Flow cytometric analyses over a time course revealed robust ROS production by macrophages expressing Janus and Dectin-1 (Figure 4.7A). In contrast, RAW264.7 cells transduced with Clecsf8 or vector controls failed to induce the respiratory burst in response to zymosan (Figure 4.7A). These results show that Janus could induce the respiratory burst in transduced macrophages and I next wished to verify that the receptor could mediate this activity in primary cells. I therefore cultured purified neutrophils on anti-hClecsf8 immobilised on plastic, to determine if specific stimulation of this receptor was able to induce a respiratory burst. Indeed, I observed that cross-linking of Clecsf8 on human neutrophils resulted in greatly increased ROS production after 30 minutes, when compared to the isotype control (Figure 4.7B). These results show that Clecsf8 is able to induce the respiratory burst in RAW264.7 macrophages and primary human neutrophils.

University of Cape Town

4.2.2.4 Clecsf8 can signal via Syk kinase

As cytokine production and induction of the respiratory burst by Dectin-1 involves intracellular signalling via Syk [38, 42] and as Clecsf8 is able to perform both these functions, I wished to determine whether Clecsf8 signals through this kinase. To this end, I isolated hClecsf8, mClecsf8 and Dectin-1 signalling complexes from transduced RAW264.7 cells by anti-HA immunoprecipitation, following treatment with or without pervanadate, which stimulates recruitment of signalling molecules [141] (Figure 4.8A). Probing with an anti-phosphotyrosine antibody revealed a phosphorylated molecule of ~70kDa in both mClecsf8 and Dectin-1 precipitates stimulated with pervanadate, which was absent in the unstimulated samples (Figure 4.8A). As it is known that Dectin-1

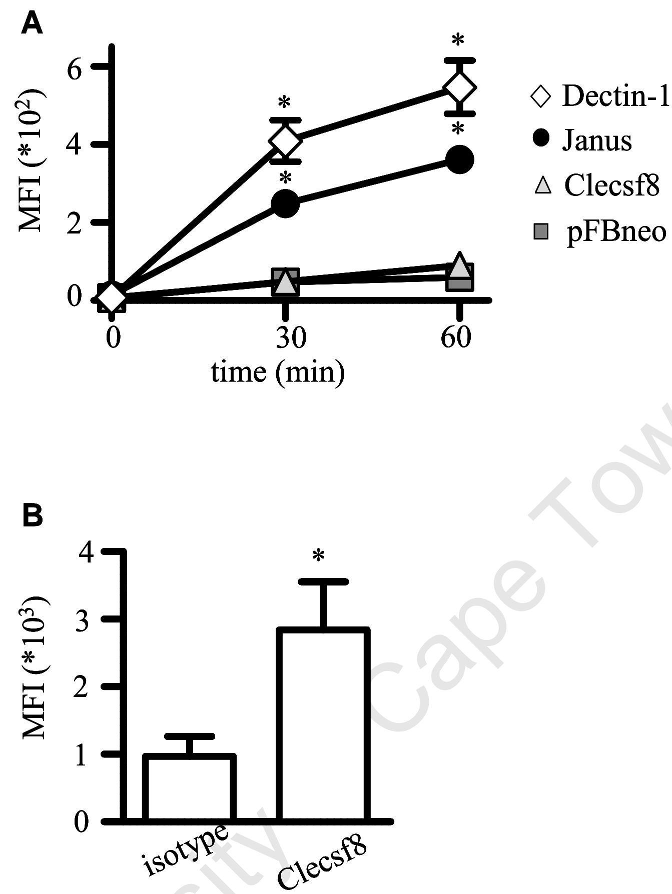


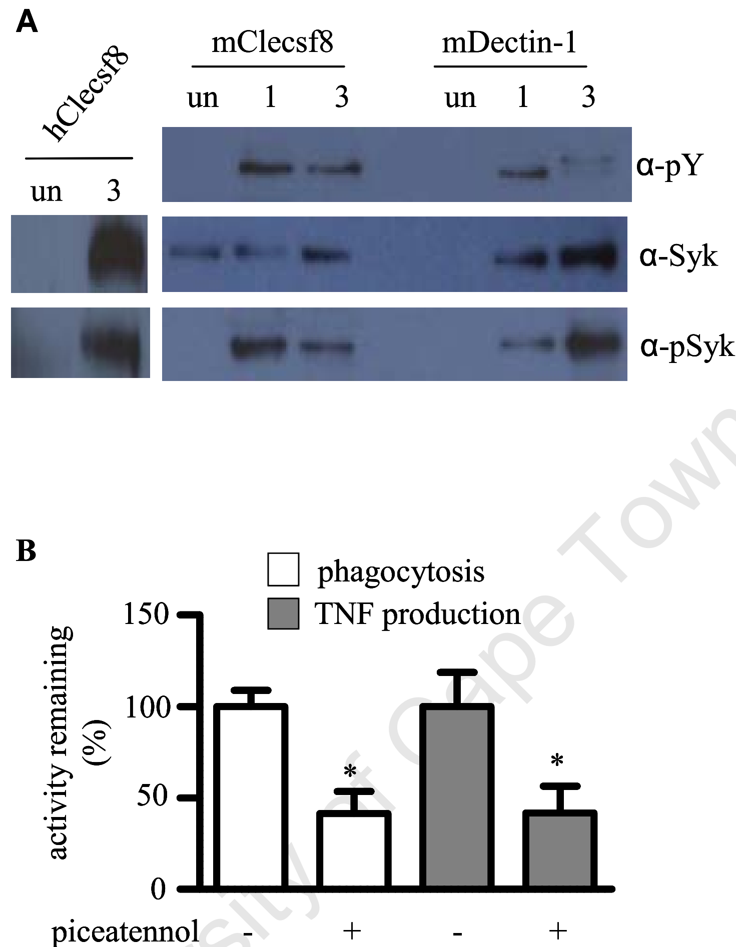
Figure 4.7 **Clecsf8 can mediate the respiratory burst.** (A) RAW264.7 cells transfected with the vector control, Janus, mClecsf8 or Dectin-1 were stimulated with zymosan for the indicated amount of time and the respiratory burst was measured by assessing the conversion of dihydrorhodamine 123 to rhodamine. (**B**) Stimulation of peripheral blood neutrophils for 30 minutes on anti-hClecsf8 or isotype coated plates. The data are expressed as mean fluorescent intensity (MFI) ± SEM of data pooled from two independent experiments. *, $p < 0.05$ versus control.

University of Cape Town

associates with Syk kinase, I next probed with an anti-Syk antibody and demonstrated that Syk could associate with both human and murine Clecsf8 (Figure 4.8A). In addition, stimulation with pervanadate resulted in the phosphorylation of Syk in all instances, which was detected with an anti-phospho-Syk antibody (Figure 4.8A). However, despite considerable effort, I was unable to verify the association of Syk with Clecsf8 in primary neutrophils, a failure which I attribute to very low levels of the receptor on the cell surface (in fact I was also unable to demonstrate immunoprecipitation of Clecsf8 from the cells, since there is no appropriate antibody available for this analysis). Thus these results suggest that Clecsf8 is able to mediate intracellular signalling via Syk kinase.

I wished to determine whether signalling via Syk kinase was responsible for the cytokine production and phagocytic abilities of Clecsf8. To this end, I included the Syk inhibitor piceatannol during zymosan stimulation of RAW264.7 cells transduced with Janus, and examined the effect on TNFα production. As expected, stimulation with zymosan resulted in TNFα production by cells expressing Janus, and this production could be significantly reduced by treatment of the cells with piceatannol (Figure 4.8B). Similarly, to examine whether Syk kinase was responsible for the phagocytic capability of hClecsf8 on neutrophils, cells were treated with piceatannol prior to phagocytosis of anti-hClecsf8 coated beads and this treatment significantly reduced the receptor's phagocytic activity. These results show that Clecsf8 is able to induce cytokine production and phagocytosis by means of signalling via Syk kinase.

University of Cape Town



University of Cape Town

Figure 4.8 **Clecsf8 can signal via Syk Kinase.** (A) Western blots of anti-HA immunoprecipitates from RAW264.7 cells transduced with hClecsf8, mClecsf8 or Dectin-1. Cells were either unstimulated (un) or stimulated for 1 or 3 minutes with pervanadate. Blots were probed with anti-phosphotyrosine (α-pY), anti-Syk and anti-phospho-Syk (α-pSyk), as indicated. (B) Characterisation of the effect of piceatannol on the ability of Clecsf8 to mediate phagocytosis in neutrophils (white bars, see Figure 4.5D) and cytokine production in Janus expressing RAW264.7 cells (grey bars, see Figure 4.7A). The data shown are mean ± SEM normalized to the untreated cells and are pooled data from at least two independent experiments. *, $p < 0.05$ compared to control cells.

4.3 Discussion

This chapter describes the search for a possible Clecsf8 ligand as well as the identification of Clecsf8 functions. Although Clecsf8 contains the nine conserved residues which are generally involved in calcium coordination and carbohydrate binding [105], no ligand was identified when the FcClecsf8 fusion protein was used to probe a carbohydrate microarray (Figure 4.2). Notably, Clecsf8 does not contain the QPD or EPN motifs which are predicted to bind galactosides and mannose, respectively [134]. In addition, although the presence of these conserved residues generally shows carbohydrate ligand recognition, it is important to note that there are a growing number of receptors which contain these residues but bind non-carbohydrate ligands, as well as receptors which lack these residues yet are able to recognise carbohydrates. Dectin-1 for example, is a non-classical C-type lectin which lacks the conserved carbohydrate binding residues, yet is able to recognise a variety of β-1,3-linked and β-1,6- linked glucans [135].

University of Cape Town

In an effort to identify an endogenous ligand, I screened a range of murine tissues using FcClecsf8 as a probe, based on a previous study to identify endogenous ligands for the C-type lectin MICL [130]. However, none of the cells from kidney, spleen, liver, lung or heart appeared to contain a Clecsf8 ligand (Figure 4.3A). There are however many other organs that I have not yet screened and the presence of an endogenous ligand can therefore not be ruled out. Importantly, Mincle and Clec9A have been shown to recognise pre-formed endogenous ligands released during necrosis [46, 95] and I therefore speculated that the same may be true for Clecsf8. The organs I screened were positive for

the Clec9A ligand, indicating the presence of dead cells. Clecsf8 however did not bind a ligand in these cells. Additionally, as I have shown Clecsf8 expression on cells in peripheral blood, it is possible that the ligand may be present in the vasculature, such as on other leukocytes, platelets or erythrocytes, or on endothelium. In a second attempt to identify an endogenous ligand, we screened cells expressing Mincle or Dectin-2 for Clecsf8 recognition as these receptors are genetically linked and encoded adjacently to Clecsf8 (Figure 4.3B). Despite the fact that other C-type lectins have been shown to recognise genetically linked receptors, specifically those encoded directly alongside each other, Clecsf8 did not recognise either of the receptors.

Importantly, many C-type lectins, including those from the Clecsf8 family, have been shown to recognise exogenous ligands from fungi, mycobacteria and viruses [25, 69, 92, 96]. The FcClecsf8 could therefore be used to probe various pathogenic and non-pathogenic microbes in future studies. Additionally, binding to ligands may also take place via other regions of a C-type lectin. For example, DCIR contains the conserved carbohydrate binding residues in its CRD but has been shown to bind to HIV-1 via its neck region and thereby facilitate viral transmission to DCs [69]. If Clecsf8 similarly happens to recognise its ligand(s) via a domain other than the CRD, the FcClecsf8 probe would not be the correct tool for ligand identification. Another possible approach to identify both endogenous and exogenous ligands in the future would be to generate a reporter cell system, which I discuss in detail in Chapter 6.

University of Cape Town

To analyse the function of Clecsf8, in the absence of a known ligand, I used two approaches. Firstly, I generated Janus, a novel chimera which contains two C-type lectin CRDs, and mimicked Clecsf8 function by stimulating the receptor with Dectin-1 ligands in transduced RAW264.7 macrophages. Secondly, I studied Clecsf8 function on primary neutrophils by cross-linking the receptor with an anti-hClecsf8 antibody. These approaches revealed exciting new insights into Clecsf8 function, such as the signalling capacity of the receptor, the ability to induce cytokine production, phagocytosis and the respiratory burst.

University of Cape Town

The functional characterisation of Clecsf8 revealed that the receptor signals via Syk kinase to induce phagocytosis, cytokine production and the respiratory burst, despite the lack of a known cytoplasmic signalling motif. These findings suggest association of Clecsf8 with a signalling adaptor molecule. Indeed, in Chapter 3 I described Clecsf8 retention in NIH3T3 cells due to the absence of the putative myeloid expressed adaptor molecule, which would associate with Clecsf8 via the receptor CRD. To characterise Clecsf8 functionally, I generated the chimeric receptor Janus which contains the CRD of Dectin-1 as well as that of Clecsf8. Janus mimicked the cellular localisation of Clecsf8 and was retained inside NIH3T3 cells. I showed that Janus could bind to zymosan particles and could therefore be used to analyse the function of Clecsf8. Importantly, the use of a chimeric receptor to study receptor functions does have potential drawbacks. As Janus contains a Dectin-1 CRD, I used the defined ligands of Dectin-1, zymosan or β-glucan, to trigger signalling via the Clecsf8 intracellular domain. These ligands were therefore responsible for chimeric receptor clustering which may have led to interactions

that are significantly different from those which occur when Clecsf8 binds its natural ligands. Nevertheless, where possible, I included functional studies in primary neutrophils and directly stimulated the receptor using cross-linking antibodies, to confirm these functions.

As mentioned, similarly to Dectin-2, Mincle and DCAR, Clecsf8 has a short cytoplasmic domain that does not contain a known signalling motif [105, 106]. Mincle, Dectin-2 and DCAR have however been shown to induce intracellular signalling via the Src family kinases and this signalling is dependent on association with FcRγ chain [25, 49, 95]. Despite the lack of a signalling motif or association with a known adaptor molecule, I have shown that Clecsf8 is able to induce intracellular signalling in RAW264.7 cells, via Syk kinase. Presumably this occurs via a novel myeloid adaptor molecule. Importantly, C-type lectin signalling through Syk in myeloid cells has also been shown for numerous other receptors, including Dectin-1, Clec9A, Clec2 and CEACAM3, which all contain or associate with, an ITAM or ITAM-like intracellular signalling motif [33, 42, 138, 142]. C-type lectin signalling through Syk has been shown to be cell specific and can result in numerous cellular outcomes, including cytokine production, phagocytosis, induction of the respiratory burst and the production of arachidonic acid [19]. Additionally, Dectin-1, Dectin-2 and Mincle signalling via the Syk pathway have been shown to induce CARD9 and NF-κB mediated pro-inflammatory responses and promote robust TH_{17} adaptive responses [25, 83, 96, 99, 143]. As Clecsf8 is able to signal via this pathway, it is possible that the receptor may also be able to couple innate to adaptive immunity.

University of Cape Town

Numerous C-type lectins expressed on the cell surface are implicated in antigen phagocytosis [35] and I show here that Clecsf8 is able to mediate phagocytosis in RAW264.7 macrophages and neutrophils. I have additionally shown that Clecsf8 mediated phagocytosis in neutrophils is partially dependent on signalling via Syk (Figure. 7C). Importantly, Dectin-1 mediated phagocytosis has been shown to require Syk only in specific cell types. In macrophages, for example, phagocytosis has been shown to occur via a Syk-independent mechanism, while in DCs, Dectin-1 mediated uptake is Syk dependent [34, 37]. Interestingly, Clec2 and CEACAM3 have both been shown to mediate phagocytosis in neutrophils and for CEACAM3, this uptake has been shown to be Syk dependent [33, 142]. Importantly, the ITAM-like motif in the cytoplasmic domain of C-type lectins such as Dectin-1 and Clec2 has been shown to be essential for mediating phagocytosis [33, 34]. It is important to consider that although Clecsf8 mediated phagocytosis is Syk dependent in neutrophils, it may not be so in other cell types. To better understand the mechanism of Clecsf8 mediated phagocytosis, it would be interesting to investigate the contribution of other enzymes, such as PI-3 kinase, the Rho GTPases and PKC, which are known to be involved in mediating the phagocytic responses of other receptors. These findings indicate that Clecsf8 is able to mediate antigen uptake by circulating cells but the physiological relevance of this, which may include pathogen clearance or presentation to T cells, remains to be determined.

In addition to phagocytosis, I have shown that Clecsf8 is able to mediate the production of the pro-inflammatory cytokine TNFα in RAW264.7 cells. As mentioned, Dectin-1 containing a truncated cytoplasmic tail or mutated ITAM motif has previously been

University of Cape Town

shown to be able to bind to zymosan particles but could not induce TNFα production [24, 39]. Indeed, other members of the Dectin-2 family, including Mincle and Dectin-2 have been shown to induce cytokine production by signalling via the FcRγ chain ITAM motif [53]. Stimulation of Mincle with mycobacterial cord factor, for example, has been shown to induce TNFα and MIP-2 in macrophages [96]. In neutrophils however, co-stimulation with TLR2 agonist and the mycobacterial cord factor, which has recently been shown to be a ligand for Mincle, induced the production of IL10 [97, 144]. I showed that Clecsf8 mediated pro-inflammatory cytokine production may be amplified by costimulation through the TLR pathway in macrophages (Figure 4.6B). It would be interesting to determine whether the receptor could mediate the production of cytokines or chemokines from neutrophils. Surprisingly, in neutrophils coactivation of TLR pathways and C-type lectin signalling via the Syk kinase pathway, has been shown to produce minimal amounts of pro-inflammatory cytokines and large amounts of anti-inflammatory IL10 [144]. It is therefore possible that Clecsf8 may act in a pro-inflammatory manner in macrophages and in a regulatory manner via co-stimulation of the TLR pathway in neutrophils.

University of Cape Town

Stimulation of Clecsf8 also triggers production of reactive oxygen species in RAW264.7 macrophages and peripheral blood neutrophils, a novel function for the Dectin-2 family of receptors. The respiratory burst is an important antimicrobial mechanism which is dependent on signalling via Syk kinase. In fact, Fcγ receptor, Dectin-1 and CEACAM3 have been shown to induce the respiratory burst in different cell types, including neutrophils, and this function is completely lost in Syk-deficient cells or upon Syk

inhibition [42, 142, 145]. An additional antimicrobial function held by neutrophils is the formation of neutrophil extracellular traps (NETs). NETs consist of a chromatin backbone embedded with antimicrobial granule proteins, such as bactericidal permeability increasing protein, neutrophil elastase and myeloperoxidase [146]. Release of NETs from neutrophils has been shown to aid in pathogen clearance by trapping and killing both bacteria and fungi, by providing a high concentration of localised antimicrobial proteins [146, 147]. Interestingly, the formation of NETs after challenge with bacteria is inhibited in Syk deficient neutrophils [148]. It is therefore possible that Clecsf8 could play a role in pathogen recognition by neutrophils to induce intracellular signalling via Syk kinase resulting in extracellular trap formation.

Speculatively, like other members in the Dectin-2 family, Clecsf8 may function as a pattern recognition receptor, although we can't exclude a possible role in the regulation of homeostasis. The expression of this receptor on neutrophils certainly implies a role in innate immunity, as these are short-lived cells which provide a first line of defence against infection and make important contributions to recruitment, activation and programming of APCs. Indeed, neutrophils function at inflammatory sites to attract monocytes and DCs and influence whether macrophages develop pro- or anti-inflammatory responses [149-151]. These functions may be carried out by the production of cytokines such as TNFα, or by cell-cell contact, such as Mac-1 recognition by the C-type lectin DC-SIGN expressed on DCs [151]. A role in immunity is also suggested by Clecsf8's ability to induce phagocytosis as well as the respiratory burst.

University of Cape Town

In summary, the work presented in this chapter characterises Clecsf8 as an activation receptor. Despite the absence of a signalling motif, I show that Clecsf8 is able to signal via Syk kinase, which is very likely to occur via association with a signalling adaptor molecule. Signalling via Syk is necessary for the phagocytic ability of Clecsf8 in neutrophils as well as TNFα production by transduced RAW264.7 macrophages. I additionally show that Clecsf8 is able to mediate the respiratory burst in both transduced macrophages and primary neutrophils. Despite the novel insights into the functions of Clecsf8 presented in this chapter, the physiological roles of the receptor are still unclear. In the chapter to follow, I will describe the characterisation of Clecsf8 deficient mice, in an effort to elucidate these functions.

University of Cape Town

Chapter 5

In vivo characterisation of Clecsf8

5.1 Introduction

Mice deficient in a specific receptor can be used as a valuable tool to study the role of molecules in controlling infection and/or maintaining homeostasis. The functions of numerous C-type lectins, including members of the Dectin-2 family, have been characterised in this manner. For example, DCIR has been shown to be a negative regulator of DC expansion, as mice deficient in DCIR have increased levels of DCs and activated T cells in the lymph nodes [70]. These mice spontaneously develop joint abnormalities and are more susceptible to collagen induced arthritis, indicating a protective role for DCIR in the prevention of autoimmune disease [70]. The role of Mincle in response to fungi has also been identified using knockout mice. Mincle deficient mice have been shown to have greater kidney fungal burden than wild type mice when infected with *Candida albicans*, and bone marrow derived macrophages from Mincle deficient mice produced lower levels of TNFα than wild type cells, in response to *Candida* yeast [92]. In a separate study, macrophages from Mincle deficient mice were also shown to have impaired inflammatory cytokine production compared to wild type cells in response to the fungus *Malassezia pachydermatis* [94]. Additionally, blocking Dectin-2 recognition of fungi in Dectin-1 deficient mice demonstrated the necessity for

University of Cape Town

Dectin-2 in driving the TH_{17} adaptive response to *Candida albicans* [83]. Indeed, Dectin-2 deficient mice displayed significantly lower survival and higher fungal burden in the kidneys than wild type mice upon infection with *C. albicans* [77]. Additionally, recognition of *C. albicans* by Dectin-2 on human DCs has been shown to specifically activate NF-κB subunit c-Rel, which is pivotal in expression of IL1β and IL23, indication a specialised function for Dectin-2 in TH_{17} immunity [152]. These are but a few examples of how the availability of a knockout mouse can add invaluable insights into the function of a receptor. As I had previously shown Clecsf8 to be an activation receptor *in vitro,* I wished to characterise the physiological function(s) of the molecule and therefore studied the Clecsf8 deficient mouse (termed "Clecsf8 knockout").

The Clecsf8 knockout (Clecsf8$^{-/-}$) mice were generated on a C57BL/6 genetic background by the Consortium for Functional Glycomics (CFG). Full details of the construction of the knockout mice can be found on the website (www.functionalglycomics.org). In brief, a targeting vector was designed which contained a selection cassette comprising a resistance gene for neomycin and thymidine kinase flanked by loxP sites, as well as a single loxP site 5' to exon 5 of the Clecsf8 gene (*Clec4d)* (Figure 5.1). These sites would allow cre-recombinase to excise the cytoplasmic, transmembrane and neck regions of *Clec4d* (Figure 5.1).

The knockout mice appear to be phenotypically normal and do not suffer any adverse effects related to the gene deficiency. Although not significantly different to wild type animals, immunological examination by the CFG showed a trend towards decreased T

University of Cape Town

and B cell proliferation in knockout mice when cells were stimulated with anti-CD3 and anti-IgM respectively and a trend towards increased mature re-circulating B cells and c-kit cells in the bone marrow of knockout mice. However, the mice appear healthy, breed well and histological examination of numerous organs showed no abnormalities. As the Dectin-2 family of receptors have been shown to play essential roles in recognising pathogens and/or maintaining homeostasis, I wished to determine whether Clecsf8 plays a part in any of these functions. In this chapter, I therefore investigated the role of the receptor in a variety of inflammatory and infectious conditions, using candidate models which were available in our laboratory.

5.2 Results

5.2.1 Genotyping

Before we used the Clecsf8$^{-/-}$ mice in any experiments, we confirmed that they were deficient in Clecsf8 by means of genotyping PCR. DNA was isolated from tail cuts of Clecsf8 knockout and wild type C56BL/6 mice and specific primers were used to amplify *Clec4d*. Genotyping of wild type mice produced a band of 550bp and Clecsf8$^{-/-}$ mice were confirmed to be homozygous as they produced a single band of 360bp (Figure 5.2).

