Characterisation of the C-type lectin receptor CLEC-2: expression, ligands and functions

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Declaration

I Diego Souto Maior Mourão Sá confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Myeloid cells express a plethora of C-type lectin receptors (CLR) that can regulate inflammatory responses. Dectin-1 belongs to a sub-family of CLRs that possesses an extracellular C-type lectin domain (CTLD) and a single YxxL intracellular motif (hemITAM) that allows signalling via Syk kinase and induction of downstream functions. Based on consensus sequences for the CTLD and hemITAM, we identified CLEC-2 as a dectin-1-like receptor. CLEC-2 was previously characterised as a Syk-coupled platelet receptor able to induce platelet aggregation when targeted by the snake venom rhodocytin and by cells expressing the endogenous protein podoplanin. I generated monoclonal antibodies against mouse CLEC-2 and found that CLEC-2 is also expressed on lymphoid and myeloid cells, including dendritic cells (DC). Notably, treatment with LPS increases CLEC-2 expression by myeloid cells and synergises with CLEC-2 signaling to induce increased secretion of IL-10 but not IL-12. This increased IL-10 production is also observed in the serum of mice administered with anti-CLEC-2 mAb and LPS, and is dependent on the presence of macrophages and DCs. Furthermore, I generated a CLEC-2 conditional KO mouse line that will provide a tool to study CLEC-2 function in myeloid cells in vivo. Collectively, these data indicate that CLEC-2 expression is not restricted to platelets and that it plays a role on the vascular development and modulation of TLR responses.

Acknowledgement

The completion of this thesis was only possible with the unconditional support of my family throughout all my studies. This thesis is dedicated to my future wife Renata Cavalieri, which always supported and pushed me forward during the difficulties.

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Abbreviations

APC	Antigen Presenting cell
BMDC	Bone-marrow derived dendritic cell
DC	Dendritic cell
FACS	Fluorescence Activated Cell Sorting
GM-CSF	Granulocyte Monocyte-Colony Stimulating Factor
FLT3L	FMS-like tyrosine kinase 3 ligand
IFN	Interferon
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
MAP	Mitogen-Activated Protein
MHC	Major Histocompatibility complex
ROS	Reactive Oxygen Species
Th1	Type 1 helper cell
Th17	Type 17 helper cell
TNF	Tumour Necrosis Factor
CD	Cluster of differentiation
ELISA	Enzyme-linked immunosorbent assay

Chapter 1. Introduction

1.1 Inflammation

Inflammation is a multi-step process by which organisms restore tissue homeostasis. This inflammatory response was characterised around 3000 years ago, as a process comprising 4 cardinal signs: redness, swelling, heat and pain. More recently, the loss of function of the tissue was added as the fifth cardinal sign of inflammation, and is the only sign that appears in all inflammatory processes. The physiological basis for these cardinal signs was described later, as a result of changes to blood flow followed by extravasation of blood components (leucocytes and plasma components) to the inflamed tissue (Medzhitov 2010).

The inflammatory response is an ancient conserved process present in all multicellular organisms. As such, the innate immune system plays important role in inflammation. Even more considering that the adaptive immune system is evolutionary “younger” and only present on jawed fishes and vertebrates. However, in vertebrates the adaptive immune system was shown to contribute to the inflammatory process as a mechanism dependent of activation by the innate immune system (Iwasaki and Medzhitov 2010).

The inflammatory response is initiated by changes in tissue homeostasis, which include different noxious conditions such as infection, trauma, toxic agents and metabolic changes. As a consequence, production of inflammatory mediators and leucocyte mobilisation happens, which subsequently leads to the elimination of the insult with tissue repair and restoration of homeostasis (see figure 1.1) (Wahl et al. 1997).

As a multi-step process, each inflammatory step is sequentially controlled and dependent on each other. For example, the resolution of inflammation is achieved by restoration of tissue homeostasis with the effective elimination of the inflammatory trigger. Together with the elimination of the insult, the restoration of tissue homeostasis needs to be completed by the removal of the recruited leucocytes. This process is greatly dependent on the phagocytosis of dead leucocytes and the emigration of phagocytes from the tissue, with several

molecular mediators involved. A significant example is the change of production of prostaglandins to the production of lipoxins. Prostaglandins are mediators that can induce vessel dilation favouring leucocyte recruitment, while the lipoxins can induce the phagocytosis of apoptotic leucocytes, reduce the vascular permeability and promote the emigration of phagocytes from the tissue via the lymphatics (Serhan and Savill 2005). An important aspect of this phagocyte emigration from the tissue is its capacity to reach secondary lymphoid organs and activate the adaptive immune system (Luster et al. 2005). Activated lymphocytes migrate to inflamed tissue where they contribute to elimination of the inflammatory trigger and resolution of inflammation.

Because of the sequential nature of the inflammatory response, the detection of the inflammatory trigger constitutes its very first step and is crucial to determine the subsequent steps and types of reactions.