University of Cape Town

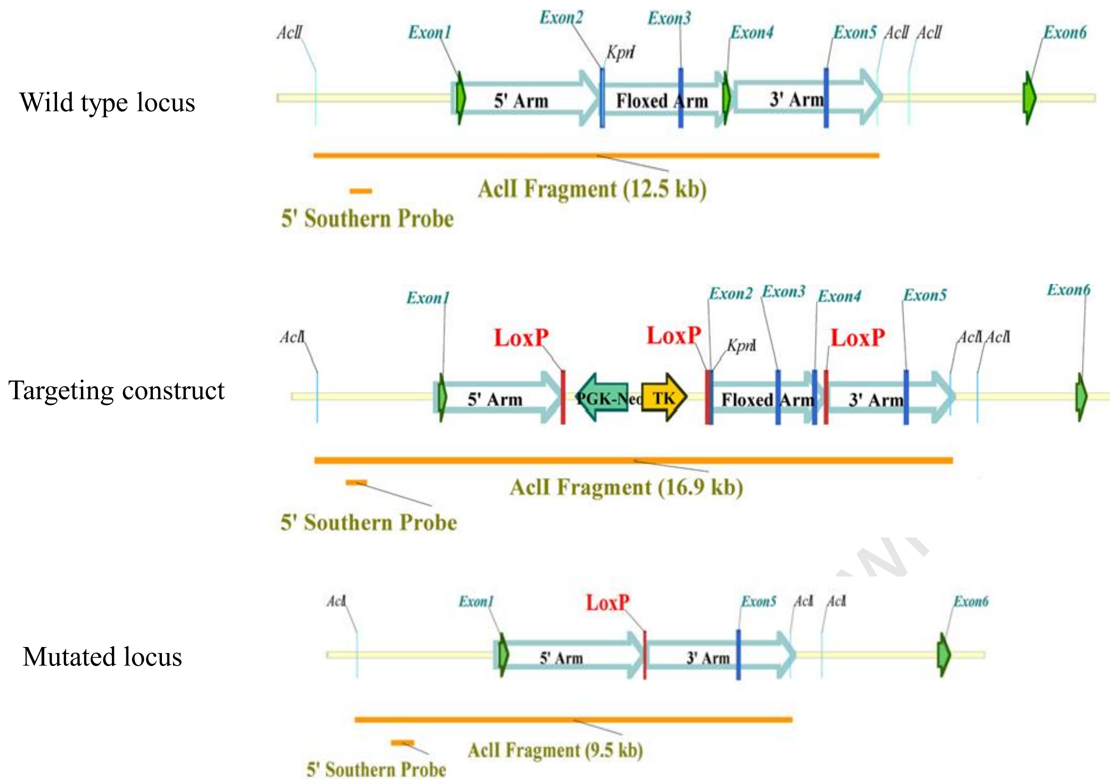


Figure 5.1 **Generation of Clecsf8$^{-/-}$ mice.** Scheme of the Clecsf8 gene locus, the targeting construct and the mutated locus. Adapted from https://www.functionalglycomics.org/static/consortium/resources/DataCoreFmcl.shtml

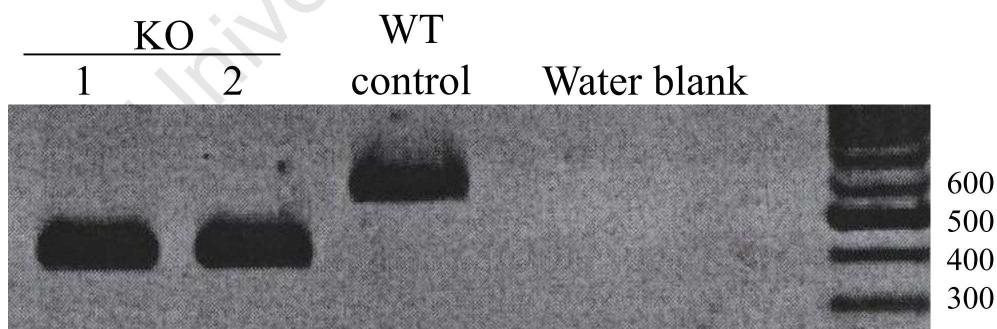


Figure 5.2 **Genotyping of Clecsf8 knockout mice.** Mice were genotyped before use in experiments. PCR of DNA extracted from tail cuts from two Clecsf8$^{-/-}$ (KO) mice produced bands of 360bp and a wild type (WT) control mouse produced a band of 550bp.

5.2.2 The role of Clecsf8 in the inflammatory response

Myeloid cells play essential roles in the propagation and resolution of inflammation and as Clecsf8 is expressed on myeloid cells including monocytes and neutrophils, I wished to determine whether the receptor played a role in recruitment of cells to sites of inflammation.

5.2.2.1 Thioglycollate induced inflammation

To determine whether Clecsf8 plays a role in inflammation, wild type and knockout mice were injected intraperitoneally with thioglycollate to induce sterile peritonitis. Peritoneal cells were isolated at early (18 h) or late (96h) time points by lavage and cell recruitment was analysed by flow cytometry. Staining cells for F4/80 and CD11b or Ly6G and 7/4 expression enabled us to identify different cell populations (Figure 5.3A) [75]. No difference was seen in the total number of cells or percentage of macrophages and neutrophils between Clecsf8 knockout and wild type mice, at either 18 or 96 hours after induction of peritonitis (Figure 5.3 B). Ninety-six hours after induction of inflammation both groups displayed a decrease in the average percentage of neutrophils and a slight increase in the average percentage of macrophages (Figure 5.3B). These results show that in the absence of Clecsf8, mice demonstrate a normal thioglycollate induced peritoneal inflammatory response.

University of Cape Town

5.2.2.2 Necrosis induced inflammation

Physiological cellular processes result in dead cells which are normally ingested and removed by phagocytes to maintain tissue homeostasis. This physiological cell death, known as apoptosis, generally does not induce inflammatory responses [153]. However, if homeostasis is not properly maintained, or if excessive cell death occurs and apoptotic cells are not efficiently cleared, the cells undergo necrosis. Necrosis is characterized by the disruption of membranes and release of cellular and nuclear components, which can act as damage signals and induce an inflammatory response. Our understanding of the receptors responsible for this recognition and the mechanisms by which they respond remains limited, however Mincle and Clec9A have recently been shown to be able to detect cells dying of necrosis and Mincle has been shown to aid in induction of the inflammatory response to these cells [46, 47, 95]. I therefore wished to determine whether Clecsf8 contributes to the inflammatory response induce by necrotic cell death. Speculatively, this could occur via recognition of necrotic cells by Clecsf8 and the subsequent production of cytokines to induce cellular recruitment, as has been suggested for Mincle. To examine whether Clecsf8 recognises necrotic cells or plays a role in inflammation I used two models, including necrotic cell induced peritonitis and whole-body irradiation.

In an effort to determine whether Clecsf8 recognises a ligand in necrotic cells, I injected heat-shocked necrotic EL4 cells into the peritoneum of wild type and knockout mice and characterised the inflammatory response 16 hours later. Peritoneal cells were isolated by

University of Cape Town

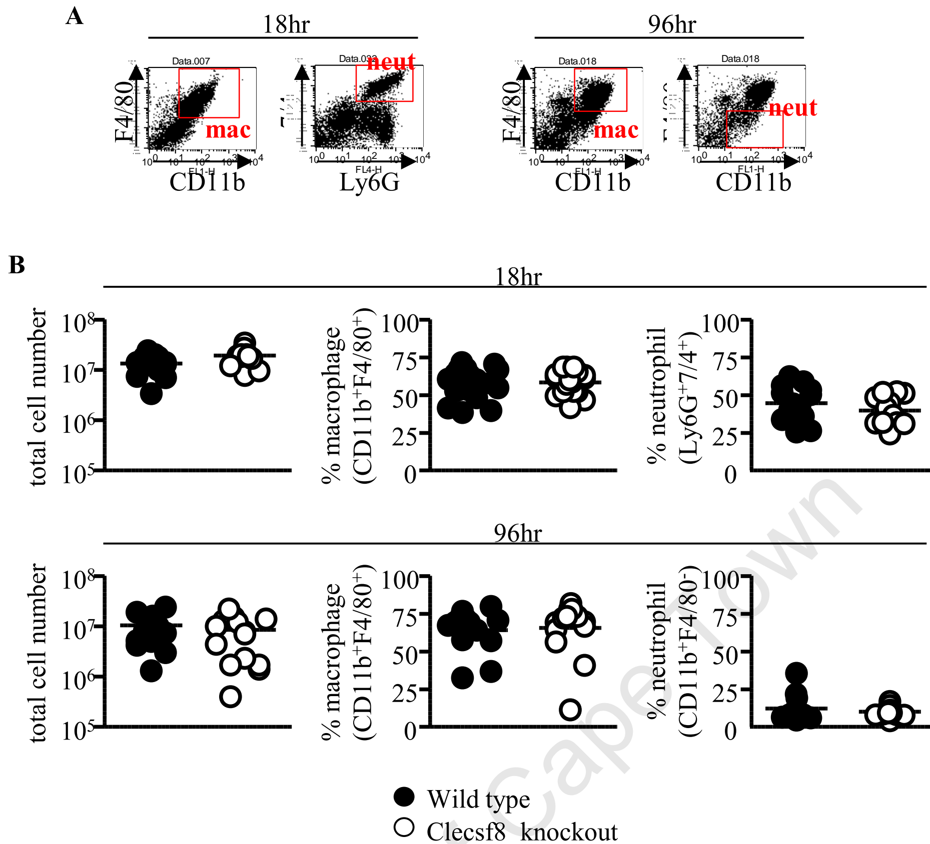


Figure 5.3 **Clecsf8$^{-/-}$ and wild type mice display similar levels of cellular recruitment during sterile peritonitis.** (A) The gating strategy used to identify neutrophils and macrophages 18 and 96 hours after thioglycollate induced sterile peritonitis. (B) Scatter plots of total peritoneal cells/ml of lavage and percentage of neutrophils and macrophages from the peritoneal cavities of Clecsf8$^{-/-}$ (open circles) and wild type (black circles), 18 and 96 hours after induction of peritonitis. Data are from 13 mice/group, pooled from two independent experiments.

University of Cape Town

lavage and analysed by flow cytometry to identify neutrophils ($7/4^{+}Ly6G^{+}$) (Figure 5.4A). Wild type and knockout mice showed similar levels of total peritoneal cells, as well as percentages of neutrophils recruited to the peritoneum (Figure 5.4A).

When mice are exposed to whole-body irradiation, massive apoptosis, which leads to necrosis, is induced in lymphoid tissues such as the thymus, which results in infiltration of neutrophils [154]. I therefore exposed wild type and knockout mice to 4Gy X-rays, harvested thymuses 12 hours later and analysed neutrophil recruitment by flow cytometry (Figure 5.4B). Irradiation resulted in significant neutrophil recruitment to the thymus of both wild type and Clecsf8 knockout mice (Figure 5.4B). However there was no difference in the percentages of neutrophils between the two groups (Figure 5.4B). Taken together, these results show that Clecsf8 does not play a role in neutrophil recruitment in response to necrotic cell death.

University of Cape Town

5.2.3 The role of Clecsf8 in infection

As discussed in Chapter 1, numerous members of the Dectin-2 family have been shown to have exogenous ligands present on microbes and to function in the immune response to infections. I therefore wished to determine whether Clecsf8 may similarly function in immunity to infection and used a variety of infection models to address this question.

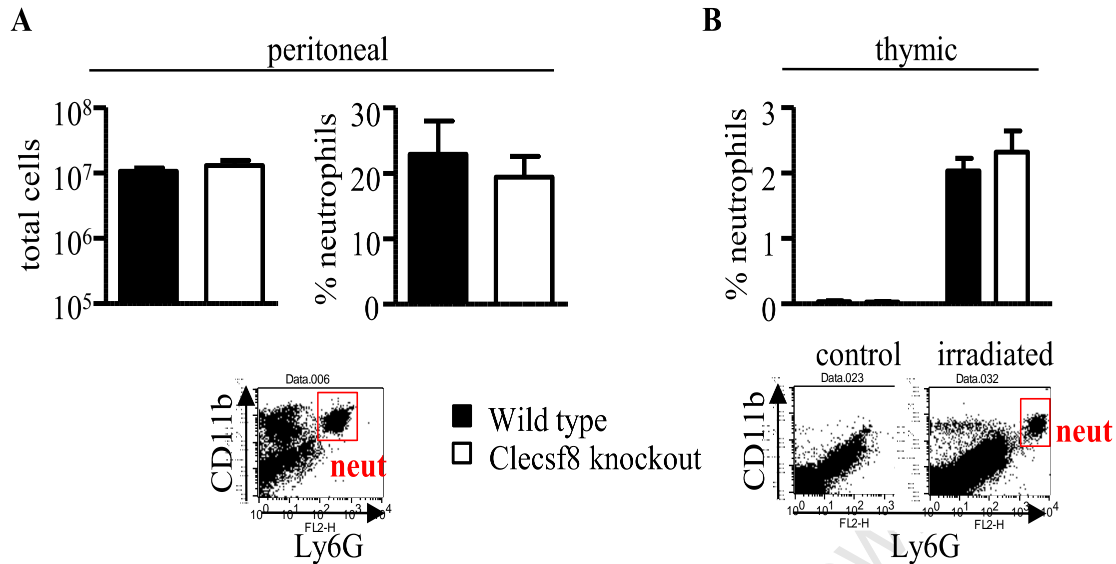


Figure 5.4 **Clecsf8$^{-/-}$ and wild type mice display similar levels of neutrophil recruitment in response to necrotic cell death.** (A) Total recruited peritoneal cell numbers and the percentage of neutrophils from Clecsf8$^{-/-}$ and wild type mice 16 hours after peritoneal challenge with necrotic EL4 cells. A representative blot of Ly6G and 7/4 expression on peritoneal cells used to identify neutrophils is shown below. Data shown are from 9 mice/group, pooled from two independent experiments. (B) Neutrophil infiltration into the thymus of Clecsf8$^{-/-}$ and wild type control or irradiated mice, as indicated. The bar graph shows percentage neutrophils in the thymus of 15 mice pooled from three independent experiments. Representative dot blots showing the CD11b^{+}Ly6G^{+} recruitment of neutrophils into the thymus of irradiated mice.

University of Cape Town

5.2.3.1 Fungal infections

The role of the innate immune response to fungal infections involves numerous PRRs playing roles in fungal recognition and clearance, including both C-type lectins and TLRs. Dectin-1 for example has been shown to recognize several species of fungi, including *Candida* spp., *Aspergillus* spp., *Saccharomyces* spp., *Pneumocystis* spp. and *Coccidiodes* spp. and to act cooperatively with TLR2 on macrophages to induce TNF production in response to fungi [24, 155-158]. Additionally, as mentioned, Mincle and Dectin-2 have both been shown to recognize fungi and play a role in anti-fungal protection [83, 92, 119].

The immune system naturally fights off fungal infections by means of phagocytic cells and their fungicidal mechanisms such as the respiratory burst, as well as by the development of adaptive TH_1 and TH_{17} responses [159]. As I had shown that Clecsf8 is an activation receptor which can induce phagocytosis and the respiratory burst, I wished to determine whether Clecsf8 may aid in controlling fungal infections. To this end, wild type and Clecsf8 knockout mice were infected intravenously with $1x10^5$ CFU *C. albicans* and their survival monitored over three weeks. Clecsf8 deficient and wild type mice were equally susceptible to infection (Figure 5.5). This data therefore indicates that Clecsf8 does not play a major role in controlling *C. albicans* infection.

5.2.3.2 Helminth infections

Dectin-2 has recently been identified as the first Syk-coupled C-type lectin to recognise a soluble egg antigen (SEA) released from the helminth *Schitostosoma mansoni* [87]. Additionally, Dectin-2 has been shown to mediate TH_2 immunity to house dust mite by

University of Cape Town

generation of cysteinyl leukotrines [86]. As TH_2 responses are essential for the resolution of helminth infections, it is possible that Dectin-2 may function in a similar manner during helminth recognition. I therefore wished to explore the possibility that Clecsf8 may function similarly to Dectin-2 and induce TH_2 immunity to helminth infections. I made use of the nematode *Nippostrongylus brasiliensis* for this investigation.

Free-living infective third stage (L3) *Nippostrongylus brasiliensis* larvae infect their hosts by penetrating the skin, entering the blood stream and migrating to the lungs. They are then coughed up and swallowed into the intestine, where they attach themselves to the intestinal epithelium and develop into egg-producing adult worms. These eggs are released and develop into L3 infective larvae outside the host. Murine intestinal worm infections characteristically induce TH_2 effector mechanisms such as TH_2 cytokine production, eosinophilia, goblet cell hyperplasia and synthesis of pathogen specific antibodies such as IgE and IgG1 [160, 161]. These effector mechanisms help to clear the infection and in an intact immune system, worms are expelled 10-12 days post infection [162].

University of Cape Town

To determine whether Clecsf8 may play a role in *Nippostrongylus brasiliensis* infection, preliminary comparative studies were performed with Clecsf8 knockout and wild type littermates infected subcutaneously with 750 L3 larvae. I examined the levels of total serum IgE and the ability of the mice to expel the worms at 10 days post infection. In comparison to naïve mice, infection with *N. brasiliensis* induced IgE production as expected[160, 161], which was present at comparative levels in the serum of wild type

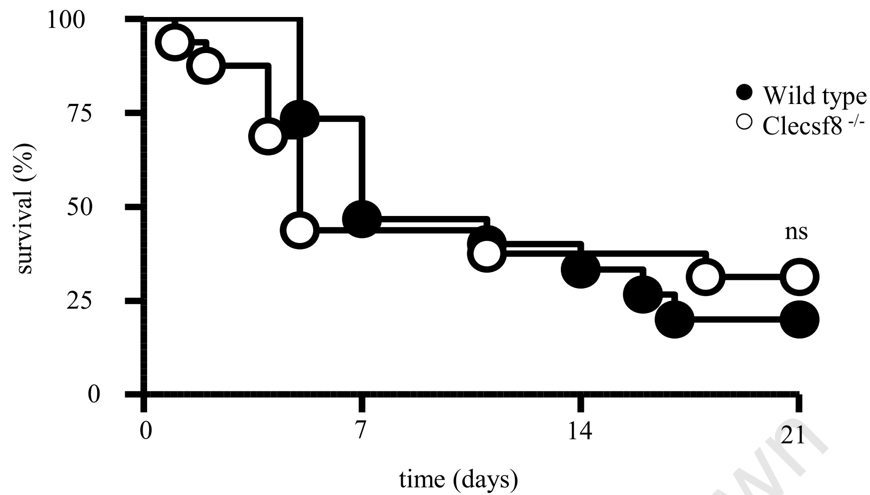


Figure 5.5 **Clecsf8 does not play a major role in anti-fungal immunity.** Survival curve of wild type (n=15) and Clecsf8$^{-/-}$ (n=16) mice infected intravenously with 1x10^{5} CFU *Candida albicans*.

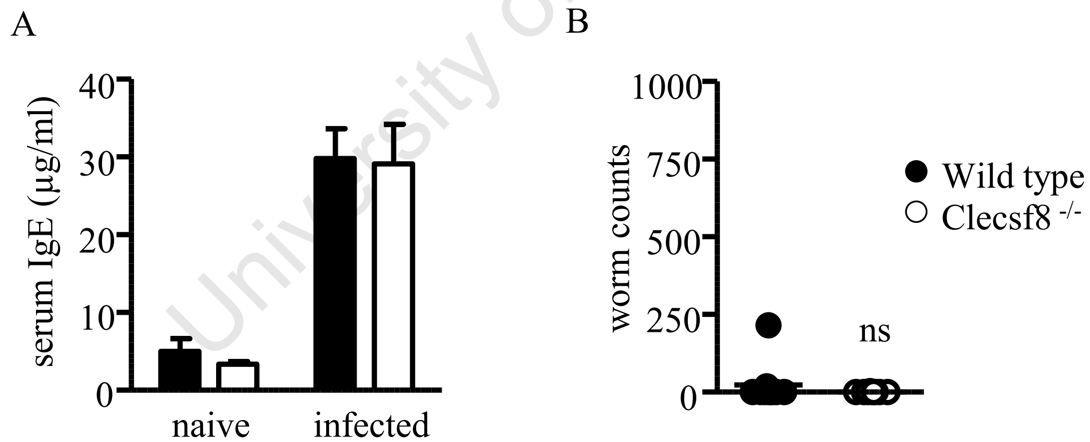


Figure 5.6 **Clecsf8 does not play a major role in immunity to *Nippostrongylus brasiliensis*.** Ten days post infection with 750 L3 *N. brasiliensis* larvae. (A) Total serum IgE levels of naïve and infected wild type and Clecsf8$^{-/-}$ mice. (B) The number of adult worms in the intestines of individual wild type and Clecsf8$^{-/-}$ mice.

University of Cape Town

And Clecsf8 knockout mice (Figure 5.6A). Additionally, wild type and Clecsf8 knockout mice were equally capable of expelling worms from the intestine (Figure 5.6B). These results suggest that Clecsf8 does not play a major role in the immune response to nematode infections.

5.2.3.3 Bacterial infections

The role of the Dectin-2 cluster of receptors in immunity to bacterial infections has been poorly characterised. In fact, only Mincle has been shown to play a role in mycobacterial infections by recognising the mycobacterial cord factor TDM, as discussed in chapter 1 [96, 97]. Investigations using a soluble Dectin-2 CRD however, have shown Dectin-2 recognition of *Mycobacterial tuberculosis* as well as LPS from *Klebsiella pneumonia* [76], however further studies are required to determine whether Dectin-2 functions in immunity to these bacteria. To determine whether Clecsf8 could play a role in anti-bacterial immunity we investigated infections with *Listeria monocytogenes* (intracellular bacterium) as well as *Staphylococcus aureas* (extracellular bacterium), as these infection models were readily available in our laboratory.

University of Cape Town

Listeria monocytogenes is an opportunistic Gram-positive intracellular bacterium, which naturally infects its host through ingestion of contaminated foods. The bacteria infect and traverse the epithelial cells of the gastrointestinal tract and disseminate into the bloodstream, which carries them to organs such as the spleen or liver. Here the bacteria are ingested by macrophages into the phagosome. The bacteria are able to escape from the phagosome by secreting virulence factors and can then replicate in the cytoplasm, as well as propel themselves through the cytosol and into the neighbouring cells by active

actin polymerisation. This ability to survive intracellularly enables *L. monocytogenes* to avoid host defence and disseminate into tissues, enhancing bacterial virulence [163].

Although C-type lectins have not been reported to recognise the bacteria, the innate immune responses triggered upon *Listeria* infection are critical for host survival, with IFN-γ and TNF being essential in resistance to infection [164, 165]. Additionally, CARD9 has been shown to be essential for innate immune responses to *Listeria*, as CARD9 deficient mice failed to clear infection and displayed altered cytokine production in comparison to wild type animals [166]**.** At the cellular level, neutrophils and monocytes/macrophages are believed to play the major role in defence against the pathogen, with production of cytokines, reactive oxygen intermediates and nitric oxide aiding in pathogen clearance [167-169].

To define the role of Clecsf8 during listeriosis, we infected Clecsf8 deficient mice and their wild type littermates intraperitoneally with a sub-lethal ($2x10^5$ CFU) or lethal ($2x10^6$ CFU) dose of *L. monocytogenes* and monitored their survival (Figure 5.7). No significant differences were found in susceptibility of the two groups to either infection. These results indicate that Clecsf8 does not play a major role in protecting against *L. monocytogenes* infection.

I next attempted to define the role of Clecsf8 in *Staphylococcus aureas* infections, which are a major cause of morbidity and mortality globally. Interestingly, PRRs such as TLR-2, the intracellular PRR NOD2 and the C-type lectin MBL, play a role in defence against *S. aureas*, as mice deficient in these receptors are more susceptible to bacterial challenge

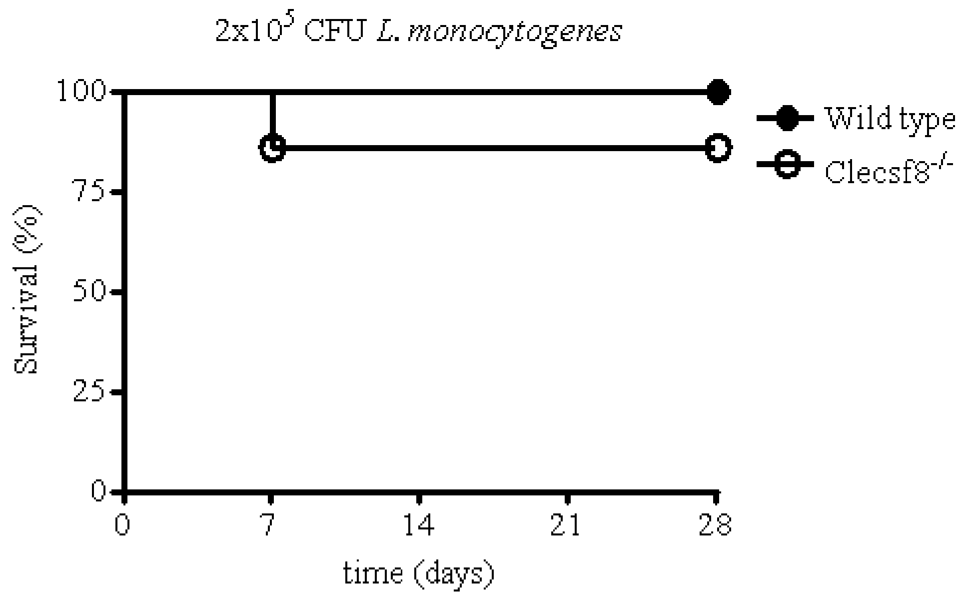
University of Cape Town

than wild type littermates [170-172]. Additionally, in a study with congenic DA rats in which the Dectin-2 family gene cluster was substituted with alleles from arthritis-resistant PVG.1AV1 rats, the congenic animals were more susceptible to intravenous *S. aureas* infection than DA rats [173]. This indicates that the Dectin-2 family of genes may influence the clinical outcome of *S. aureas* infection. However, which receptors of this family are involved remains unknown. I therefore challenged mice intravenously with $2x10^7$ CFU *S. aureas* and monitored their survival over time. There was however no difference in the susceptibility to infection between the two groups (Figure 5.8), indicating that Clecsf8 does not play a major role in immunity to *S. aureas*.

University of Cape Town

5.3 Discussion

As the Dectin-2 family of receptors has been shown to function in immunity to pathogens as well as in maintaining homeostasis, I wished to determine whether Clecsf8 could also function in this manner. I therefore investigated the role of Clecsf8 in fungal, bacterial and helminth infections. However, none of the infections examined revealed significant differences between the wild type and Clecsf8 deficient animals. It is important to note that the recognition of microbes is generally very specific, whereby receptors may recognise only a certain strain or morphology of the pathogen. For example, Dectin-2 shows preferential binding to hyphal rather than yeast components of certain fungi and Mincle recognition of *C. albicans*, is likely to be strain specific [25, 92, 94]. Therefore, although we have studied Clecsf8 recognition of representative fungal, nematode and bacterial pathogens, we can most certainly not rule out the fact that the receptor may still



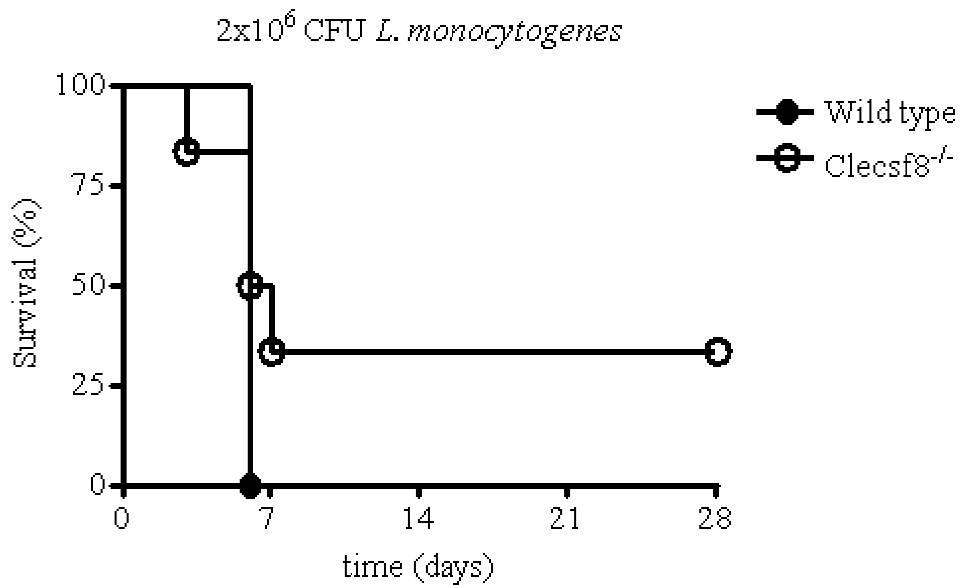


Figure 5.7 **Clecsf8 does not play a major role in immunity to *Listeria monocytogenes*.** Survival curves of wild type and Clecsf8$^{-/-}$ mice (n=6) infected intraperitoneally with low dose ($2x10^5$ CFU) and high dose ($2x10^6$ CFU) *L. monocytogenes.*

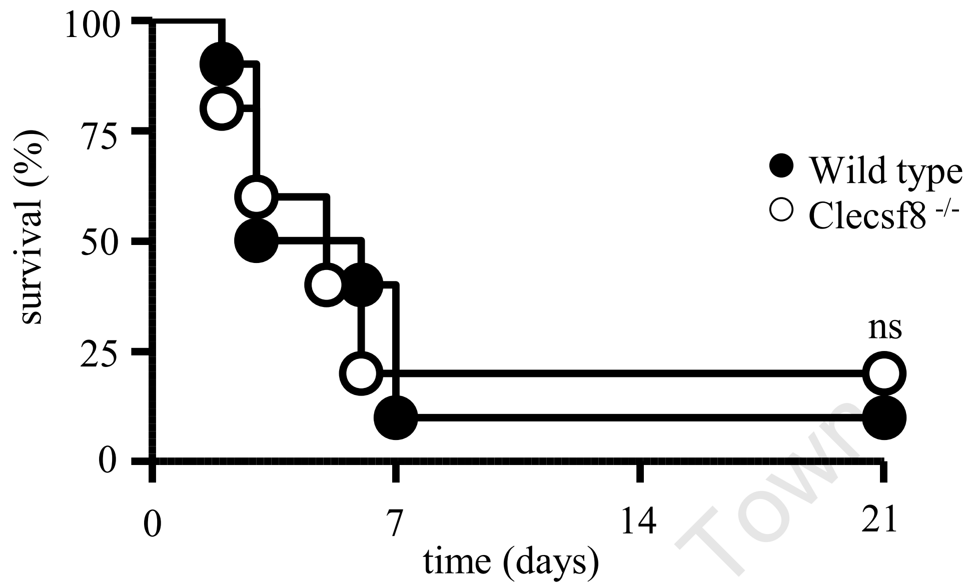


Figure 5.8 **Clecsf8 does not play a major role in immunity to *Staphylococcus aureas*.** Survival curves of wild type (n=6) and Clecsf8$^{-/-}$ (n=7) mice infected intravenously with 2x10^7 CFU *S. aureas*.

University of Cape Town

recognise other pathogens. Additionally, we have not yet studied the recognition of viruses by Clecsf8, as will be discussed in Chapter 6. Another possibility is that Clecsf8, like other members of the Dectin-2 family, recognises endogenous ligand(s) and functions in controlling homeostasis. Although I observed no effect of Clecsf8 deficiency in sterile peritonitis or response to necrotic cell death and was not able to identify an endogenous ligand for the receptor (Chapter 4), further studies in homeostasis are required. In conclusion, I have shown that although Clecsf8 is an activation receptor which can mediate cytokine production, phagocytosis and the respiratory burst, I have not found a role for the receptor in the infection models described here, nor have I observed an effect of Clecsf8 deficiency in sterile peritonitis, response to necrotic cell death or the development of spontaneous autoimmune arthritis (data not shown). Thus despite these analyses, I still have no insights into the physiological role of Clecsf8 and future directions to elucidate these functions will be discussed in Chapter 6.

University of Cape Town

Chapter 6

General summary and future directions

6.1 Review of the aims of this project

The aim of this thesis was to functionally characterise the C-type lectin, Clecsf8. To this end my objectives were to:

- Characterise human Clecsf8 expression in peripheral blood
- Characterise human and murine Clecsf8 localisation in transfected cells
- Determine whether Clecsf8 associates with an adaptor molecule
- Identify ligands of Clecsf8
- Determine whether Clecsf8 could induce cellular responses such as cytokine production, phagocytosis and the respiratory burst
- Define the signalling capacity of Clecsf8
- Characterise the role of Clecsf8 *in vivo* by investigating the receptor's functions in inflammation and infection

Here I will give a final summary of the key findings for these objectives, which have previously been discussed in detail within the relevant chapters. I will additionally suggest future directions for the project.

University of Cape Town

6.2 Summary of key findings

Clecsf8 is a poorly characterised member of the Dectin-2 cluster of C-type lectin receptors and was originally thought to be expressed exclusively by macrophages. I show here that Clecsf8 is expressed on circulating $CD14^{+}CD16^{-}$ monocytes and neutrophils in human peripheral blood. I also noted that expression of the receptor became down-regulated upon differentiation of monocytes into macrophages or DCs *in vitro*, suggesting that Clecsf8 may only be expressed and function on circulating cells. I examined the effects of a variety of cytokines and TLR ligands on Clecsf8 expression in both monocytes and neutrophils and found that certain pro-inflammatory cytokines and microbial components could marginally up-regulate expression. Like other members of the Dectin-2 family, which require association with an adaptor molecule for cell surface expression, Clecsf8 is retained intracellularly in non-myeloid cells. However, I demonstrated that Clecsf8 does not associate with known adaptor molecules including DAP10, DAP12 or FcRγ chain. Surprisingly, we found that the CRD of Clecsf8 was responsible for intracellular retention.

University of Cape Town

To functionally characterise Clecsf8 I made use of RAW264.7 cells transfected with a chimeric receptor with defined ligands, as well as cross-linking Clecsf8 on the surface of primary neutrophils and demonstrated that Clecsf8 functions as an activation receptor. Despite the absence of a known intracellular signalling motif, I have shown that Clecsf8 was able to induce signalling via Syk kinase in myeloid cells and that it could induce pro-inflammatory cytokine production, phagocytosis and the respiratory burst.

Although I had determined these exciting functions of Clecsf8, I wished to identify the ligand and determine the physiological role of the receptor. Despite screening both endogenous and exogenous molecules, I was unable to identify a ligand for Clecsf8. I carried out extensive *in vivo* characterisation of the Clecsf8 knockout mouse but did not find any defects in their ability to resist infection with fungi, nematodes or intracellular and extracellular bacteria. Additionally, Clecsf8 deficiency had no effect in murine models of sterile peritonitis or response to necrotic cell death.

6.3 Future directions

6.3.1 Generate anti-mClecsf8 monoclonal antibody

Although I have shown that hClecsf8 is expressed on peripheral blood monocytes and neutrophils, further characterisation of expression of the receptor in murine cells and tissues was not possible due to the lack of a specific antibody. It would therefore be useful to generate a monoclonal antibody against mClecsf8. I have attempted to do this three times but have been unsuccessful. I immunised Clecsf8$^{-/-}$ mice with the FcClecsf8 fusion protein and generated hybridomas by fusing splenocytes with myeloma cells. I tested conditioned supernatants by ELISA and flow cytometry on live RAW264.7 cells and permeabilised NIH3T3 cells. Unfortunately, I was not able to identify a positive hybridoma using these screening methods. I speculate that this may be due to the presence of endogenous Clecsf8 on RAW264.7 cells, as Balch et al. identified Clecsf8

University of Cape Town

mRNA in these cells [105], which may result in high background staining. Additionally, screening using NIH3T3 cells may not have worked due to the fact that many antibodies do not function under fixed and permeabilised conditions. Due to time constraints, I could not attempt another fusion. Future studies would need to identify a more sensitive method of screening for positive hybridomas.

Although I characterised the expression of hClecsf8 in peripheral blood, it would be important to further characterise the expression of both the human and mouse receptor in other cell types as well as tissues. This could be carried out using a specific monoclonal antibody, by means of flow cytometry or immunofluorescent microscopy for example. In addition to characterising the expression of mClecsf8, this tool could possibly be used to study the function of mClecsf8 by cross-linking the receptor on primary cells, as performed for hClecsf8. The antibody could also be used to block Clecsf8 function *in vivo*, as was performed to demonstrate the role of Mincle as an inflammatory receptor for necrotic cell death [95]. Additionally, a specific antibody could be used to target antigens to cells that modulate immune responses. If Clecsf8 was found to be expressed on DCs in tissues for example, anti-mClecsf8 could possibly be used to deliver antigens to DCs which could result in presentation to T cells. Targeting molecules to a specific receptor has also been exploited as an immunotherapeutic strategy, but whether this may be true for Clecsf8 remains to be determined.

University of Cape Town

6.3.2 Ligand screening and physiological function

To elucidate the function of Clecsf8, it will be important to identify the receptor's ligand(s). One mechanism would be to continue screening with the FcClecsf8 fusion protein, looking at murine cells that have not yet been screened, such as bone marrow and peripheral blood cells, for example. The protein could also be used to screen for exogenous ligands, such as live and heat killed bacteria, fungi and viruses. Importantly, it would also be advisable to study different morphologies of microbes, as some receptors show preferential binding to different morphological forms. Dectin-2 for example, exhibits preferential recognition of hyphal over conidial forms of fungi [53].

Another possible approach to identify both endogenous and exogenous ligands in the future would be to generate a reporter cell system. This system uses BWZ.36 cells which are transfected with a chimeric receptor consisting of the extracellular and transmembrane regions of the C-type lectin of interest coupled to CD3δ chain. The cells contain a β-gal reporter construct which is activated via NFAT. Therefore, ligand binding would induce signalling via the CD3δ chain ITAM motifs and trigger expression of IL-2 and β-galactosidase. This system has successfully been used in our laboratory and by others, to identify ligands for C-type lectins [130, 174]. Indeed, the endogenous ligand of Mincle was identified using a similar reporter cell line [95]. A possible limitation of this method however, may be that the reporter construct would require the putative adaptor molecule for expression on the surface of BWZ.36 cells. Identification of the adaptor and co-transfections would therefore be necessary.

University of Cape Town

Importantly, both the above screening methods are artificial systems and if Clecsf8 functions as a complex to recognise its ligand, the necessary cooperating molecules may not be present for ligand binding. An alternative would be to study Clecsf8-ligand interactions by means of biochemical techniques, such as immunoprecipitations using the FcClecsf8 fusion protein for example.

Although I have shown that Clecsf8 functions as an activation receptor in RAW264.7 macrophages and neutrophils, I have not yet elucidated the true physiological role of the receptor. Once the expression pattern of the receptor has been investigated in more detail, it may be worthwhile to demonstrate Clecsf8-mediated activation in different cell types. However, a natural ligand for Clecsf8, rather than an antibody or chimeric receptor, is required for a better demonstration of function(s).

University of Cape Town

Additionally, it is necessary to further characterise the Clecsf8$^{-/-}$ mice. Although I have investigated infections with certain pathogens, it is important to remember that pathogen recognition is often extremely specific. As I have only considered one strain of each pathogen we cannot rule out the possibility that Clecsf8 may function in controlling infections with other organisms. As Mincle has recently been identified as a receptor for *Mycobacterium tuberculosis* [96], we are currently investigating the role of Clecsf8 in MTB infection. Additionally, C-type lectins play a role in viral recognition, whether it be in aiding viral infections, such as is seen with DCIR recognition of HIV-1 or induction of virally induced pathologies, such as is seen for Clec5A activation by dengue virus, which

results in an intense cytokine storm and vascular leakage [69, 175]. Indeed, a wide range of viruses are recognised by C-type lectins, including Hepatitis B virus, Herpes simplex virus, Influenza virus and Ebola virus to name a few, and it would therefore be necessary to explore the recognition of viruses by Clecsf8.

Besides recognition of non-self structures, it is also possible that Clecsf8 may recognise an endogenous self ligand and play a role in immune homeostasis. Indeed, numerous C-type lectins contribute to maintaining homeostasis. DC-SIGN, for example recognises ICAM-3 on T cells, thereby establishing initial contact between the DC and T cell [176]. In serum, the mannose receptor contributes to lysosomal hydrolases clearance [177], as well as to the levels of the hormone lutropin [178]. In the Dectin-2 family specifically, DCIR$^{-/-}$ mice spontaneously develop autoimmune diseases due to excessive expansion of DCs [70] and Mincle recognises a nuclear protein released during necrotic cell death, resulting in neutrophil recruitment [95]. Although Clecsf8 deficiency did not have an effect on the response to necrotic cell death and the Clecsf8$^{-/-}$ mice did not spontaneously develop arthritis, we should not exclude the possibility that Clecsf8 may function in immune homeostasis.

University of Cape Town

6.3.3 Adaptor molecule identification

As I have shown that Clecsf8 is likely to associate with an adaptor molecule for cell surface expression and signalling, the identification of this adaptor would shed great insight into Clecsf8 functions. As association with the putative adaptor molecule occurs

via Clecsf8's CRD, it could be helpful to identify which residues are involved in this association by means of introducing mutations to the receptor CRD. Additionally, as the adaptor molecule is putatively responsible for coupling of the receptor to Syk and downstream functional effects, it would be useful to test this hypothesis. This would be possible by investigating whether stimulation of the chimera which contains the Dectin-1 CRD and Clecsf8 body but lacks the Clecsf8 CRD is still able to induce responses such as cytokine production or phagocytosis, thereby disclosing information about the dependence of Clecsf8 on the CRD-mediated adaptor association.

As I know that Clecsf8 is expressed on the surface of transduced RAW264.7 macrophages, I can assume that the adaptor molecule is expressed endogenously in these cells. One method of identifying this molecule would therefore be to generate a retroviral RAW264.7 cDNA expression library and to screen this library for a molecule that transports Janus to the surface of NIH3T3 cells. The transport of Janus to the cell surface can be screened by flow cytometry or fluorescence microscopy. For fluorescence microscopy, Janus expression can be identified by the ability of the cell to bind FITC-zymosan particles, via the Dectin-1 CRD. Positive ligand-binding cells could then be isolated and enriched and genomic DNA isolated from the cells. PCR could be used to recover the proviral DNA insert, which could then be sent for sequencing to identify the associated molecules. Alternatively, one could perform immunoprecipitations of mClecsf8 from transduced RAW264.7 cells (or hClecsf8 from neutrophils) as described in Chapter 3. These immunoprecipitates could then be analysed by SDS-PAGE and mass spectrometry to identify associated molecules.

University of Cape Town

Possible candidates for adaptor molecules could include other C-type lectins. For example, NKG2A and NKG2C are both dependent on association with the C-type lectin CD94 for expression on the cell surface [179, 180]. The CD94/NKG2A complex signals in an inhibitory fashion in NK cells via two ITIMs in the NKG2A cytoplasmic tail [181, 182]. The CD94/NKG2C complex however, requires the additional presence of DAP12 for efficient expression on the cell surface [183]. Additionally, NKG2C does not contain a signalling motif but receptor ligation results in NK cell activation, which is likely to take place by signalling via DAP12 [181, 183].

Once the adaptor molecule has been identified, it would be important to study the functional significance of the molecule. The availability of a specific knockout mouse would aid the physiological characterisation of the adaptor and aid in our understanding of Clecsf8 functions.

University of Cape Town

6.4 Conclusion

In conclusion, this thesis describes the characterisation of Clecsf8 as an activation receptor expressed on myeloid cells. *In vitro* analyses have revealed that Clecsf8 signals via Syk kinase to induce pro-inflammatory cytokine production, phagocytosis and the respiratory burst, functions which are likely to be mediated through interaction with an unidentified adaptor molecule. Although these findings suggest a role in innate immunity, the role of Clecsf8 *in vivo* could not be elucidated. The identification of the associating

adaptor molecule, and the ligands and physiological role of Clecsf8 are the immediate issues which require further attention.

References

1. Lander, E.S., et al., *Initial sequencing and analysis of the human genome.* Nature, 2001. **409**(6822): p. 860-921.
2. Medzhitov, R. and C. Janeway, Jr., *Innate immune recognition: mechanisms and pathways.* Immunol Rev, 2000. **173**: p. 89-97.
3. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity.* Cell, 2006. **124**(4): p. 783-801.
4. Creagh, E.M. and L.A. O'Neill, *TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity.* Trends Immunol, 2006. **27**(8): p. 352-7.
5. Drickamer, K., *Two distinct classes of carbohydrate-recognition domains in animal lectins.* J Biol Chem, 1988. **263**(20): p. 9557-60.
6. Drickamer, K., *C-type lectin-like domains.* Curr Opin Struct Biol, 1999. **9**(5): p. 585-90.
7. Weis, W.I., M.E. Taylor, and K. Drickamer, *The C-type lectin superfamily in the immune system.* Immunol Rev, 1998. **163**: p. 19-34.
8. Drickamer, K. and A.J. Fadden, *Genomic analysis of C-type lectins.* Biochem Soc Symp, 2002(69): p. 59-72.
9. Zelensky, A.N. and J.E. Gready, *The C-type lectin-like domain superfamily.* Febs J, 2005. **272**(24): p. 6179-217.
10. Koppel, E.A., et al., *Distinct functions of DC-SIGN and its homologues L-SIGN (DC-SIGNR) and mSIGNR1 in pathogen recognition and immune regulation.* Cell Microbiol, 2005. **7**(2): p. 157-65.
11. Neth, O., et al., *Mannose-binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition.* Infect Immun, 2000. **68**(2): p. 688-93.
12. Cambi, A., M. Koopman, and C.G. Figdor, *How C-type lectins detect pathogens.* Cell Microbiol, 2005. **7**(4): p. 481-8.
13. Ewart, K.V., et al., *The ice-binding site of Atlantic herring antifreeze protein corresponds to the carbohydrate-binding site of C-type lectins.* Biochemistry, 1998. **37**(12): p. 4080-5.
14. Geider, S., et al., *Pancreatic lithostathine as a calcite habit modifier.* J Biol Chem, 1996. **271**(42): p. 26302-6.
15. Mann, K. and F. Siedler, *The amino acid sequence of ovocleidin 17, a major protein of the avian eggshell calcified layer.* Biochem Mol Biol Int, 1999. **47**(6): p. 997-1007.
16. Natarajan, K., et al., *Structure and function of natural killer cell receptors: multiple molecular solutions to self, nonself discrimination.* Annu Rev Immunol, 2002. **20**: p. 853-85.
17. Sano, H., et al., *Analysis of chimeric proteins identifies the regions in the carbohydrate recognition domains of rat lung collectins that are essential for interactions with phospholipids, glycolipids, and alveolar type II cells.* J Biol Chem, 1998. **273**(8): p. 4783-9.

University of Cape Town

18. Yokoyama, W.M. and B.F. Plougastel, *Immune functions encoded by the natural killer gene complex.* Nat Rev Immunol, 2003. **3**(4): p. 304-16.
19. Brown, G.D., *Dectin-1: a signalling non-TLR pattern-recognition receptor.* Nat Rev Immunol, 2006. **6**(1): p. 33-43.
20. Reth, M., *Antigen receptor tail clue.* Nature, 1989. **338**(6214): p. 383-4.
21. Lanier, L.L., *Up on the tightrope: natural killer cell activation and inhibition.* Nat Immunol, 2008. **9**(5): p. 495-502.
22. Long, E.O., *Negative signaling by inhibitory receptors: the NK cell paradigm.* Immunol Rev, 2008. **224**: p. 70-84.
23. Karre, K., et al., *Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy.* Nature, 1986. **319**(6055): p. 675-8.
24. Brown, G.D., et al., *Dectin-1 mediates the biological effects of beta-glucans.* J Exp Med, 2003. **197**(9): p. 1119-24.
25. Sato, K., et al., *Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses.* J Biol Chem, 2006. **281**(50): p. 38854-66.
26. Ivashkiv, L.B., *Cross-regulation of signaling by ITAM-associated receptors.* Nat Immunol, 2009. **10**(4): p. 340-7.
27. Gross, O., et al., *Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity.* Nature, 2006. **442**(7103): p. 651-6.
28. Hara, H., et al., *The adaptor protein CARD9 is essential for the activation of myeloid cells through ITAM-associated and Toll-like receptors.* Nat Immunol, 2007. **8**(6): p. 619-29.
29. Long, E.O., *Regulation of immune responses through inhibitory receptors.* Annu Rev Immunol, 1999. **17**: p. 875-904.
30. Barrow, A.D. and J. Trowsdale, *You say ITAM and I say ITIM, let's call the whole thing off: the ambiguity of immunoreceptor signalling.* Eur J Immunol, 2006. **36**(7): p. 1646-53.
31. Pinheiro da Silva, F., et al., *Inhibitory ITAMs: a matter of life and death.* Trends Immunol, 2008. **29**(8): p. 366-73.
32. Aderem, A. and D.M. Underhill, *Mechanisms of phagocytosis in macrophages.* Annu Rev Immunol, 1999. **17**: p. 593-623.
33. Kerrigan, A.M., et al., *CLEC-2 is a phagocytic activation receptor expressed on murine peripheral blood neutrophils.* J Immunol, 2009. **182**(7): p. 4150-7.
34. Herre, J., et al., *Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages.* Blood, 2004. **104**(13): p. 4038-45.
35. Kerrigan, A.M. and G.D. Brown, *C-type lectins and phagocytosis.* Immunobiology, 2009.
36. Bonifacino, J.S. and L.M. Traub, *Signals for sorting of transmembrane proteins to endosomes and lysosomes.* Annu Rev Biochem, 2003. **72**: p. 395-447.
37. Rogers, N.C., et al., *Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins.* Immunity, 2005. **22**(4): p. 507-17.
38. Dennehy, K.M., et al., *Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors.* Eur J Immunol, 2008. **38**(2): p. 500-6.

University of Cape Town

39. Gantner, B.N., et al., *Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2.* J Exp Med, 2003. **197**(9): p. 1107-17.
40. Skrzypek, F., et al., *Dectin-1 is required for human dendritic cells to initiate immune response to Candida albicans through Syk activation.* Microbes Infect, 2009. **11**(6-7): p. 661-70.
41. Taylor, P.R., et al., *Dectin-1 is required for beta-glucan recognition and control of fungal infection.* Nat Immunol, 2007. **8**(1): p. 31-8.
42. Underhill, D.M., et al., *Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production.* Blood, 2005. **106**(7): p. 2543-50.
43. Segal, B.H. and L.R. Romani, *Invasive aspergillosis in chronic granulomatous disease.* Med Mycol, 2009. **47 Suppl 1**: p. S282-90.
44. Speert, D.P., *Bacterial infections of the lung in normal and immunodeficient patients.* Novartis Found Symp, 2006. **279**: p. 42-51; disussion 51-5, 216-9.
45. Ravichandran, K.S. and U. Lorenz, *Engulfment of apoptotic cells: signals for a good meal.* Nat Rev Immunol, 2007. **7**(12): p. 964-74.
46. Sancho, D., et al., *Identification of a dendritic cell receptor that couples sensing of necrosis to immunity.* Nature, 2009. **458**(7240): p. 899-903.
47. Shrimpton, R.E., et al., *CD205 (DEC-205): a recognition receptor for apoptotic and necrotic self.* Mol Immunol, 2009. **46**(6): p. 1229-39.
48. Kono, H. and K.L. Rock, *How dying cells alert the immune system to danger.* Nat Rev Immunol, 2008. **8**(4): p. 279-89.
49. Kanazawa, N., et al., *Dendritic cell immunoactivating receptor, a novel C-type lectin immunoreceptor, acts as an activating receptor through association with Fc receptor gamma chain.* J Biol Chem, 2003. **278**(35): p. 32645-52.
50. Dzionek, A., et al., *BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood.* J Immunol, 2000. **165**(11): p. 6037-46.
51. Dzionek, A., et al., *BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction.* J Exp Med, 2001. **194**(12): p. 1823-34.
52. Fernandes, M.J., et al., *Identification and characterization of the gene for a novel C-type lectin (CLECSF7) that maps near the natural killer gene complex on human chromosome 12.* Genomics, 2000. **69**(2): p. 263-70.
53. Graham, L.M. and G.D. Brown, *The Dectin-2 family of C-type lectins in immunity and homeostasis.* Cytokine, 2009. **48**(1-2): p. 148-55.
54. Arce, I., et al., *Molecular and genomic characterization of human DLEC, a novel member of the C-type lectin receptor gene family preferentially expressed on monocyte-derived dendritic cells.* Eur J Immunol, 2001. **31**(9): p. 2733-40.
55. Cao, W., et al., *BDCA2/Fc epsilon RI gamma complex signals through a novel BCR-like pathway in human plasmacytoid dendritic cells.* PLoS Biol, 2007. **5**(10): p. e248.
56. Rock, J., et al., *CD303 (BDCA-2) signals in plasmacytoid dendritic cells via a BCR-like signalosome involving Syk, Slp65 and PLCgamma2.* Eur J Immunol, 2007. **37**(12): p. 3564-75.
57. Jahn, P.S., et al., *BDCA-2 signaling inhibits TLR-9-agonist-induced plasmacytoid dendritic cell activation and antigen presentation.* Cell Immunol.

University of Cape Town

58. Wu, P., et al., *TLR9/TLR7-triggered downregulation of BDCA2 expression on human plasmacytoid dendritic cells from healthy individuals and lupus patients.* Clin Immunol, 2008. **129**(1): p. 40-8.
59. Farkas, L., et al., *Plasmacytoid dendritic cells (natural interferon- alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions.* Am J Pathol, 2001. **159**(1): p. 237-43.
60. Riboldi, E., et al., *Engagement of BDCA-2 blocks TRAIL-mediated cytotoxic activity of plasmacytoid dendritic cells.* Immunobiology, 2009. **214**(9-10): p. 868-76.
61. Bates, E.E., et al., *APCs express DCIR, a novel C-type lectin surface receptor containing an immunoreceptor tyrosine-based inhibitory motif.* J Immunol, 1999. **163**(4): p. 1973-83.
62. Kanazawa, N., et al., *DCIR acts as an inhibitory receptor depending on its immunoreceptor tyrosine-based inhibitory motif.* J Invest Dermatol, 2002. **118**(2): p. 261-6.
63. Klechevsky, E., et al., *Cross-priming CD8+ T cells by targeting antigens to human dendritic cells through DCIR.* Blood.
64. Meyer-Wentrup, F., et al., *Targeting DCIR on human plasmacytoid dendritic cells results in antigen presentation and inhibits IFN-alpha production.* Blood, 2008. **111**(8): p. 4245-53.
65. Richard, M., et al., *The expression pattern of the ITIM-bearing lectin CLECSF6 in neutrophils suggests a key role in the control of inflammation.* J Leukoc Biol, 2002. **71**(5): p. 871-80.
66. Richard, M., et al., *The ITIM-bearing CLECSF6 (DCIR) is down-modulated in neutrophils by neutrophil activating agents.* Biochem Biophys Res Commun, 2003. **310**(3): p. 767-73.
67. Richard, M., et al., *Granulocyte macrophage-colony stimulating factor reduces the affinity of SHP-2 for the ITIM of CLECSF6 in neutrophils: a new mechanism of action for SHP-2.* Mol Immunol, 2006. **43**(10): p. 1716-21.
68. Meyer-Wentrup, F., et al., *DCIR is endocytosed into human dendritic cells and inhibits TLR8-mediated cytokine production.* J Leukoc Biol, 2009. **85**(3): p. 518-25.
69. Lambert, A.A., et al., *The C-type lectin surface receptor DCIR acts as a new attachment factor for HIV-1 in dendritic cells and contributes to trans- and cis-infection pathways.* Blood, 2008. **112**(4): p. 1299-307.
70. Fujikado, N., et al., *Dcir deficiency causes development of autoimmune diseases in mice due to excess expansion of dendritic cells.* Nat Med, 2008. **14**(2): p. 176-80.
71. Eklow, C., et al., *Cellular distribution of the C-type II lectin dendritic cell immunoreceptor (DCIR) and its expression in the rheumatic joint: identification of a subpopulation of DCIR+ T cells.* Ann Rheum Dis, 2008. **67**(12): p. 1742-9.
72. Fernandes, M.J., et al., *Characterization of a novel receptor that maps near the natural killer gene complex: demonstration of carbohydrate binding and expression in hematopoietic cells.* Cancer Res, 1999. **59**(11): p. 2709-17.

University of Cape Town

73. Ariizumi, K., et al., *Cloning of a second dendritic cell-associated C-type lectin (dectin-2) and its alternatively spliced isoforms.* J Biol Chem, 2000. **275**(16): p. 11957-63.
74. Bonkobara, M., et al., *Epidermal Langerhans cell-targeted gene expression by a dectin-2 promoter.* J Immunol, 2001. **167**(12): p. 6893-900.
75. Taylor, P.R., et al., *Dectin-2 is predominantly myeloid restricted and exhibits unique activation-dependent expression on maturing inflammatory monocytes elicited in vivo.* Eur J Immunol, 2005. **35**(7): p. 2163-74.
76. McGreal, E.P., et al., *The carbohydrate-recognition domain of Dectin-2 is a C-type lectin with specificity for high mannose.* Glycobiology, 2006. **16**(5): p. 422-30.
77. Saijo, S., et al., *Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against Candida albicans.* Immunity. **32**(5): p. 681-91.
78. Aragane, Y., et al., *Involvement of dectin-2 in ultraviolet radiation-induced tolerance.* J Immunol, 2003. **171**(7): p. 3801-7.
79. Bonkobara, M., et al., *Ultraviolet-B radiation upregulates expression of dectin-2 on epidermal Langerhans cells by activating the gene promoter.* Photochem Photobiol, 2005. **81**(4): p. 944-8.
80. Willment, J.A., S. Gordon, and G.D. Brown, *Characterization of the human beta - glucan receptor and its alternatively spliced isoforms.* J Biol Chem, 2001. **276**(47): p. 43818-23.
81. Carter, R.W., et al., *Induction of CD8+ T cell responses through targeting of antigen to Dectin-2.* Cell Immunol, 2006. **239**(2): p. 87-91.
82. Suram, S., et al., *Pathways regulating cytosolic phospholipase A2 activation and eicosanoid production in macrophages by Candida albicans.* J Biol Chem.
83. Robinson, M.J., et al., *Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection.* J Exp Med, 2009. **206**(9): p. 2037-51.
84. Bi, L., et al., *CARD9 Mediates Dectin-2-induced I{kappa}B{alpha} Kinase Ubiquitination Leading to Activation of NF-{kappa}B in Response to Stimulation by the Hyphal Form of Candida albicans.* J Biol Chem. **285**(34): p. 25969-77.
85. Barrett, N.A., et al., *Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells.* J Immunol, 2009. **182**(2): p. 1119-28.
86. Barrett, N.A., et al., *Dectin-2 mediates Th2 immunity through the generation of cysteinyl leukotrienes.* J Exp Med. **208**(3): p. 593-604.
87. Ritter, M., et al., *Schistosoma mansoni triggers Dectin-2, which activates the Nlrp3 inflammasome and alters adaptive immune responses.* Proc Natl Acad Sci U S A. **107**(47): p. 20459-64.
88. Gavino, A.C., et al., *Identification and expression profiling of a human C-type lectin, structurally homologous to mouse dectin-2.* Exp Dermatol, 2005. **14**(4): p. 281-8.
89. Kanazawa, N., et al., *Molecular cloning of human dectin-2.* J Invest Dermatol, 2004. **122**(6): p. 1522-4.
90. Xie, J., et al., *Human Dectin-1 isoform E is a cytoplasmic protein and interacts with RanBPM.* Biochem Biophys Res Commun, 2006. **347**(4): p. 1067-73.

University of Cape Town

91. Matsumoto, M., et al., *A novel LPS-inducible C-type lectin is a transcriptional target of NF-IL6 in macrophages.* J Immunol, 1999. **163**(9): p. 5039-48.
92. Wells, C.A., et al., *The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to Candida albicans.* J Immunol, 2008. **180**(11): p. 7404-13.
93. Screpanti, I., et al., *Lymphoproliferative disorder and imbalanced T-helper response in C/EBP beta-deficient mice.* Embo J, 1995. **14**(9): p. 1932-41.
94. Yamasaki, S., et al., *C-type lectin Mincle is an activating receptor for pathogenic fungus, Malassezia.* Proc Natl Acad Sci U S A, 2009. **106**(6): p. 1897-902.
95. Yamasaki, S., et al., *Mincle is an ITAM-coupled activating receptor that senses damaged cells.* Nat Immunol, 2008. **9**(10): p. 1179-88.
96. Ishikawa, E., et al., *Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle.* J Exp Med, 2009. **206**(13): p. 2879-88.
97. Schoenen, H., et al., *Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate.* J Immunol. **184**(6): p. 2756-60.
98. Hunter, R.L., et al., *Multiple roles of cord factor in the pathogenesis of primary, secondary, and cavitary tuberculosis, including a revised description of the pathology of secondary disease.* Ann Clin Lab Sci, 2006. **36**(4): p. 371-86.
99. Werninghaus, K., et al., *Adjuvanticity of a synthetic cord factor analogue for subunit Mycobacterium tuberculosis vaccination requires FcRgamma-Syk-Card9-dependent innate immune activation.* J Exp Med, 2009. **206**(1): p. 89-97.
100. Davidsen, J., et al., *Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from M. tuberculosis (trehalose 6,6'-dibehenate)-a novel adjuvant inducing both strong CMI and antibody responses.* Biochim Biophys Acta, 2005. **1718**(1-2): p. 22-31.
101. Khader, S.A., et al., *IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge.* Nat Immunol, 2007. **8**(4): p. 369-77.
102. Flornes, L.M., et al., *Identification of lectin-like receptors expressed by antigen presenting cells and neutrophils and their mapping to a novel gene complex.* Immunogenetics, 2004. **56**(7): p. 506-17.
103. Lorentzen, J.C., et al., *Association of arthritis with a gene complex encoding C-type lectin-like receptors.* Arthritis Rheum, 2007. **56**(8): p. 2620-32.
104. Ichioka, M., et al., *Increased Expression of Macrophage-Inducible C-type Lectin in Adipose Tissue of Obese Mice and Humans.* Diabetes. **60**(3): p. 819-26.
105. Balch, S.G., et al., *Cloning of a novel C-type lectin expressed by murine macrophages.* J Biol Chem, 1998. **273**(29): p. 18656-64.
106. Arce, I., et al., *The human C-type lectin CLECSF8 is a novel monocyte/macrophage endocytic receptor.* Eur J Immunol, 2004. **34**(1): p. 210-20.
107. Haskard, D.O. and R.C. Landis, *Interactions between leukocytes and endothelial cells in gout: lessons from a self-limiting inflammatory response.* Arthritis Res, 2002. **4 Suppl 3**: p. S91-7.
108. Mollenkopf, H.J., K. Hahnke, and S.H. Kaufmann, *Transcriptional responses in mouse lungs induced by vaccination with Mycobacterium bovis BCG and*

University of Cape Town

infection with Mycobacterium tuberculosis. Microbes Infect, 2006. **8**(1): p. 136-44.
109. Tang, Z., et al., *Gene expression profiling during the transition to failure in TNF-alpha over-expressing mice demonstrates the development of autoimmune myocarditis.* J Mol Cell Cardiol, 2004. **36**(4): p. 515-30.
110. Wandstrat, A. and E. Wakeland, *The genetics of complex autoimmune diseases: non-MHC susceptibility genes.* Nat Immunol, 2001. **2**(9): p. 802-9.
111. Willment, J.A., et al., *The human beta-glucan receptor is widely expressed and functionally equivalent to murine Dectin-1 on primary cells.* Eur J Immunol, 2005. **35**(5): p. 1539-47.
112. Morita, S., T. Kojima, and T. Kitamura, *Plat-E: an efficient and stable system for transient packaging of retroviruses.* Gene Ther, 2000. **7**(12): p. 1063-6.
113. Marshall, A.S., et al., *Identification and characterization of a novel human myeloid inhibitory C-type lectin-like receptor (MICL) that is predominantly expressed on granulocytes and monocytes.* J Biol Chem, 2004. **279**(15): p. 14792-802.
114. Graham, L.M., et al., *Soluble Dectin-1 as a tool to detect beta-glucans.* J Immunol Methods, 2006. **314**(1-2): p. 164-9.
115. Palma, A.S., et al., *Ligands for the beta-glucan receptor, Dectin-1, assigned using "designer" microarrays of oligosaccharide probes (neoglycolipids) generated from glucan polysaccharides.* J Biol Chem, 2006. **281**(9): p. 5771-9.
116. Schallus, T., et al., *Malectin: a novel carbohydrate-binding protein of the endoplasmic reticulum and a candidate player in the early steps of protein N-glycosylation.* Mol Biol Cell, 2008. **19**(8): p. 3404-14.
117. Chen, C.J., et al., *Identification of a key pathway required for the sterile inflammatory response triggered by dying cells.* Nat Med, 2007. **13**(7): p. 851-6.
118. Uchimura, E., et al., *Transient infiltration of neutrophils into the thymus in association with apoptosis induced by whole-body X-irradiation.* J Leukoc Biol, 2000. **67**(6): p. 780-4.
119. Bugarcic, A., et al., *Human and mouse macrophage-inducible C-type lectin (Mincle) bind Candida albicans.* Glycobiology, 2008. **18**(9): p. 679-85.
120. Kataoka, H., et al., *Biosynthesis and post-translational processing of lectin-like oxidized low density lipoprotein receptor-1 (LOX-1). N-linked glycosylation affects cell-surface expression and ligand binding.* J Biol Chem, 2000. **275**(9): p. 6573-9.
121. Kato, Y., Y. Adachi, and N. Ohno, *Contribution of N-linked oligosaccharides to the expression and functions of beta-glucan receptor, Dectin-1.* Biol Pharm Bull, 2006. **29**(8): p. 1580-6.
122. Lanier, L.L., *DAP10- and DAP12-associated receptors in innate immunity.* Immunol Rev, 2009. **227**(1): p. 150-60.
123. Blank, U., et al., *Complete structure and expression in transfected cells of high affinity IgE receptor.* Nature, 1989. **337**(6203): p. 187-9.
124. Lanier, L.L., et al., *Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells.* Nature, 1998. **391**(6668): p. 703-7.
125. Wu, J., et al., *An activating immunoreceptor complex formed by NKG2D and DAP10.* Science, 1999. **285**(5428): p. 730-2.

University of Cape Town

126. Underhill, D.M. and H.S. Goodridge, *The many faces of ITAMs.* Trends Immunol, 2007. **28**(2): p. 66-73.
127. Chu, C.L., et al., *Increased TLR responses in dendritic cells lacking the ITAM-containing adapters DAP12 and FcRgamma.* Eur J Immunol, 2008. **38**(1): p. 166-73.
128. Hamerman, J.A., et al., *Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12.* Nat Immunol, 2005. **6**(6): p. 579-86.
129. Geissmann, F., S. Jung, and D.R. Littman, *Blood monocytes consist of two principal subsets with distinct migratory properties.* Immunity, 2003. **19**(1): p. 71-82.
130. Pyz, E., et al., *Characterisation of murine MICL (CLEC12A) and evidence for an endogenous ligand.* Eur J Immunol, 2008. **38**(4): p. 1157-63.
131. Willment, J.A., et al., *Dectin-1 expression and function are enhanced on alternatively activated and GM-CSF-treated macrophages and are negatively regulated by IL-10, dexamethasone, and lipopolysaccharide.* J Immunol, 2003. **171**(9): p. 4569-73.
132. Diefenbach, A., et al., *Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D.* Nat Immunol, 2002. **3**(12): p. 1142-9.
133. Rabinovich, B., et al., *NKG2D splice variants: a reexamination of adaptor molecule associations.* Immunogenetics, 2006. **58**(2-3): p. 81-8.
134. Drickamer, K., *Engineering galactose-binding activity into a C-type mannose-binding protein.* Nature, 1992. **360**(6400): p. 183-6.
135. Brown, G.D. and S. Gordon, *Immune recognition. A new receptor for beta-glucans.* Nature, 2001. **413**(6851): p. 36-7.
136. Welte, S., et al., *Mutual activation of natural killer cells and monocytes mediated by NKp80-AICL interaction.* Nat Immunol, 2006. **7**(12): p. 1334-42.
137. Spreu, J., et al., *Interaction of C-type lectin-like receptors NKp65 and KACL facilitates dedicated immune recognition of human keratinocytes.* Proc Natl Acad Sci U S A. **107**(11): p. 5100-5.
138. Huysamen, C., et al., *CLEC9A is a novel activation C-type lectin-like receptor expressed on BDCA3+ dendritic cells and a subset of monocytes.* J Biol Chem, 2008. **283**(24): p. 16693-701.
139. Yang, Z. and J.S. Marshall, *Zymosan treatment of mouse mast cells enhances dectin-1 expression and induces dectin-1-dependent reactive oxygen species (ROS) generation.* Immunobiology, 2009. **214**(4): p. 321-30.
140. Vowells, S.J., et al., *Flow cytometric analysis of the granulocyte respiratory burst: a comparison study of fluorescent probes.* J Immunol Methods, 1995. **178**(1): p. 89-97.
141. Swarup, G., S. Cohen, and D.L. Garbers, *Inhibition of membrane phosphotyrosyl-protein phosphatase activity by vanadate.* Biochem Biophys Res Commun, 1982. **107**(3): p. 1104-9.
142. Sarantis, H. and S.D. Gray-Owen, *The specific innate immune receptor CEACAM3 triggers neutrophil bactericidal activities via a Syk kinase-dependent pathway.* Cell Microbiol, 2007. **9**(9): p. 2167-80.

University of Cape Town

143. LeibundGut-Landmann, S., et al., *Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17.* Nat Immunol, 2007. **8**(6): p. 630-8.
144. Zhang, X., et al., *Coactivation of Syk kinase and MyD88 adaptor protein pathways by bacteria promotes regulatory properties of neutrophils.* Immunity, 2009. **31**(5): p. 761-71.
145. Kiefer, F., et al., *The Syk protein tyrosine kinase is essential for Fcgamma receptor signaling in macrophages and neutrophils.* Mol Cell Biol, 1998. **18**(7): p. 4209-20.
146. Brinkmann, V., et al., *Neutrophil extracellular traps kill bacteria.* Science, 2004. **303**(5663): p. 1532-5.
147. Urban, C.F., et al., *Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms.* Cell Microbiol, 2006. **8**(4): p. 668-76.
148. Van Ziffle, J.A. and C.A. Lowell, *Neutrophil-specific deletion of Syk kinase results in reduced host defense to bacterial infection.* Blood, 2009. **114**(23): p. 4871-82.
149. Bennouna, S., et al., *Cross-talk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection.* J Immunol, 2003. **171**(11): p. 6052-8.
150. Tsuda, Y., et al., *Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant Staphylococcus aureus.* Immunity, 2004. **21**(2): p. 215-26.
151. van Gisbergen, K.P., et al., *Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN.* J Exp Med, 2005. **201**(8): p. 1281-92.
152. Gringhuis, S.I., et al., *Selective C-Rel activation via Malt1 controls anti-fungal T(H)-17 immunity by dectin-1 and dectin-2.* PLoS Pathog. **7**(1): p. e1001259.
153. Cambi, A. and C. Figdor, *Necrosis: C-type lectins sense cell death.* Curr Biol, 2009. **19**(9): p. R375-8.
154. Iyoda, T., et al., *Neutrophils accelerate macrophage-mediated digestion of apoptotic cells in vivo as well as in vitro.* J Immunol, 2005. **175**(6): p. 3475-83.
155. Steele, C., et al., *Alveolar macrophage-mediated killing of Pneumocystis carinii f. sp. muris involves molecular recognition by the Dectin-1 beta-glucan receptor.* J Exp Med, 2003. **198**(11): p. 1677-88.
156. Steele, C., et al., *The beta-glucan receptor dectin-1 recognizes specific morphologies of Aspergillus fumigatus.* PLoS Pathog, 2005. **1**(4): p. e42.
157. Viriyakosol, S., et al., *Innate immunity to the pathogenic fungus Coccidioides posadasii is dependent on Toll-like receptor 2 and Dectin-1.* Infect Immun, 2005. **73**(3): p. 1553-60.
158. Netea, M.G., et al., *Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors.* J Clin Invest, 2006. **116**(6): p. 1642-50.
159. Romani, L., *Immunity to fungal infections.* Nat Rev Immunol, 2004. **4**(1): p. 1-23.
160. Coffman, R.L., et al., *B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells.* J Immunol, 1986. **136**(12): p. 4538-41.

University of Cape Town

161. Coffman, R.L., et al., *Antibody to interleukin-5 inhibits helminth-induced eosinophilia in mice.* Science, 1989. **245**(4915): p. 308-10.
162. Finkelman, F.D., et al., *Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites.* Immunol Rev, 2004. **201**: p. 139-55.
163. Pamer, E.G., *Immune responses to Listeria monocytogenes.* Nat Rev Immunol, 2004. **4**(10): p. 812-23.
164. Buchmeier, N.A. and R.D. Schreiber, *Requirement of endogenous interferon-gamma production for resolution of Listeria monocytogenes infection.* Proc Natl Acad Sci U S A, 1985. **82**(21): p. 7404-8.
165. Havell, E.A., *Evidence that tumor necrosis factor has an important role in antibacterial resistance.* J Immunol, 1989. **143**(9): p. 2894-9.
166. Hsu, Y.M., et al., *The adaptor protein CARD9 is required for innate immune responses to intracellular pathogens.* Nat Immunol, 2007. **8**(2): p. 198-205.
167. Schwegmann, A., et al., *Protein kinase C delta is essential for optimal macrophage-mediated phagosomal containment of Listeria monocytogenes.* Proc Natl Acad Sci U S A, 2007. **104**(41): p. 16251-6.
168. Wu, W., et al., *CARD9 facilitates microbe-elicited production of reactive oxygen species by regulating the LyGDI-Rac1 complex.* Nat Immunol, 2009. **10**(11): p. 1208-14.
169. Yin, J. and T.A. Ferguson, *Identification of an IFN-gamma-producing neutrophil early in the response to Listeria monocytogenes.* J Immunol, 2009. **182**(11): p. 7069-73.
170. Deshmukh, H.S., et al., *Critical role of NOD2 in regulating the immune response to Staphylococcus aureus.* Infect Immun, 2009. **77**(4): p. 1376-82.
171. Takeuchi, O., K. Hoshino, and S. Akira, *Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection.* J Immunol, 2000. **165**(10): p. 5392-6.
172. Shi, L., et al., *Mannose-binding lectin-deficient mice are susceptible to infection with Staphylococcus aureus.* J Exp Med, 2004. **199**(10): p. 1379-90.
173. Guo, J.P., et al., *The rat antigen-presenting lectin-like receptor complex influences innate immunity and development of infectious diseases.* Genes Immun, 2009. **10**(3): p. 227-36.
174. Iizuka, K., et al., *Genetically linked C-type lectin-related ligands for the NKRP1 family of natural killer cell receptors.* Nat Immunol, 2003. **4**(8): p. 801-7.
175. Chen, S.T., et al., *CLEC5A is critical for dengue-virus-induced lethal disease.* Nature, 2008. **453**(7195): p. 672-6.
176. Geijtenbeek, T.B., et al., *Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses.* Cell, 2000. **100**(5): p. 575-85.
177. Lee, S.J., et al., *Mannose receptor-mediated regulation of serum glycoprotein homeostasis.* Science, 2002. **295**(5561): p. 1898-901.
178. Mi, Y., S.D. Shapiro, and J.U. Baenziger, *Regulation of lutropin circulatory half-life by the mannose/N-acetylgalactosamine-4-SO4 receptor is critical for implantation in vivo.* J Clin Invest, 2002. **109**(2): p. 269-76.

University of Cape Town

179. Lazetic, S., et al., *Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits.* J Immunol, 1996. **157**(11): p. 4741-5.
180. Brooks, A.G., et al., *NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor.* J Exp Med, 1997. **185**(4): p. 795-800.
181. Houchins, J.P., et al., *Natural killer cell cytolytic activity is inhibited by NKG2-A and activated by NKG2-C.* J Immunol, 1997. **158**(8): p. 3603-9.
182. Le Drean, E., et al., *Inhibition of antigen-induced T cell response and antibody-induced NK cell cytotoxicity by NKG2A: association of NKG2A with SHP-1 and SHP-2 protein-tyrosine phosphatases.* Eur J Immunol, 1998. **28**(1): p. 264-76.
183. Lanier, L.L., et al., *Association of DAP12 with activating CD94/NKG2C NK cell receptors.* Immunity, 1998. **8**(6): p. 693-701.

University of Cape Town

Appendix I: Publications

Publications contributed to during this thesis:

LM Graham, V Gupta, G Schafer, D Reid, M Kimberg, K Dennehy, W Horsnell, R Guler, M Campanero-Rhodes, A Palma, T Feizi, S Kim, P Sobieszczuk, J Willment, and GD Brown. ***The C-type lectin receptor CLECSF8 (CLEC4D) is expressed by myeloid cells and triggers cellular activation through Syk kinase.*** Under review.

Marakalala, MJ, LM Graham and GD Brown. ***The role of Syk-coupled C-type lectin receptors in the immunity to Mycobacterium tuberculosis infections.*** Clinical and Developmental Immunology. Accepted 16 December 2010.

Graham, LM and GD Brown. ***The Dectin-2 family of C-type lectins in immunity and homeostasis.*** 2009. Cytokine 48(1-2):148-55.

Graham, L.M., and G.D. Brown. ***Innate recognition of Aspergillus fumigatus by the mammalian immune system***. *In* Latgé, J.P. and W. Steinbach (ed.), Aspergillus fumigatus and Aspergillosis. American Society for Microbiology, Washington, D.C. ISBN: 978-1-55581-438-0

University of Cape Town

The C-type lectin receptor CLECSF8 (CLEC4D) is expressed by myeloid cells and triggers cellular activation through Syk kinase.

Lisa M. Graham[1], Vandana Gupta[1], Georgia Schafer[1], Delyth M. Reid[2], Matti Kimberg[1], Kevin M. Dennehy[1¥], William G. Hornsell[1], Reto Guler[1], Maria A. Campanero-Rhodes3[¥], Angelina S. Palma[3¥], Ten Feizi[3], Stella K. Kim[4], Peter Sobieszczuk[5], Janet A. Willment[1,2] and Gordon D. Brown[1,2*]

[1]Institute of Infectious Disease and Molecular Medicine, CLS, Faculty of Health Sciences, University of Cape Town, Observatory, 7925, Cape Town, South Africa, [2]Section of Immunology and Infection, Institute of Medical Sciences, University of Aberdeen, Aberdeen, AB25 2ZD, United Kingdom, [3]Glycosciences Laboratory, Imperial College London, Middlesex, HA1 3UJ, UK, [4]School of Dentistry, University of California at San Francisco, San Francisco, CA 94143, USA, and [5]Transgenic Animal Core Facility, Sylvester Comprehensive Cancer Center, University of Miami, Miami, FL 33136, USA.

University of Cape Town

Running title: Characterisation of CLECSF8

*Corresponding author information: Dr Gordon D. Brown, Section of Immunology and Infection, Division of Applied Medicine, Institute of Medical Sciences, Foresterhill, University of Aberdeen, Aberdeen AB25 2ZD, United Kingdom. (gordon.brown@abdn.ac.uk)

Word count text: 4992
Word count abstract: 185
Figure count: 7
Reference count: 38

[¥]Current address:
Kevin M. Dennehy: Institut für Medizinische Virologie, Universitätsklinikum Tübingen 72076 Tübingen, Germany.,

Angelina S. Palma: REQUIMTE/Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal.,

Maria A Campanero-Rhodes: Instituto de Química-Física "Rocasolano", CSIC, and Ciber of Respiratory Diseases (CIBERES) Serrano 119, 28006 Madrid, Spain

University of Cape Town

Abstract

CLECSF8 is a poorly characterised member of the “Dectin-2 cluster” of C-type lectin receptors and was originally thought to be expressed exclusively by macrophages. We show here that CLECSF8 is primarily expressed by peripheral blood neutrophils and monocytes, but that expression of this receptor is lost upon differentiation of monocytes into macrophages or dendritic cells. Like the other members of the Dectin-2 family, which require association of their transmembrane domains with signalling adaptors for surface expression, CLECSF8 is retained intracellularly when expressed in non-myeloid cells. However, we demonstrate that CLECSF8 does not associate with any known signalling adaptor molecule, including DAP10, DAP12, or the FcRγ chain, and we found that the C-type lectin domain of CLECSF8 was responsible for its intracellular retention. Although CLECSF8 does not contain a signalling motif in its cytoplasmic domain, we show that this receptor is capable of inducing signalling via Syk kinase in myeloid cells and that it can induce phagocytosis, pro-inflammatory cytokine production and the respiratory burst. These data therefore indicate that CLECSF8 functions as an activation receptor on myeloid cells through an association with a novel adaptor molecule.

University of Cape Town

Introduction

C-type lectin receptors (CLRs) form a superfamily of molecules which contain at least one C-type lectin domain (CTLD)[1]. These receptors recognise a wide range of ligands varying from endogenous molecules to conserved (largely carbohydrate-based) structures found in microbes called pathogen associated molecular patterns (PAMPs)[2]. C-type lectins function in diverse ways and have been found to play essential roles in both immunity and homeostasis. The transmembrane receptors may contain cytoplasmic signalling motifs that enable intracellular signalling upon ligand binding, yet receptors lacking these motifs can also trigger intracellular signalling by associating with adaptors such as the FcRγ chain, DAP10 or DAP12[3,4]. The association with these adaptors is normally mediated by charged residues in or near the transmembrane domains of these CLRs[3].

Our research focuses on C-type lectins expressed by myeloid cells, particularly those of the Dectin-1 and Dectin-2 clusters[3,5,6]. Of relevance here is the Dectin-2 cluster, comprising of Dectin-2, DCIR, DCAR, BDCA-2, Mincle and CLECSF8. All these type II transmembrane receptors are encoded by six exons and share a common structure, consisting of a single extracellular CTLD, a stalk region of varying length and a transmembrane region[3]. Other than DCIR, which contains an immunoreceptor tyrosine-based inhibitory motif (ITIM), all the other receptors have short cytoplasmic tails that lack signalling motifs. Mincle, Dectin-2, DCAR and BDCA-2 have been shown to associate with FcRγ chain, an adaptor containing an immunoreceptor tyrosine-based activation motif (ITAM)[3].

Characterisation of the receptors in the “Dectin-2 cluster” over the last few years has revealed exciting new insights into the functions of these molecules. Dectin-2, for example, has been shown to recognise fungi[7-10], while Mincle recognises mycobacteria, fungi and an endogenous ligand that is released during necrotic cell death[11-13]. Importantly, both of these receptors signal through association with the FcRγ chain and can directly induce innate inflammation and the development of adaptive immunity. CLECSF8 (CLEC4D), which also lacks signalling motifs in its short cytoplasmic tail and is presumed to associate with signalling adaptors, is the least characterized member of this family. Murine CLECSF8 (mCLECSF8) was first identified by

University of Cape Town

differential display PCR and shown at the mRNA level to be expressed in a macrophage restricted manner, with highest transcript levels found in peritoneal macrophages, bone marrow and spleen and lower levels in lung and lymph nodes[14]. Human CLECSF8 (hCLECSF8) also demonstrated a monocyte/macrophage restricted expression pattern at the mRNA level, with transcript expression found to be down-regulated upon culture *in vitro* and up-regulated by IL6, TNF, IFNγ and IL10[15]. hCLECSF8 was additionally shown to be capable of mediating endocytosis[15], but the function of this receptor is still unknown. Here we have characterised the expression and function of murine and human CLECSF8 using both primary cells and transfected cell lines.

University of Cape Town

Material and Methods

Primary cells

Peripheral blood leukocytes (PBLs) were isolated from healthy volunteers as previously described[16]. For the generation of macrophages and DCs, human PBMCs were isolated from buffy coats (generously provided by the Western Province Blood Bank, Cape Town) and differentiated as previously described[16]. To isolate neutrophils from peripheral blood, leukocytes were separated by centrifugation over a two-layer (62% and 75%) Percoll Plus (GE Healthcare) gradient, at 20°C for 25min (10min at 200 x *g* and 15min at 400 x *g*, without interruption). The neutrophils were collected from the interface between the two Percoll solutions and washed with PBS containing 5mM glucose and 0.2% BSA. After centrifugation for 7min at 250 x *g* at 4°C, contaminating erythrocytes were lysed by hypotonic treatment for 30s with 0.2% NaCl, followed by restoration of isotonicity with 1.6% NaCl. After centrifugation, neutrophils (~95% pure) were resuspended in RPMI and counted.

To test the effect of cytokines and TLR agonists on CLECSF8 expression, PBLs were plated at $5x10^5$ cells/well in 24-well plates and stimulated for 6 hours with IL4 (20ng/ml), IL6 (80ng/ml), IL10 (20ng/ml), TNF (10ng/ml), IFNγ (10ng/ml), Pam3CSK4 (TLR1/2; 100ng/ml), LPS (TLR4; 100ng/ml), flagellin (TLR5; 20ng/ml) and FSL-1 (TLR2/6; 20ng/ml). All TLR agonists were from Invitrogen and cytokines from R&D Systems. Cells were then analysed by flow cytometry as described below.

Cell lines and growth conditions

NIH3T3 fibroblasts, RAW264.7 macrophages, A20 B cells, and Plat-E ecotropic retroviral packaging cells were maintained in DMEM or RPMI 1640 (Gibco) supplemented with 10% FCS (Gibco), 2mM L-glutamine, 100U/ml penicillin and 0.1mg/ml streptomycin (Cambrex), and cultured at 37°C with 5% CO_2.

University of Cape Town

Generation of constructs and transduced cell lines

The complete mCLECSF8, hCLECSF8, Mincle and Dectin-2 open reading frames were isolated by PCR and cloned into the pFBneo retroviral vector (Stratagene) containing an HA tag, as described previously for MICL[17]. The receptor chimeras were generated using overlapping extension PCR such that the final constructs translated as: D1$^{\text{CTLD}}$/SF8$^{\text{TM-cyto}}$ ($^{\text{SF8-76}}$TGGTW$^{\text{SF8-80}}$/$^{\text{D1-110}}$QTTGG $^{\text{D1-73}}$); SF8$^{\text{CTLD}}$/D1$^{\text{TM-cyto}}$ ($^{\text{D1-112}}$TGGFSQ$^{\text{D1-117}}$/$^{\text{SF8-74}}$GATGG$^{\text{SF8-78}}$); Janus ($^{\text{SF8-215}}$WKPSK$^{\text{SF8-219}}$/$^{\text{D1-69}}$FWRHN$^{\text{D1-73}}$); D1$^{\text{CTLD}}$/Min$^{\text{TM-cyto}}$ ($^{\text{MIN-80}}$CPLNWK$^{\text{MIN-85}}$/$^{\text{D1-113}}$GGFSQS$^{\text{D1-118}}$); Min$^{\text{CTLD}}$/D1$^{\text{TM-cyto}}$ ($^{\text{D1-119}}$CLPNWI$^{\text{D1- 124}}$ / $^{\text{MIN-74}}$GSVKNC$^{\text{MIN-79}}$); D1$^{\text{CTLD}}$/D2$^{\text{TM-cyto}}$ ($^{\text{D2-80}}$PNHWK$^{\text{D2-84}}$/$^{\text{D1-113}}$GGFSQSC$^{\text{D1-119}}$); D2$^{\text{CTLD}}$/D1$^{\text{TM-cyto}}$ ($^{\text{D1-119}}$CLPNWI$^{\text{D1-124}}$/$^{\text{D2-73}}$EKMWGC$^{\text{D2-78}}$) and D1$^{\text{CTLD}}$/D2$^{\text{TM-cyto}}$ ($^{\text{D2-80}}$PNHWK$^{\text{D2-84}}$/$^{\text{D1-113}}$GGFSQSC$^{\text{D1-119}}$). DNA constructs encoding DAP10, DAP12 and FcRγ were a kind gift from Phil Taylor (University of Cardiff) and NKG2D, from Brian Rabinovich (University of Texas). All constructs were verified by sequencing.

To generate stable cell lines, constructs were packaged into virions using Plat-E ecotropic cells, and the various cell lines were transduced as previously described[17]. All cell lines were used as nonclonal populations to reduce founder effects and were generated at least twice to confirm phenotype.

University of Cape Town

FACS analysis

FACS was performed on live cells according to conventional protocols at 4°C in the presence of 2mM NaN_3. Cells were blocked with PBS containing 5mM EDTA, 0.5% BSA and 5% heat-inactivated rabbit serum (murine cells) or murine serum (PBLs) and 50ug/ml human IgG (*in vitro* cultured human cells). For permeabilisation of cells prior to staining, cells were fixed with 1% formaldehyde at room temperature and permeabilised by the addition of 0.5% saponin. After staining with primary antibodies for 1h at 4°C, cells were washed and stained with the relevant secondary antibody. Cells were fixed with 1% formaldehyde prior to analysis.

The following antibodies were used in these assays: anti-HA (clone 16B12, Covance), anti-hCLECSF8 (MAB2806; R&D systems), CD14-PE, CD16-FITC, CD3-FITC, CD4-FITC, CD8-FITC, CD86-PE (all from Serotec), CD19-PE, CD56-PE, HLA-DR-FITC and DC-SIGN-FITC (all from BD Pharmingen); as well as irrelevant PE- or FITC-labelled or unlabelled mouse IgG_1 (BD Pharmingen), IgG_{2b} and IgG_3 (Serotec) control antibodies. The secondary antibodies used were goat anti-mouse PE and donkey anti-mouse APC (both from Jackson Immunoresearch).

Phagocytosis assays

University of Cape Town

Phagocytosis was quantified in transduced RAW264.7 cells by seeding cells at $5x10^5$ cells/well in 12-well plates the day before the assay. Some cells were treated with 5μM Cytochalasin D (Calbiochem) for 40min before and throughout the assay to inhibit phagocytosis. After washing, FITC-zymosan (Molecular Probes) was added at a ratio of 10 particles/cell and left to settle for 1h at 4^oC, to facilitate synchronised phagocytosis. After washing to remove unbound particles, cells were incubated at 37^oC for 30min. External zymosan was detected with anti-zymosan antibodies, as described[18]. For microscopy, cells were seeded onto acid-treated coverslips at $3x10^4$ cells/well in 24-well plates the day prior to the experiment. FITC-zymosan binding and phagocytosis was allowed to take place, as described above. Cells were fixed and permeabilised, and actin was stained with 1μM TRITC-phalloidin (Sigma-Aldrich). Coverslips were mounted with Vectashield (Vector Laboratories) and cells were examined by fluorescence microscopy on a Zeiss Axiovert 40.

Phagocytosis by neutrophils was similarly determined, except that FITC-labelled Dynabeads (Invitrogen) coated with either anti-hCLECSF8 or an isotype control were added at 2 beads/cell and permitted to bind at 10^oC for 30min. Cells were treated with 5μM Cytochalasin D or 50μM piceatannol (Sigma) for 40 min prior to and during the assay, where indicated. Phagocytosis was allowed to occur at 37^oC for 30 min. External beads were detected with a goat anti-mouse-PE antibody. Cells which had bound or internalised FITC-beads were gated and the percentage of phagocytosis was

determined by comparing the PE^- and the PE^+ populations, as described previously[19].

Fluorescent zymosan binding and cytokine production assays

Fluorescent zymosan binding and TNF production by transduced RAW264.7 cells were determined as previously described[20]. Soluble β-glucan (100μg/ml) or piceatannol (50μM) were added 20 min or 40 min prior to the assay, respectively, where indicated. Synergism between TLR4 and CLECSF8 was similarly determined, except that the transduced RAW264.7 cells were stimulated with either 100μg/ml particulate β-glucan or 10ng/ml LPS alone or in combination, as indicated. After incubation for 3h at 37°C, TNF in the supernatants was measured by ELISA (BD Biosciences).

Respiratory burst assays

For analysis of the respiratory burst in transduced RAW264.7 macrophages, cells were loaded with 20μM dihydrorhodamine 123 (Sigma) and incubated with 50μg/ml particulate β-glucan for the indicated length of time. In neutrophils, ROS production was measured by cross-linking CLECSF8 on the cell surface. Briefly, on the day prior to the experiment, 24-well poly-L-lysine plates (BD Biocoat) were activated with 2.5% gluteraldehyde (Sigma), followed by coating with 50μg/ml cross-linking sheep anti-mouse IgG (Jackson Immunoresearch) and subsequent blocking of free aldehyde groups with 0.2M glycine. 10μg/ml anti-hCLECSF8 or isotype control antibody was then added, allowed to bind over night and washed before use. Freshly isolated neutrophils were loaded with 20μM dihydrorhodamine 123, added to the wells and incubated at 37°C for 30min. ROS production was determined by measuring the conversion of dihydrorhodamine 123 to rhodamine using flow cytometry.

Cell lysates, immunoprecipitations and Western blotting

Signalling complexes were immunoprecipitated from transduced RAW264.7 cells by coating $2x10^7$ cells/time point with 10μg/ml anti-HA for 2h with rotation at 4°C. Cells were then stimulated with pervanadate for 1 or 3min and lysed

University of Cape Town

as previously described[21]. Lysates were added to pan mouse IgG Dynabeads (Invitrogen) pre-blocked with PBS containing 0.5% BSA and rotated for 2h at 4°C. After extensive washing, immunoprecipitates were analysed by Western blotting. To confirm transductions with the adaptor molecules and association of the adaptors with the relevant receptor, A20 cell lysates were pre-cleared by rotation with Protein A Sepharose beads (GE Healthcare) for 30min at 4°C and then the beads removed by centrifugation for 10min. Anti-FcRγ, anti-DAP10 or anti-DAP12 were added to the lysates and rotated for 2h at 4°C before addition of Protein A Sepharose beads for an additional hour. Beads were washed extensively with lysis buffer and immunoprecipitates were analysed by Western blotting. To prepare cellular extracts, cells were lysed and prepared as previously described[21]. For Western blotting, proteins were detected with anti-phosphotyrosine (clone 4G10), anti-Syk, anti-phospho-Syk (Cell Signalling Technology), anti-HA (clone 16B12, Covance), anti-DAP10 and DAP12 (kind gift from Toshiyuki Takai, Tohoku University), or anti-FcRγ (Millipore), followed by appropriate HRP-linked secondary antibodies (Jackson Immunoresearch).

Statistics

Comparisons between two groups were performed using the students *t*-test. Comparisons between multiple groups were performed using one way ANOVA, with Dunnetts post test. All experiments were performed in duplicate or triplicate, and independently repeated at least twice. *; $p < 0.05$.

University of Cape Town

Results

Expression of CLECSF8 on human peripheral blood cells

To characterise CLECSF8, we first examined the cellular expression profile of this receptor on human PBLs[15] by flow cytometry. For these analyses we made use of a commercially available mAb to hCLECSF8, which specifically stained transduced cells expressing this receptor (Supplemental Figure 1). Using this antibody, as well as discriminating cells based on their size, granularity and with lineage-specific markers, we detected hCLECSF8 expression on $CD16^+$ neutrophils as well as on the $CD14^+CD16^-$ inflammatory monocytes[22] (Figure 1A). However, hCLECSF8 was not detected on $CD14^{low}CD16^+$ monocytes, $CD16^-$ eosinophils, $CD8^+$ or $CD4^+$ T cells, $CD19^+$ B cells nor on $CD56^+$ NK cells.

We then investigated changes in hCLECSF8 expression upon differentiation of monocytes into macrophages and DCs *in vitro*[16]. The differentiation of monocytes into macrophages, which can be characterised by the down-regulation of CD14 and up-regulation of HLA-DR, resulted in the loss of expression of hCLECSF8 (Figure 1B). Similarly, CLECSF8 expression was not detected on monocytes cultured with GM-CSF and IL4 to induce a DC-like phenotype (characterised by increased DC-SIGN and decreased CD14 expression), nor on DC's matured with LPS (characterised by increased CD86 and loss of CD14 expression).

Expression of hCLECSF8 mRNA was previously shown to be modulated by cytokines and other agents[15], so we examined the effects of a variety of cytokines and TLR agonists on hCLECSF8 expression in both monocytes and neutrophils following a 6h stimulation of PBL *in vitro.* Although we found a significant increase in hCLECSF8 expression on monocytes treated with LPS, and on neutrophils treated with TNF, IFNγ, PAM_3CSK_4 and LPS, the effect of these agents on receptor levels were relatively modest (< 2 fold) (Figure 1C). Overall, these data demonstrate that hCLECSF8 is expressed on a population of circulating monocytes and neutrophils, but is down-regulated following differentiation of monocytes into macrophages or

University of Cape Town

DCs, and can be marginally influenced by certain pro-inflammatory cytokines and microbial components.

CLECSF8 does not associate with known adaptors for expression at the cell surface.

Many of the Dectin-2 cluster of receptors associate with the FcRγ signalling adaptor for transport to the cell surface[3], thus we next examined the expression profile of haemagglutinin (HA)-tagged human and murine CLECFS8 in transduced NIH3T3 fibroblasts, A20 B-cells, and RAW264.7 macrophages. As described for other receptors in this cluster, we observed that both human and murine CLECSF8-HA were retained intracellularly when expressed in NIH3T3 or A20 cells, but were located on the cell surface when expressed in RAW264.7 macrophages (Figure 2A). This suggested that CLECSF8 associates with a myeloid-expressed adaptor for expression at the cell surface. Western blot analyses of lysates from the transfected RAW264.7 cells indicated that murine CLECSF8 was expressed as a monomer of ~24kDa (equivalent to its predicted molecular weight[14]; Figure 2B). Cells expressing the unrelated human C-type lectin CLEC9A, which dimerises through disulphide linkages[21], were included as a control.

To determine which adaptor was involved in mediating the surface expression of CLECSF8, we co-transduced mCLECSF8-HA expressing A20 cells along with murine DAP10, DAP12 or the FcRγ chain. As positive controls for these experiments, we included Mincle, which associates with the FcRγ chain, or NKG2D, which associates with DAP10 or DAP12[13,23]. None of the CLRs, were detected on the cell surface of transduced A20 cells in the absence of an adaptor molecule (Figure 2C). However, while co-expression with the appropriate adaptor induced surface expression of Mincle and NKG2D, as expected[13,23], none of the adaptors resulted in surface expression of mCLECSF8-HA. Western blot analyses of immunoprecipitations of the adaptors from the various cell lysates confirmed expression of the various constructs as well as their association with the relevant receptors (Figure 2D). These results suggest that, unlike other members of this family, CLECSF8 does not associate with DAP10, DAP12 or FcRγ.

University of Cape Town

The intracellular retention of CLECSF8 requires the CTLD

The association of the signalling adaptors with other members of the Dectin-2 cluster involves charged residues in the transmembrane domain or cytoplasmic tail[3]. As CLECSF8 lacks these residues and did not associate with any of the known adaptors, we sought to determine which domain of CLECSF8 was responsible for its intracellular retention in NIH3T3 cells. For these experiments, we generated chimeric receptors whereby various domains of mCLECSF8 were replaced with that of an unrelated C-type lectin, Dectin-1 (Cartoon representations of these chimeras are shown in Supplemental Figure 2). We chose to use Dectin-1 for these experiments, as this receptor does not associate with, nor require, an adaptor for cell surface expression[24]. Furthermore the specificity of the Dectin-1 CTLD for β-glucans has been well documented[24-26], allowing the chimeric receptors to be used in functional analyses (see below).

We generated two chimeras; the first comprising of the CTLD of Dectin-1 coupled to the transmembrane and intracellular regions of CLECSF8 (termed $D1^{CTLD}/SF8^{TM\text{-}cyto}$), and the second consisting of the CLTD of CLECSF8 coupled to the transmembrane and intracellular regions of Dectin-1 (termed $SF8^{CTLD}/D1^{TM\text{-}cyto}$). The expression of both of these chimeras was then examined in transduced NIH3T3 and RAW264.7 cells. We found that the chimera $SF8^{CTLD}/D1^{TM\text{-}cyto}$, containing the CTLD of mCLECSF8, displayed the same expression profile as full-length mCLECSF8, in that it was retained intracellularly in NIH3T3 cells but expressed at the cell surface of RAW264.7 cells (Figure 3A). In contrast, the chimera $D1^{CTLD}/SF8^{TM\text{-}cyto}$ was expressed on the surface of both cell types. As expected[24], full-length Dectin-1 was expressed on the surface of both NIH3T3 and RAW264.7 cells (data not shown). These findings demonstrate that the CTLD of mCLECSF8 is responsible for its intracellular retention in NIH3T3 cells.

As intracellular retention has not previously been shown to depend on the CTLD, we wondered whether any other members of the Dectin-2 cluster of receptors possessed similar characteristics. We therefore generated HA-tagged full-length Mincle and Dectin-2, as well as chimeras similar to those

University of Cape Town

described above (see Supplemental Figure 2), and examined the expression profile of each of these receptors in transduced NIH3T3 and RAW264.7 cells. Full-length Mincle and Dectin-2 displayed the same profile as full-length mCLECSF8, in that they were expressed on the surface of RAW264.7 macrophages but retained intracellularly in NIH3T3 cells (Figure 3B). However, in contrast to CLECSF8, chimeras containing the CTLD of Dectin-1 coupled to the transmembrane and intracellular regions of Mincle ($D1^{CTLD}/Min^{TM\text{-}cyto}$) or Dectin-2 ($D1^{CTLD}/D2^{TM\text{-}cyto}$) were retained intracellularly in NIH3T3 cells, whereas chimeras consisting of the transmembrane and intracellular regions of Dectin-1 coupled to the CTLD of Mincle ($Min^{CTLD}/D1^{TM\text{-}cyto}$) or Dectin-2 ($D2^{CTLD}/D1^{TM\text{-}cyto}$) were expressed at the cell surface (Figure 3C). All chimeras were expressed on the surface of RAW264.7 cells (Figure 3C). These results therefore demonstrate that the intracellular retention of CLECSF8 occurs through a novel mechanism involving its CTLD.

CLECSF8 is a phagocytic receptor

As the ligand of CLECSF8 is currently unknown, we set about characterising the functions of this receptor using a chimeric receptor containing the CTLD of the β-glucan receptor Dectin-1. While we have used this strategy successfully to characterise the functions of other C-type lectins, such as CLEC9A[21], the expression profile of CLECSF8 (discussed above) suggested that the CTLD of this receptor was important for its intracellular retention and may be involved in its association with any putative signalling adaptor. Thus, we generated a novel chimeric receptor termed Janus, consisting of the HA-tagged CTLD of Dectin-1 fused to the entire mCLECSF8 receptor (Figure 4A). As we had shown for full length CLECSF8, we could demonstrate that Janus was retained intracellularly when expressed in NIH3T3 cells, and on the surface of RAW264.7 cells (Figure 4B).

We then examined the ability of RAW264.7 cells transduced with Janus, CLECSF8 or Dectin-1 to bind and internalize the β-glucan rich particle, zymosan. As expected, the expression of Dectin-1 on these cells conferred the ability to bind zymosan in a β-glucan dependent manner (Figure 4C). Similar levels of zymosan binding were also obtained by the Janus expressing

University of Cape Town

cells, demonstrating functionality of the chimeric receptor. Cells expressing full-length CLECSF8 or vector-only control cells, showed background levels of zymosan binding (Figure 4C). We then evaluated the ability of these cells to internalize these particles by flow cytometry and fluorescence microscopy, in the presence or absence of cytochalasin D to inhibit actin polymerisation (Figure 4D and E). Using these methods, we could demonstrate that RAW264.7 cells expressing Dectin-1 were able to internalize zymosan particles in an actin-dependent manner, as expected[18], and we also observed a similar uptake of particles with cells expressing the Janus chimeric receptor. As a control for these experiments, we included RAW264.7 cells expressing a truncated version of Dectin-1, which lacks the cytoplasmic tail and signalling motifs[18], and observed that these cells were able to bind, but not to internalize, the zymosan particles (Figure 4D and E, and data not shown). These results therefore suggest that CLECSF8 functions as a phagocytic receptor.

To examine whether CLECSF8 could function similarly on primary cells, we explored the ability of PMNs to ingest anti-hCLECSF8 coated FITC-labelled ~4.5µm Dynabeads. We could demonstrate that these beads bound specifically to RAW264.7 cells expressing hCLECSF8 (Figure 4F). Furthermore, these beads were bound and internalized by PMNs to a much greater extent, than the isotype control beads. As we had observed for the RAW264.7 cells, internalisation of these beads occurred in an actin dependent manner, as it could be inhibited by treatment with cytochalasin D (Figure 4G). Overall, these data therefore indicate that CLECSF8 functions as a phagocytic receptor.

University of Cape Town

CLECSF8 induces the production of TNF

Many CLRs can induce the production of cytokines[3,6], thus we next determined if the stimulation of CLECSF8 would also induce this type of response. For these experiments, we made use of the RAW264.7 macrophages expressing Janus and explored their ability to induce the production of the pro-inflammatory cytokine TNF, following stimulation with zymosan, using an approach we had previously used to characterise Dectin-

1[20] and CLEC9A[21]. In comparison to the vector only controls, the expression of Janus was found to induce TNF in response to zymosan (Figure 5A, grey bars). The ability of soluble β-glucans to inhibit this response (black bars) demonstrated the dependency on the Dectin-1 CTLD for ligand recognition in the chimeric receptor. Cells expressing full length Dectin-1 were also able to induce TNF, as expected[20], and served as a control in these experiments.

We and others have previously shown that the production of TNF by macrophages in response to zymosan requires collaborative signalling between Dectin-1 and TLR2[20,27,28]. To determine whether cytokine production induced through the chimeric CLECSF8 receptor also involved collaborative signalling with the TLRs, we stimulated Janus expressing RAW264.7 cells with combinations of specific receptor agonists. Stimulation with particulate β-glucan (to target the Dectin-1 CTLD of the chimera) or LPS (to target TLR4) alone, induced low levels of TNF, however, when added together the stimuli were strongly synergistic (Figure 5B). Thus, these data show that stimulation of CLECSF8 can induce pro-inflammatory cytokine production and that signalling induced by this receptor can act synergistically with the TLRs.

University of Cape Town

CLECSF8 can signal via Syk kinase

We have previously shown that the collaborative signalling between the TLRs and Dectin-1 requires Syk kinase[27], thus we explored the possibility that this kinase was also involved in the signalling mediated by CLECSF8. To this end, we first examined the effect of piceatannol, a Syk kinase inhibitor, on CLECSF8-mediated TNF production (in transduced RAW264.7 macrophages) and phagocytosis (in PMNs) and in both cases we observed that the presence of piceatannol significantly inhibited these responses (Figure 6A).

To demonstrate that CLECSF8 was capable of signalling via Syk-kinase more directly, we isolated signalling complexes from RAW264.7 cells expressing hCLECSF8, mCLECSF8 or Dectin-1 by immunoprecipitation, following treatment of the cells with or without pervanadate. Probing Western blots of the immunoprecipitates with an anti-phosphotyrosine antibody revealed a tyrosine-phosphorylated molecule of ~70kDa in both CLECSF8 and Dectin-1 precipitates from pervanadate stimulated cells, which was

identified to be Syk kinase (Figure 6B). Furthermore, probing duplicate blots with an anti-phospho-Syk antibody demonstrated that the active form of this kinase was associated with these receptors, following pervanadate stimulation (Figure 6B). However, despite considerable effort, we were unable to verify the association of Syk with CLECSF8 in PMNs (data not shown), a failure which we attribute to the very low levels of expression of this receptor on these primary cells (in fact we were also unable to demonstrate immunoprecipitation of CLECSF8 itself from these cells, since there is no appropriate antibody available for this analysis). Thus, these results suggest that CLECSF8 is able to mediate intracellular signalling via Syk kinase.

CLECSF8 can induce the respiratory burst

The ability of neutrophils to induce the respiratory burst is an important antimicrobial mechanism, which can be stimulated by some C-type lectins, including Dectin-1[29,30]. We therefore examined the ability of CLECSF8 to induce the respiratory burst by stimulating RAW264.7 macrophages expressing Janus (the CLECSF8 chimera) or Dectin-1 with zymosan, and observed that the stimulation of these cells resulted in a robust respiratory burst response (Figure 7A). In contrast, vector only control cells, or cells expressing full-length CLECSF8, failed to respond in this manner to zymosan.

To demonstrate that CLECSF8 was able to induce this response in primary cells we cultured purified PMN on anti-hCLECSF8 immobilized on plastic, to determine if specific stimulation of this receptor was able to induce a respiratory burst. Indeed, we observed that cross-linking of this receptor resulted in greatly increased ROS production when compared to the isotype control (Figure 7B). Thus, these results demonstrate that CLECSF8 is capable of inducing the respiratory burst in leukocytes.

University of Cape Town

Discussion

Recent interest in the Dectin-2 cluster of C-type lectins has provided significant new insights into the underlying mechanisms of immunity and homeostasis[3]. Here we have characterised CLECSF8, one of the least well understood members of this group and provide evidence that this receptor is expressed only by selected populations of myeloid cells and is capable of triggering cellular activation. Originally described as a monocyte/macrophage-restricted receptor[14,15], we observed that expression of CLECSF8 was limited to $CD14^{+}$ $CD16^{-}$ monocytes in peripheral blood, which are the subset thought to be recruited to sites of inflammation where they differentiate into macrophages and DC[31]. Interestingly, we found that expression of this receptor became down-regulated upon differentiation of monocytes into macrophages or DC's *in vitro*, suggesting that CLECSF8 may only be expressed and functional on circulating monocytes. We also found that CLECSF8 was expressed on circulating neutrophils, demonstrating that expression of this receptor was not restricted to monocytes, although we did not detect it on any other PBL population.

Using a variety of approaches in both primary cells and in transfected cell lines, we demonstrated that CLECSF8 can induce phagocytosis, cytokine production and the respiratory burst. Thus, like several other members of the Dectin-2 cluster, CLECSF8 is capable of triggering cellular activation. How this receptor mediates these activities is still unclear, as CLECSF8 lacks recognisable signalling motifs in its cytoplasmic tail. All of the other Dectin-2 cluster activation receptors associate with the FcRγ chain, which triggers intracellular signalling through Syk kinase[3,4]. Although we could demonstrate that signalling from CLECSF8 involved Syk kinase, this receptor did not associate with the FcRγ chain, nor other adaptors which have been implicated in C-type lectin signalling, such as DAP12 or DAP10[2].

These adaptors, in addition to mediating intracellular signalling, are required for expression of the C-type receptors at the cell surface[3]. With most C-type lectins, this association is mediated by a positively charged residue in the transmembrane domain[13,32,33]. However, with Dectin-2, the association with FcRγ requires a membrane proximal region of the cytoplasmic tail[34]. Unexpectedly, we found that surface expression of CLECSF8 involved the

University of Cape Town

CTLD, and not the stalk, transmembrane or cytoplasmic tail regions. This finding was consistent with our observation that CLECSF8 did not associate with a typical adaptor protein, and suggested that this receptor may interact with a novel signalling partner. While the nature of this protein is still unclear, it could be another C-type lectin, reminiscent of the association between NGK2A and CD94, which is required for efficient surface expression of this complex[35]. Given the ability of CLECSF8 to signal via Syk kinase, it is likely that this adaptor molecule possess an ITAM or ITAM-like motif, or itself associates with an adaptor, such as occurs in the CD94/NKG2C complex[36]. However, we did not detect any adaptor association with CLECSF8 following immunoprecipitation of this receptor from transfected RAW264.7 macrophages (data not shown). Given that most activation receptors associate with such adaptor chains, our results suggest that CLECSF8 may associate with an ITAM-like containing activation receptor. Possible candidates include Dectin-1, CLEC-2, CLEC9A and CEACAM3, however the expression profile of the latter three receptors do not fit with that described for CLECSF8, and we have tested and excluded the possibility that the adaptor molecule is Dectin-1 (data not shown).

Despite the novel insights into the functions of CLECSF8 described here, the ligands and physiological role of this receptor are still unclear. All of the Dectin-2 cluster of receptors, except CLECSF8, are “classical” C-type lectins, possessing the conserved residues required for binding carbohydrates. CLECSF8, on the other hand, retains the residues required for calcium coordination, but lacks the conserved triplet motif that is normally associated with sugar recognition[3]. Indeed, screening a carbohydrate microarray, using a soluble CLECSF8-Fc fusion protein as a probe, did not reveal any ligands for this receptor (Supplemental Figure 3). Given the diverse repertoire of molecules that have been identified as ligands for C-type lectin receptors, in general, and the Dectin-2 cluster of receptors, in particular, it is impossible to predict the nature of the ligand(s) of CLECSF8.

Similar to other members of the family, which have been shown to recognise fungi, mycobacteria and viruses, CLECSF8 may play a role in pathogen recognition[8-12,37]. Expression of CLECSF8 on circulating neutrophils, in particular, suggests a role in innate immunity, since these

University of Cape Town

short-lived cells play crucial roles in the first line of defence against invading pathogens. Indeed, the ability of CLECSF8 to induce pro-inflammatory cytokine production, phagocytosis and the respiratory burst are all suggestive of a role in pathogen recognition and clearance. However, we have extensively characterised the CLESCF8$^{-/-}$ mice available from the Consortium for Functional Glycomics, and have not found any defects in their ability to resist infection with pathogenic fungi (*Candida albicans*), extracellular bacteria (*Staphylococcus aureus*), intracellular bacteria (*Listeria monocytogenes*), or nematodes (*Nippostrongylus brasiliensis*) (Supplemental Figure 4).

Another possibility is that CLECSF8, like other members of the Dectin-2 family, recognises endogenous ligand(s) and functions in controlling homeostasis. Mincle, for example, recognises a nuclear protein (SAP130) released from necrotic cells, resulting in the production of pro-inflammatory cytokines and infiltration of neutrophils to the site of necrosis[13]. Furthermore, the inhibitory receptor DCIR has been shown to play a role in preventing autoimmune arthritis by controlling DC expansion, although the endogenous ligand of this receptor remains unknown[38]. However, we observed no effect of CLECSF8 deficiency in murine models of sterile peritonitis (Supplemental Figure 5), response to necrotic cell death (Supplemental Figure 6), experimental autoimmune uveoretinitis (Supplemental Figure 7) or the development of spontaneous autoimmune arthritis (data not shown). Thus, despite extensive analysis, we still have no insights into the physiological role of CLECSF8.

In summary, we have demonstrated that CLECSF8 functions as an activation receptor and that this activity is likely to be mediated through a novel interaction involving the CTLD, and an unidentified adaptor molecule which induces the intracellular signalling. The identification of this adaptor, and the ligands and physiological role of CLECSF8, are the immediate issues which require further attention and are currently under further investigation.

University of Cape Town

Acknowledgments

We thank Phil Taylor and Brian Rabinovich for constructs, Julie Taylor for help with histology, and the staff of our animal facilities for the care of the animals used in these studies. Wengang Chai and Yibing Zhang for collaboration in the generation and mass spectrometric analyses of oligosaccharides and oligosaccharide-NGL probes, and Robert Childs for the microarrays.This work was funded by Wellcome Trust, National Research Foundation, Deutscher Akademischer Austauschdienst, the University of Cape Town, UK Research Councils' Basic Technology Initiative 'Glycoarrays' (GRS/79268) and the UK Medical Research Council. A.S.P is a fellow of the Fundação para a Ciência e Tecnologia (SFRH/BPD/26515/2006, Portugal) and MAC of the Ministerio de Ciencia e Innovación (JCI-2007-123-160, Spain).

Authorship

Contribution: L.M.G, V.G., G.S., D.M.R., M. K., W.G.H, M.A.C. and A.S.P. performed research and analysed data. T.F., K.M.D., J.A.W. and G.D.B. designed research and analysed data. P. S. and S.K. contributed vital reagents. L.M.G and G.D.B wrote the paper.

Conflict of Interest disclosure: The authors declare no competing financial interests.

University of Cape Town

References

1. Drickamer K. C-type lectin-like domains. Curr Opin Struct Biol. 1999;9:585-590.

2. Robinson MJ, Sancho D, Slack EC, LeibundGut-Landmann S, Reis e Sousa C. Myeloid C-type lectins in innate immunity. Nat Immunol. 2006;7:1258-1265.

3. Graham LM, Brown GD. The Dectin-2 family of C-type lectins in immunity and homeostasis. Cytokine. 2009;48:148-155.

4. Kerrigan AM, Brown GD. Syk-coupled C-type lectin receptors that mediate cellular activation via single tyrosine based activation motifs. Immunol Rev. 2010;234:335-352.

5. Zelensky AN, Gready JE. The C-type lectin-like domain superfamily. FEBS J. 2005;272:6179-6217.

6. Huysamen C, Brown GD. The fungal pattern recognition receptor, Dectin-1, and the associated cluster of C-type lectin-like receptors. FEMS Microbiol Lett. 2009;290:121-128.

7. Wells CA, Salvage-Jones JA, Li X, et al. The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to Candida albicans. J Immunol. 2008;180:7404-7413.

8. Yamasaki S, Matsumoto M, Takeuchi O, et al. C-type lectin Mincle is an activating receptor for pathogenic fungus, Malassezia. Proc Natl Acad Sci U S A. 2009;106:1897-1902.

9. Saijo S, Ikeda S, Yamabe K, et al. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against Candida albicans. Immunity. 2010;32:681-691.

University of Cape Town

10. Robinson MJ, Osorio F, Rosas M, et al. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. J Exp Med. 2009;206:2037-2051.

11. Ishikawa E, Ishikawa T, Morita YS, et al. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. J Exp Med. 2009;206:2879-2888.

12. Schoenen H, Bodendorfer B, Hitchens K, et al. Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. J Immunol. 2010;184:2756-2760.

13. Yamasaki S, Ishikawa E, Sakuma M, Ogata K, Saito T. Mincle is an ITAM-couples activating receptor that senses damaged cells. Nat Immunol. 2008;9:1179-1188.

14. Balch SG, McKnight AJ, Seldin MF, Gordon S. Cloning of a novel C-type lectin expressed by murine macrophages. J Biol Chem. 1998;273:18656-18664.

15. Arce I, Martinez-Munoz L, Roda-Navarro P, Fernandez-Ruiz E. The human C-type lectin CLECSF8 is a novel monocyte/macrophage endocytic receptor. Eur J Immunol. 2004;34:210-220.

16. Willment JA, Marshall AS, Reid DM, et al. The human beta-glucan receptor is widely expressed and functionally equivalent to murine Dectin-1 on primary cells. Eur J Immunol. 2005;35:1539-1547.

17. Marshall AS, Willment JA, Lin HH, Williams DL, Gordon S, Brown GD. Identification and Characterization of a Novel Human Myeloid Inhibitory

University of Cape Town

C-type Lectin-like Receptor (MICL) That Is Predominantly Expressed on Granulocytes and Monocytes. J Biol Chem. 2004;279:14792-14802.

18. Herre J, Marshall AJ, Caron E, et al. Dectin-1 utilizes novel mechanisms for yeast phagocytosis in macrophages. Blood. 2004;104:4038-4045.
19. Kerrigan AM, Dennehy KM, Mourao-Sa D, et al. CLEC-2 is a phagocytic activation receptor expressed on murine peripheral blood neutrophils. J Immunol. 2009;182:4150-4157.
20. Brown GD, Herre J, Williams DL, Willment JA, Marshall ASJ, Gordon S. Dectin-1 mediates the biological effects of beta-glucan. J Exp Med. 2003;197:1119-1124.
21. Huysamen C, Willment JA, Dennehy KM, Brown GD. CLEC9A is a novel activation C-type lectin-like receptor expressed on BDCA3+ dendritic cells and a subset of monocytes. J Biol Chem. 2008;283:16693-16701.
22. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity. 2003;19:71-82.
23. Gilfillan S, Ho EL, Cella M, Yokoyama WM, Colonna M. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. Nat Immunol. 2002;3:1150-1155.
24. Brown GD. Dectin-1: a signalling non-TLR pattern-recognition receptor. Nat Rev Immunol. 2006;6:33-43.
25. Palma AS, Feizi T, Zhang Y, et al. Ligands for the beta-glucan receptor, Dectin-1, assigned using "designer" microarrays of

University of Cape Town

oligosaccharide probes (neoglycolipids) generated from glucan polysaccharides. J Biol Chem. 2006;281:5771-5779.

26. Adams EL, Rice PJ, Graves B, et al. Differential high affinity interaction of Dectin-1 with natural or synthetic glucans is dependent upon primary structure and is influenced by polymer chain length and side chain branching. J Pharmacol Exp Ther. 2008;325:115-123.

27. Dennehy KM, Ferwerda G, Faro-Trindade I, et al. Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. Eur J Immunol. 2008;38:500-506.

28. Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM. Collaborative Induction of Inflammatory Responses by Dectin-1 and Toll-like Receptor 2. J Exp Med. 2003;197:1107-1117.

29. Underhill DM, Rossnagle E, Lowell CA, Simmons RM. Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. Blood. 2005;106:2543-2550.

30. Kennedy AD, Willment JA, Dorward DW, Williams DL, Brown GD, DeLeo FR. Dectin-1 promotes fungicidal activity of human neutrophils. Eur J Immunol. 2007;37:467-478.

31. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. Science. 2010;327:656-661.

32. Underhill DM, Goodridge HS. The many faces of ITAMs. Trends Immunol. 2007;28:66-73.

33. Kanazawa N, Tashiro K, Inaba K, Miyachi Y. Dendritic cell immunoactivating receptor, a novel C-type lectin immunoreceptor, acts

University of Cape Town

as an activating receptor through association with Fc receptor gamma chain. J Biol Chem. 2003;278:32645-32652.

34. Sato K, Yang XL, Yudate T, et al. Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. J Biol Chem. 2006;281:38854-38866.

35. Brooks AG, Posch PE, Scorzelli CJ, Borrego F, Coligan JE. NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. J Exp Med. 1997;185:795-800.

36. Lanier LL, Corliss B, Wu J, Phillips JH. Association of DAP12 with activating CD94/NKG2C NK cell receptors. Immunity. 1998;8:693-701.

37. Lambert AA, Gilbert C, Richard M, Beaulieu AD, Tremblay MJ. The C-type lectin surface receptor DCIR acts as a new attachment factor for HIV-1 in dendritic cells and contributes to trans- and cis-infection pathways. Blood. 2008;112:1299-1307.

38. Fujikado N, Saijo S, Yonezawa T, et al. Dcir deficiency causes development of autoimmune diseases in mice due to excess expansion of dendritic cells. Nat Med. 2008;14:176-180.

University of Cape Town

Figure Legends

Figure 1. CLECSF8 expression on human peripheral blood leukocytes. (**A**) Flow cytometric analysis of peripheral blood, showing the gating of granulocytes, monocytes and lymphocytes and staining with specific cell markers and anti-hCLECSF8, as indicated. (**B**) Analysis of hCLECSF8 expression on monocyte-derived macrophages and DC. HLA-DR, CD14, DC-SIGN and CD86 were included as markers of macrophage and DC maturation, as described in the text. The filled grey histograms represent the isotype controls and the black histograms represent specific antibody staining, as indicated. The data are representative of at least five independent donors. (**C**) Freshly isolated peripheral blood monocytes (open bars) and neutrophils (grey bars) were treated with various cytokines and TLR agonists and hCLECSF8 expression was analysed by flow cytometry. The data were normalised to untreated control value and shown as the mean ± SEM of pooled data from at least five independent donors. *, $p < 0.05$. flag, flagellin.

University of Cape Town

Figure 2. CLECSF8 does not associate with known adaptor molecules. (**A**) Anti-HA flow cytometric analyses of NIH3T3, RAW264.7 and A20 B cells transduced with HA-tagged murine or human CLECSF8. (**B**) Anti-HA Western blot analyses of RAW264.7 cells expressing HA-tagged mCLECSF8 or hCLEC9A under non-reducing (-βMe) and reducing (+βMe) conditions. (**C**) Flow cytometric analyses of A20 B cells transduced with HA-tagged mCLECSF8, Mincle or NKG2D with or without FcRγ, DAP10 or DAP12. Grey filled histograms represent vector control cells, solid black histograms represent surface expression, and dashed histograms represent total (intracellular and surface) expression. (**D**) Western blot analyses of anti-FcRγ, anti-DAP10 or anti-DAP12 immunoprecipitations from the transduced A20 B cells, showing presence of the adaptor molecules and the C-type lectin receptors (probed with anti-HA), as indicated. N.D., not done.

Figure 3. The intracellular retention of CLECSF8 is dependent on the CTLD. (**A**) Anti-HA flow cytometric analyses of the HA-tagged mCLECSF8

chimeras expressed in RAW264.7 and NIH3T3 cells, as indicated. (**B**) Anti-HA flow cytometic analyses of NIH3T3 and RAW264.7 cells expressing Mincle or Dectin-2, as indicated (**C**) Flow cytometric analyses of the various Mincle/Dectin-1 and Dectin-2/Dectin-1 chimeras in NIH3T3 and RAW264.7 cells, as indicated. Grey filled histograms represent vector control cells, solid black histograms represent surface expression, and dashed histograms represent total (intracellular and surface) expression.

Figure 4. CLECSF8 is a phagocytic receptor. (**A**) Cartoon representation of Janus, consisting of the CTLD of Dectin-1 coupled to full-length CLECSF8. (**B**) Flow cytometic analysis of Janus expression in transduced RAW264.7 and NIH3T3 cells. (**C**) Quantification of zymosan binding by RAW264.7 cells transduced with vector control, CLECSF8, Janus or Dectin-1, in the presence (grey bars) or absence (white bars) of β-glucan (βGlu), as indicated. RFU, relative fluorescence units. (**D**) FACS based phagocytosis assay showing the extent of zymosan internalisation (grey histograms) by RAW264.7 cells expressing Dectin-1, Janus or truncated Dectin-1. The bars indicate the percentage of cells with internalised zymosan. Cytochalasin D (CytoD; unfilled histograms) was included as a control to inhibit phagocytosis. (**E**) Fluorescence microscopy showing the interaction of TRITC-phallodin stained RAW264.7 cells expressing Dectin-1, Janus or truncated Dectin-1 with FITC-labelled zymosan (green particles). (**F**) Binding of anti-hCLECSF8 or isotype-coated FITC-dynabeads to RAW264.7 cells, as indicated. (**G**) Binding and internalization of anti-hCLECSF8 or isotype-coated FITC-dynabeads to peripheral blood neutrophils, in the presence (grey bars) or absence (white bars) of cytochalasin D (CytoD). Shown are the mean ± SD and the data are representative of at least three independent experiments. *, $p < 0.05$.

University of Cape Town

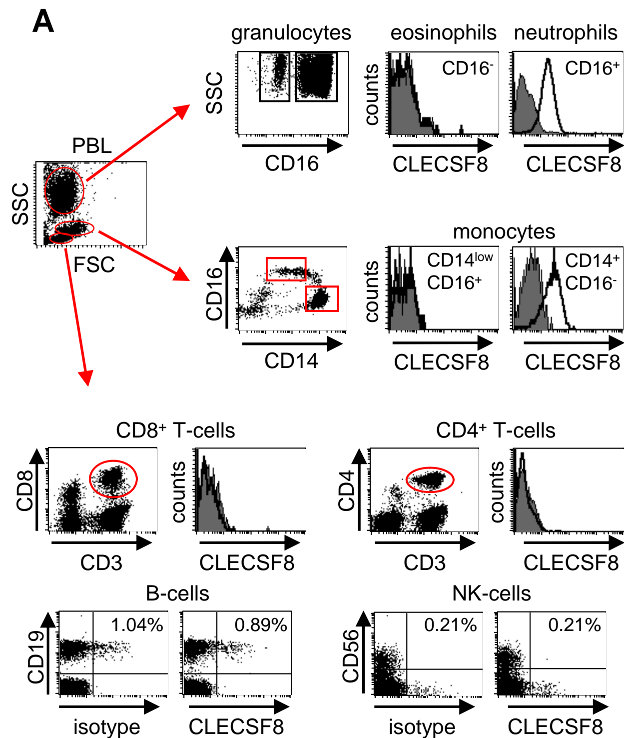
Figure 5. CLECSF8 can induce proinflammatory cytokine production. (**A**) Induction of TNF in RAW264.7 cells transfected with vector control, Janus or Dectin-1 following stimulation with zymosan (zy) in the presence (black bars) or absence (grey bars) of β-glucan (β-glu). Unstimulated (un) cells are included as a control. *, $p < 0.05$ compared to control cells. (**B**) TNF production by RAW264.7 cells transfected with Janus, stimulated with

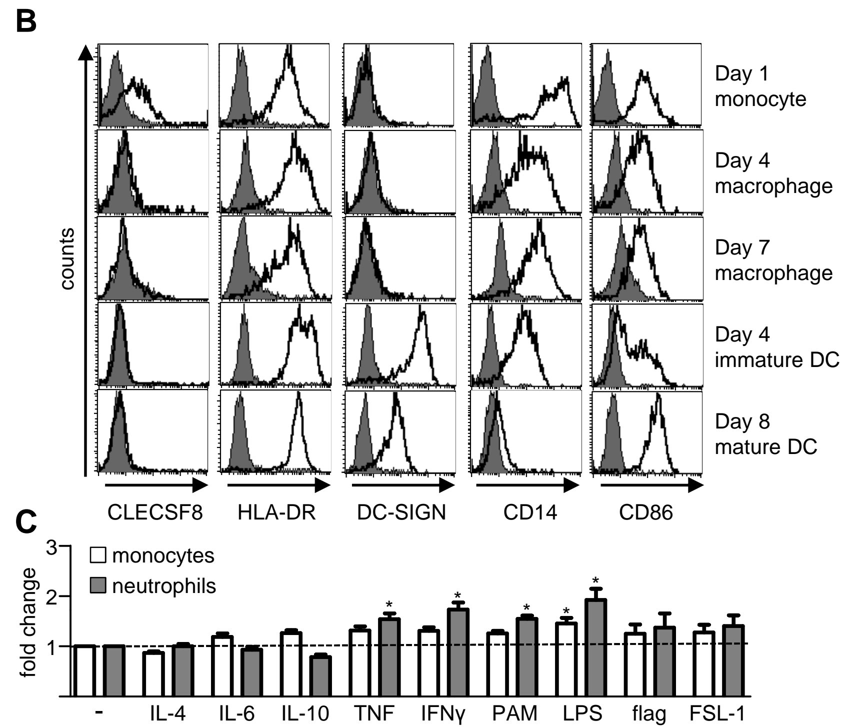
100μg/ml β-glucan or 10ng/ml LPS alone or in combination, as indicated. The data shown are mean ± SD and are representative of at least three independent experiments.

Figure 6. CLECSF8 can signal via Syk Kinase. (**A**) Characterisation of the effect of piceatannol on the ability of CLECSF8 to mediate phagocytosis in PMNs (white bars, see Figure 4) and cytokine production in Janus expressing RAW264.7 cells (grey bars, see Figure 5). The data shown are mean ± SEM normalized to the untreated cells and are pooled data from at least two independent experiments. *, $p < 0.05$ compared to control cells. (**B**) Western blots of anti-HA immunoprecipitates from RAW264.7 cells transduced with hCLECSF8, mCLECSF8 or Dectin-1. Cells were either unstimulated (un) or stimulated for 1 or 3 minutes with pervanadate. Blots were probed with anti-phosphotyrosine (α-pY), anti-Syk and anti-phospho-Syk (α-pSyk), as indicated.

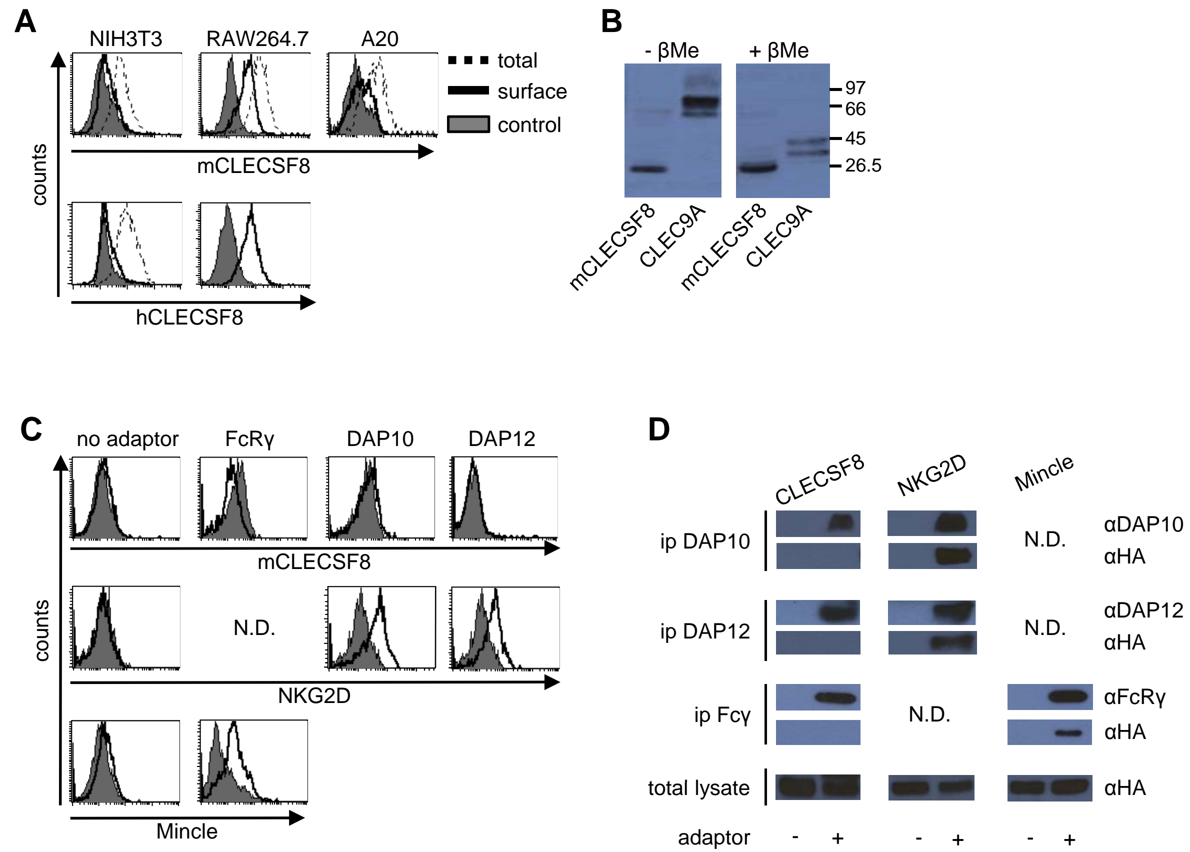
Figure 7. CLECSF8 can induce the respiratory burst. (**A**) RAW264.7 cells transfected with the vector control, Janus, mCLECSF8 or Dectin-1 were stimulated with zymosan for the indicated amount of time and the respiratory burst was measured by assessing the conversion of dihydrorhodamine 123 to rhodamine. (**B**) Stimulation of peripheral blood neutrophils for 30 minutes on anti-CLECSF8 or isotype coated plates. The data are expressed as mean fluorescent intensity (MFI) ± SEM of data pooled from two independent experiments. *, $p < 0.05$ versus control.

University of Cape Town

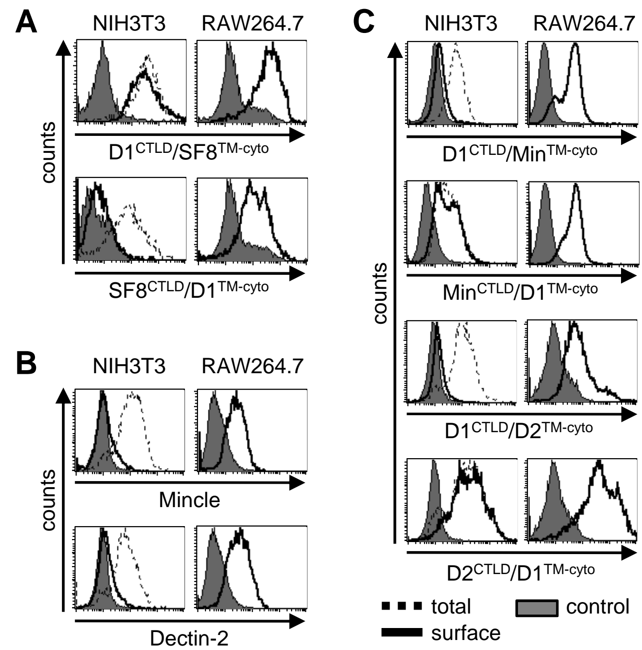




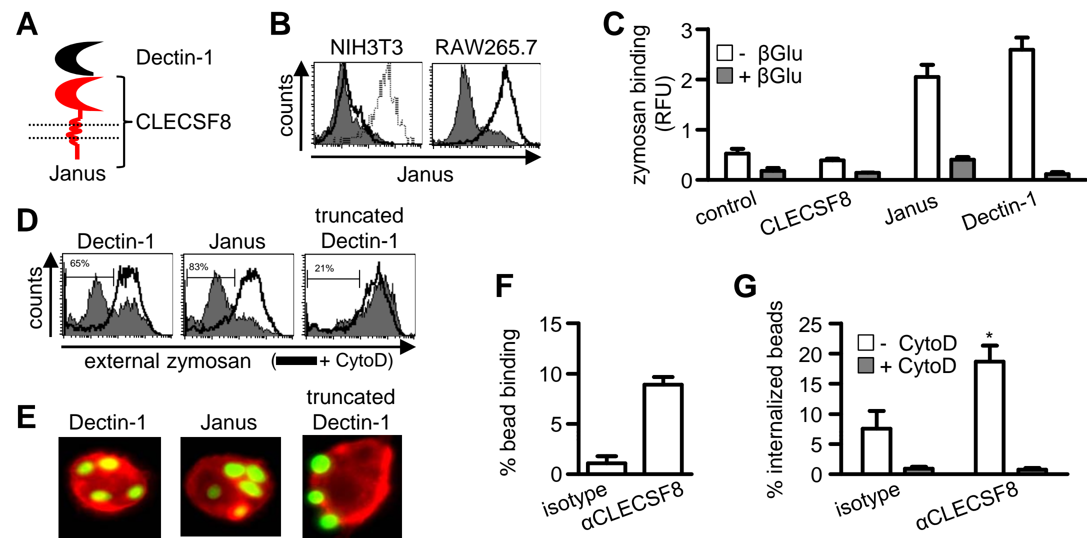
Graham et al. Figure 1



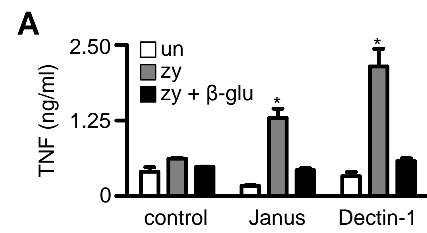
Graham et al. Figure 2

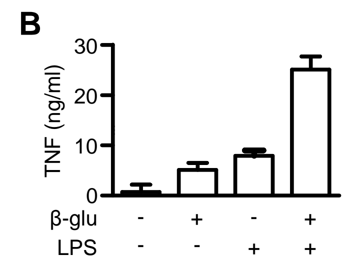


Graham et al. Figure 3



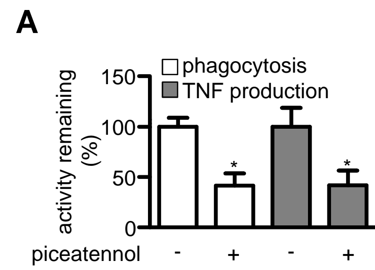
Graham et al. Figure 4



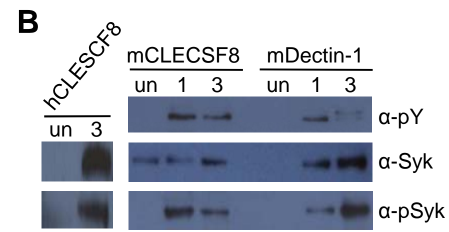


Graham et al. Figure 5

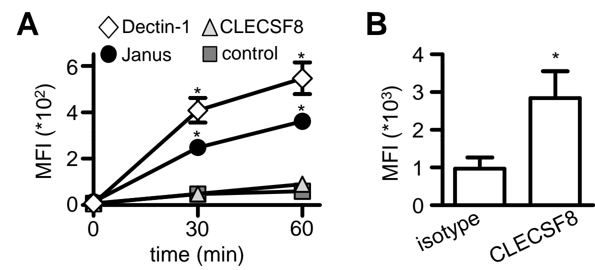
A



B



Graham et al. Figure 6



Graham et al. Figure 7

Supplemental Methods

Microarray analyses

Soluble Fc-chimeric proteins containing the CTLD of Dectin-1[1] and mCLESF8 (5'ggtaccatcattactttttacgc3' forward and 5'gaattcctcgagcttcgagggcttccaatt3' reverse) were generated by PCR and cloning into pSECTAG-Fc, essentially as described previously [1]. The oligosaccharide probes are lipid-linked (neoglycolipids or glycosylceramides). The repertoire of probe sequences was essentially as described (Supplemental Table 6 in Schallus et al 2008[2]), with the exception that the tri- and di-glucosylated high-mannose N-glycan sequences and certain chain lengths of the glucan oligosaccharide sequences are not featuring in the present analysis. Of note is that in an independent analysis (data not shown) these probes did not elicit binding signals with CLECSF8 or Dectin-1. The probes were printed in duplicate on nitrocellulose-coated glass slides at 2 and 5 fmol/spot. Binding signals at 5 fmol/spot (with error bars) are shown for 326 oligosaccharides arrayed, and were detected with the Fc-chimeric proteins as described previously [3]. The murine CLECSF8 and Dectin-1 were tested at 20 and 5 µg/ml, respectively.

Mice

C57BL/6 or CLECSF8$^{-/-}$ mice were obtained from the specific-pathogen-free facilities of the Universities of Cape Town or Aberdeen. The CLECSF8$^{-/-}$ mice were generated on a C57BL/6 background by the Consortium for Functional Glycomics (CFG). Full details of the generation of these animals and their phenotypic analysis can be obtained from the following CFG websites:

https://www.functionalglycomics.org/static/consortium/resources/DataCoreFmcl.shtml

https://www.functionalglycomics.org/glycomics/publicdata/phenotyping.jsp

All animal experimentation conformed to institutional guidelines for animal care and welfare. All experiments utilized at least 6 mice per group.

University of Cape Town

Infection with *Candida Albicans*

C. albicans SC5314 was streaked onto on Sabouraud dextrose (SAB) agar for isolation of individual colonies. Colonies were inoculated into 5ml SAB broth and cultured in a shaking incubator for 24h culture at 30°C. Cultures were washed twice with PBS and diluted to the required concentration. Eight to ten week old female wild-type or CLECSF8$^{-/-}$ mice were anaesthetized intraperitoneally and weighed before infection. Live *C. albicans* ($1x10^5$ CFU) was administered intravenously in a final volume of 100µg/ml PBS. Mice were weighed and monitored daily and were sacrificed once they had lost 20% of their body weight or if they became moribund, as determined by an animal welfare scoring sheet. Experiments continued for a maximum of 21 days.

Infection with *Listeria monocytogenes*

L. monocytogenes strain EGD was inoculated into tryptic soy broth (TSB) and incubated in a shaking incubator overnight at 37°C. The overnight culture was diluted 1/10 in TSB and allowed to grow with agitation at 37°C until an OD of 0.5 was reached. The culture was washed twice with PBS and diluted to the required concentration. Wild-type and CLECSF8$^{-/-}$ female mice (9-10 weeks old) were infected intraperitoneally with 2x10^{6} CFU/mouse in 0.2ml PBS. Mice were weighed and monitored daily and were sacrificed once they had lost 20% of their body weight or if they became moribund, as determined by an animal welfare scoring sheet. Experiments continued for a maximum of 21 days.

Infection with *Staphylococcus aureus*

S. aureus was inoculated into 5ml Luria Bertani (LB) medium and incubated with shaking at 37°C overnight. 1 ml of culture was inoculated into 1l LB medium and cultured for a further 6 hr before washing twice with PBS and storing at -80°C. The CFU concentration was determined by serial dilution on LB agar and stocks were diluted in PBS to the required concentration. Male wild-type and CLECSF8$^{-/-}$ mice (10-11 weeks old) were infected intravenously with 2x10^{7} CFU *S. aureus* in 100µl PBS. Mice were weighed and monitored daily and were sacrificed once they had lost 20% of their body weight or if they became moribund, as determined by an animal welfare scoring sheet. Experiments continued for a maximum of 21 days.

University of Cape Town

Infection with *Nippostrongylus brasiliensis*

Male and female wild-type and CLECSF8$^{-/-}$ mice (7 weeks old) were infected subcutaneously with 750 *N. brasiliensis* L3 larvae and killed 10 days post infection. Following this intestinal worm burdens were quantified by direct counting from dissected small intestines. Serum IgE levels were analysed by ELISA.

Sterile peritonitis

To induce sterile peritonitis CLECSF8$^{-/-}$ and wild-type mice were injected intraperitoneally with 1ml of 4% thioglycollate broth, 18 or 96 hours prior to peritoneal lavage. Inflammatory cells were collected by peritoneal lavage with ice-cold 5mM EDTA in PBS. FACS was performed according to conventional protocols at 4°C, as described [4]. Antibodies used were F4/80-PE (Serotec), Ly6G-PE, CD11b-FITC, 7/4-biotin and streptavidin-APC (BD).

Peritoneal inflammation in response to necrotic EL4 cells

EL4 cells were heat-shocked to induce necrosis as previously described [5]. Mice (11-12 weeks old) were injected intraperitonaelly with $7x10^6$-$1x10^7$ necrotic EL4 cells in 0.15ml PBS and 16 hours after challenge the numbers of peritoneal cells and percentage of neutrophils were analysed as described [5].

Neutrophil infiltration induced by thymocyte death

Female mice (9-10 weeks old) were whole body irradiated with X-rays (4Gy). The thymus was removed 12 hours post irradiation and the percentage of thymic neutrophils was analysed by flow cytometery using CD11b-FITC and Ly6G-PE (BD Pharmingen).

Induction of EAU

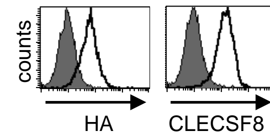
Experimental autoimmune uveoretinitis (EAU) was induced in 7 week old C57BL/6 and seven CLECSF8$^{-/-}$ mice by immunisation subcutaneously in the hind limbs with 500µg of interphotoreceptor retinal binding peptide 1–20 (GPTHLFQPSLVLDMAKVLLD; Sigma-Aldrich, Cambridge, UK) emulsified in complete Freunds's adjuvant, and supplemented with 2.5 mg/ml *Mycobacterium tuberculosis* strain H37Ra (H37Ra, Difco Laboratories, Detroit, MI, USA). 1µg Bordetella pertussis toxin was administered intraperitoneally at the time of immunisation [6]. All procedures adopted conformed to the Home Office Regulations for Animal Experimentation (UK) and to the Association for Research in Vision and Ophthalmology Statement for the use of Animals in Ophthalmic and Vision Research. After 28 days the eyes were embedded in OCT compound and frozen sections stained with haematoxylin for histological scoring of disease severity according to the method of Caspi et al. [7].

University of Cape Town

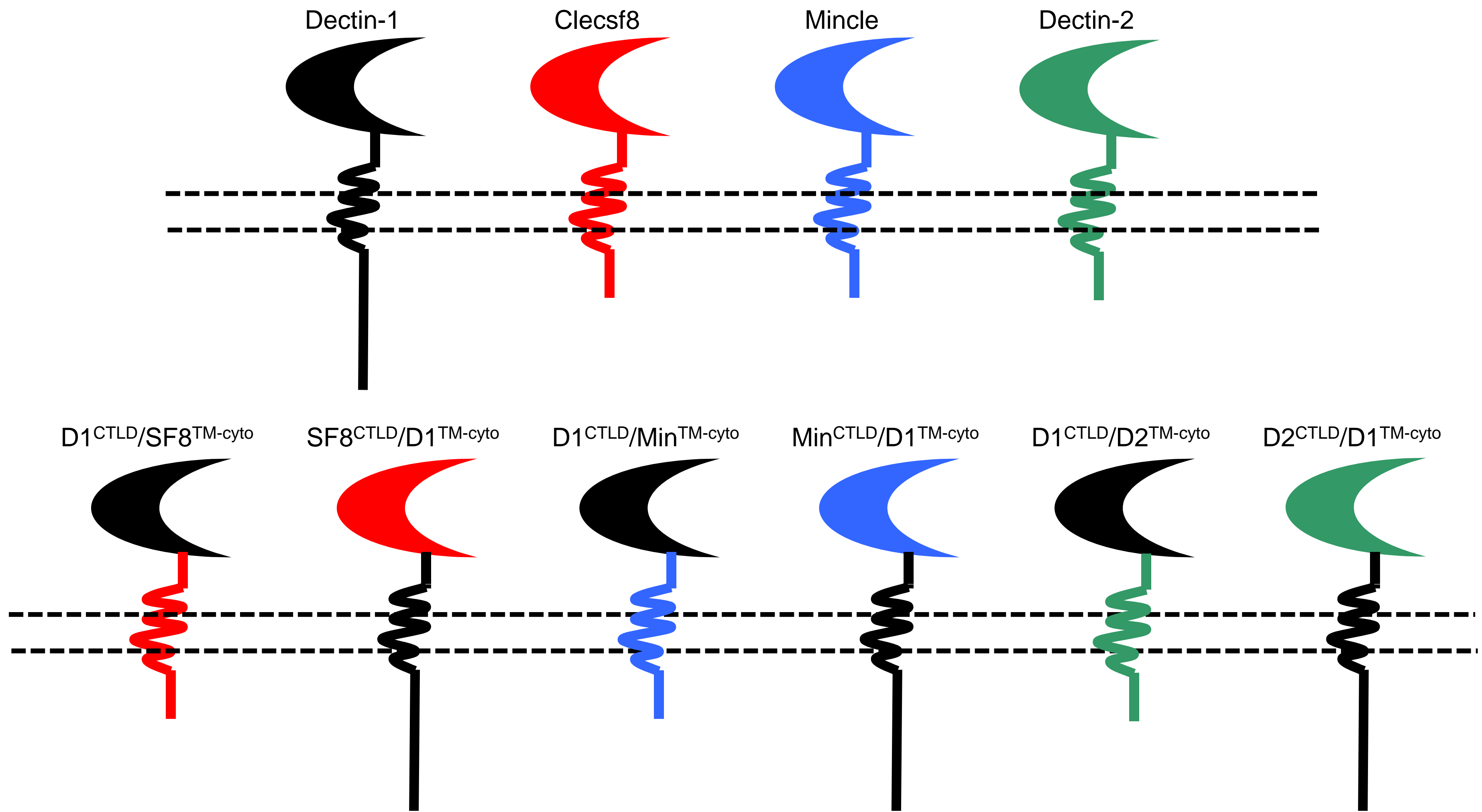
Supplemental References

1. Graham LM, Tsoni SV, Willment JA, et al. Soluble Dectin-1 as a tool to detect beta-glucans. J Immunol Methods. 2006;314:164-169.
2. Schallus T, Jaeckh C, Feher K, et al. Malectin: a novel carbohydrate-binding protein of the endoplasmic reticulum and a candidate player in the early steps of protein N-glycosylation. Mol Biol Cell. 2008;19:3404-3414.
3. Palma AS, Feizi T, Zhang Y, et al. Ligands for the beta-glucan receptor, Dectin-1, assigned using "designer" microarrays of oligosaccharide probes (neoglycolipids) generated from glucan polysaccharides. J Biol Chem. 2006;281:5771-5779.
4. Taylor PR, Brown GD, Geldhof AB, Martinez-Pomares L, Gordon S. Pattern recognition receptors and differentiation antigens define murine myeloid cell heterogeneity ex vivo. Eur J Immunol. 2003;33:2090-2097.
5. Chen CJ, Kono H, Golenbock D, Reed G, Akira S, Rock KL. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. Nat Med. 2007;13:851-856.
6. Avichezer D, Silver PB, Chan CC, Wiggert B, Caspi RR. Identification of a new epitope of human IRBP that induces autoimmune uveoretinitis in mice of the H-2b haplotype. Invest Ophthalmol Vis Sci. 2000;41:127-131.
7. Caspi RR, Roberge FG, Chan CC, et al. A new model of autoimmune disease. Experimental autoimmune uveoretinitis induced in mice with two different retinal antigens. J Immunol. 1988;140:1490-1495.

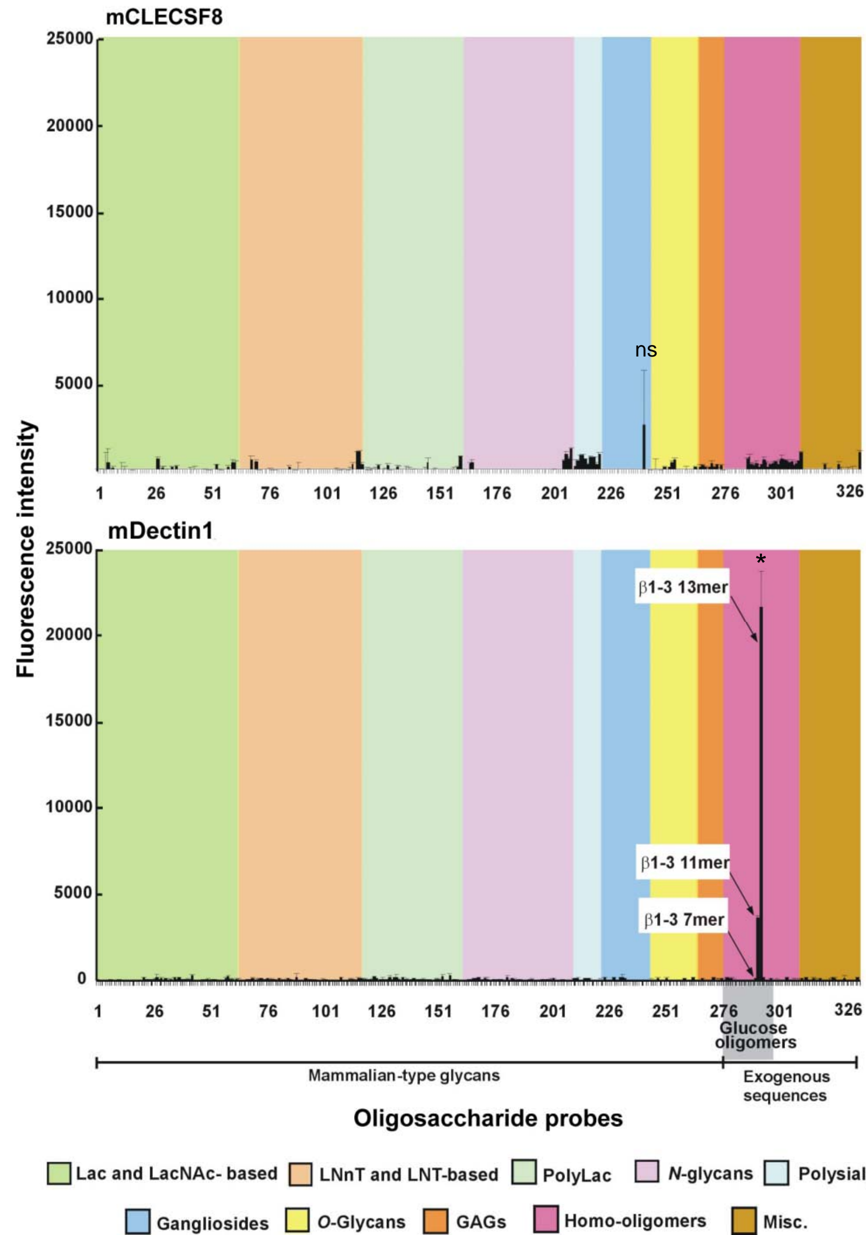
University of Cape Town



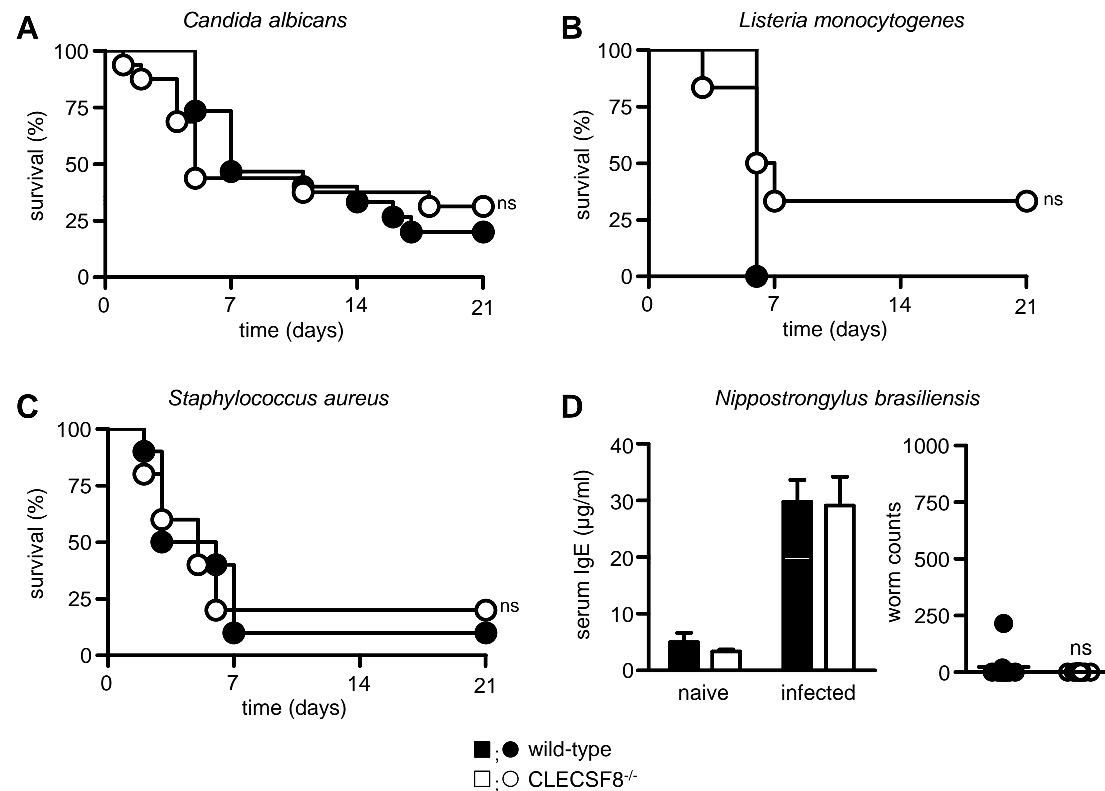
Supplemental Figure 1. Specificity of the anti-hCLECSF8 antibody. Flow cytometric analyses of RAW264.7 cells transduced with HA-tagged hCLECSF8 and stained with anti-HA or anti-hCLECSF8 (unfilled histogram). The vector only control cells are presented as filled histograms.



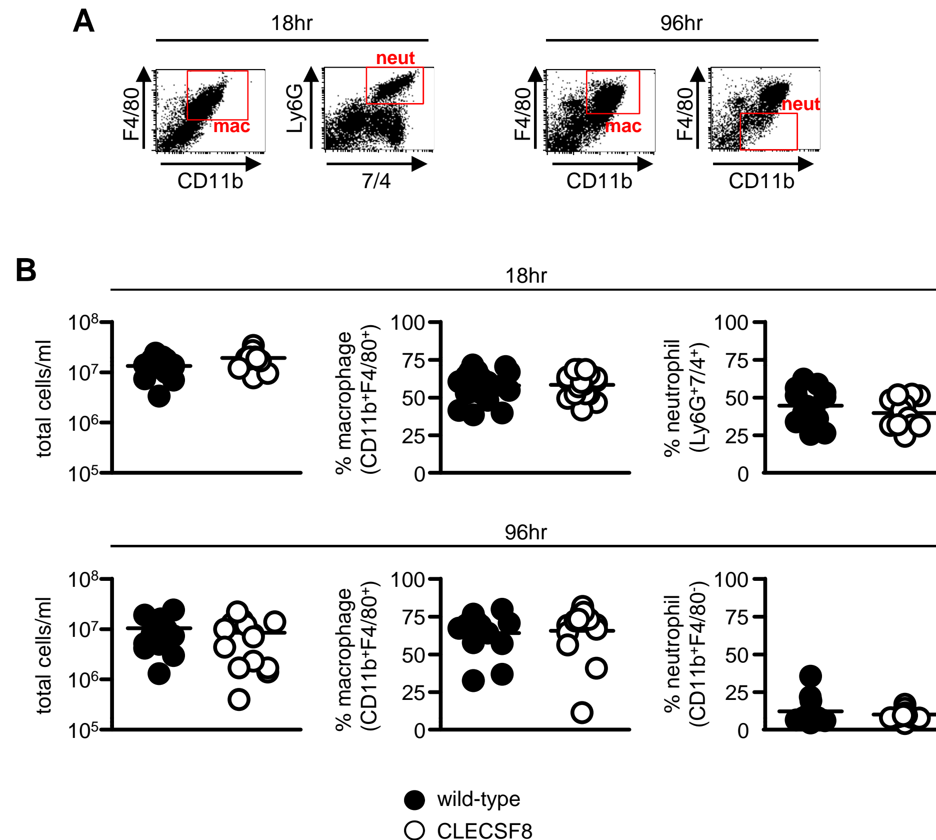
Supplemental Figure 2. Cartoon representations of the C-type lectin receptors and the chimeras used in these experiments.



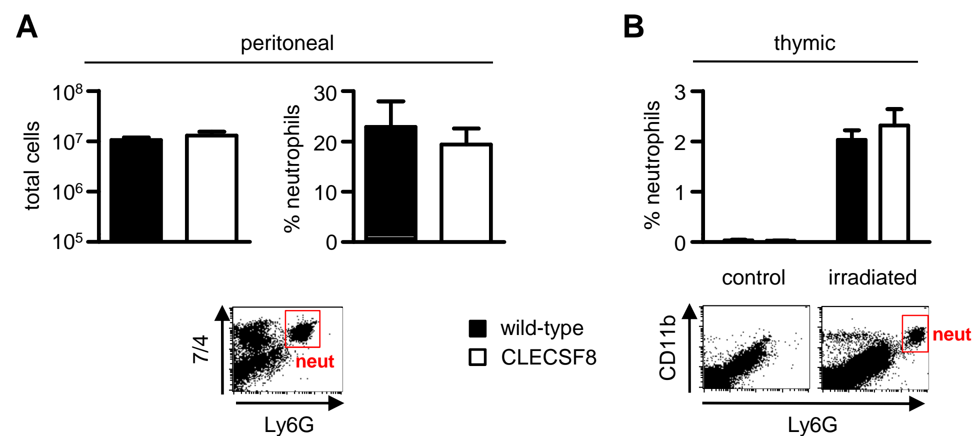
Supplemental Figure 3: Carbohydrate microarray analyses of ligands for mCLECSF8 and mDectin-1. Carbohydrate microarrays were probed with soluble mCLECSF8- and mDectin-1-Fc chimeric proteins, as described in the supplemental materials and methods. Binding signals are expressed as numerical scores, means of fluorescence intensities of duplicate values recorded at 5 fmol oligosaccharide probe/spot. The error bars represent half of the difference between the two values. None of the oligosaccharide probes included in the microarrays elicited significant binding signals with CLECSF8. This is in contrast with Dectin-1, which gave binding to β1-3-linked glucose sequences (11 and 13mers), as expected. 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, polylactosamine; GAGs, glycosaminoglycans; and Misc., miscellaneous.



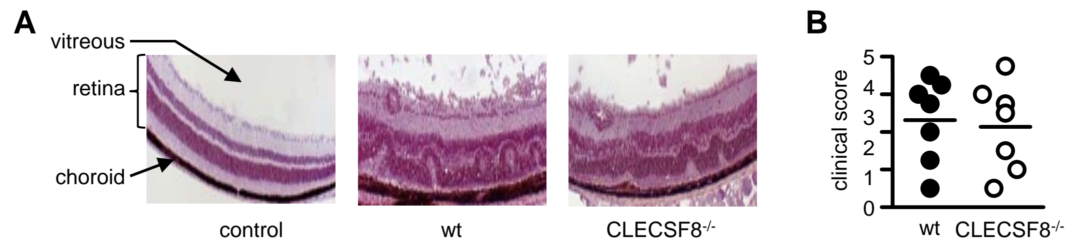
Supplemental Figure 4. Characterisation of various infection models in CLECSF8$^{-/-}$ mice. (**A**) Survival curves of wild-type (n=15) and CLECSF8$^{-/-}$ (n=16) mice infected intravenously with 1x10^5 CFU *C. albicans*. (**B**) Survival curves of wild-type and CLECSF8$^{-/-}$ mice (n=6) infected intraperitoneally with 2x10^6 CFU *L. monocytogenes.* (**C**) Survival curves of wild-type (n=6) and CLECSF8$^{-/-}$ (n=7) mice infected intravenously with 2x10^7 CFU *S. aureus.* (**D**) Ten days post infection with 750 L3 *N. brasiliensis* larvae, total serum IgE levels of naïve and infected wild-type and CLECSF8$^{-/-}$ mice were determined by ELISA. The number of adult worms in the intestine was determined for individual mice.



Supplemental Figure 5. CLECSF8$^{-/-}$ and wild-type mice display similar levels of cellular recruitment during sterile peritonitis. (**A**) The gating strategy used to identify neutrophils and macrophages 18 and 96 hours after thioglycollate induced sterile peritonitis. (**B**) Scatter plots of total peritoneal cells/ml of lavage and percentage of neutrophils and macrophages from the peritoneal cavities of CLECSF8$^{-/-}$ (open circles) and wild-type mice (black circles), 18 and 96 hours after induction of peritonitis. Data shown are from 13 mice/group, pooled from two independent experiments



Supplemental Figure 6. CLECSF8$^{-/-}$ and wild-type mice display similar levels of neutrophil recruitment in response to necrotic cell death. (**A**) Total recruited peritoneal cell numbers and the percentage of neutrophils from CLECSF8$^{-/-}$ (white bars) and wild-type (black bars) mice 16 hours after intraperitoneal challenge with necrotic EL4 cells. A representative dot blot of Ly6G and 7/4 expression on peritoneal cells used to identify neutrophils is shown below. Data shown are from nine mice/group, pooled from two independent experiments. (**B**) Neutrophil infiltration into the thymus of CLECSF8$^{-/-}$ (white bars) and wild-type (black bars) control or irradiated mice, as indicated. The bar graph shows percent neutrophils in the thymus of 15 mice pooled from three independent experiments. Representative dot blots showing the CD11b^{+} Ly6G^{+} recruitment of neutrophils into the thymus of irradiated mice.



Supplemental Figure 7: Characterisation of EAU in wild-type (wt) and CLECSF8$^{-/-}$ mice. (**A**) Haematoxylin stained frozen sections of wt and CLECSF8$^{-/-}$ eyes show similar features of EAU including retinal folds, vitritis, vasculitis and inflammatory cell infiltration of the retinal layers. Images were captured using the x10 objective. (**B**) Scoring of multiple sections per eye did not reveal any statistically significant different between wt and CLECSF8$^{-/-}$ mice.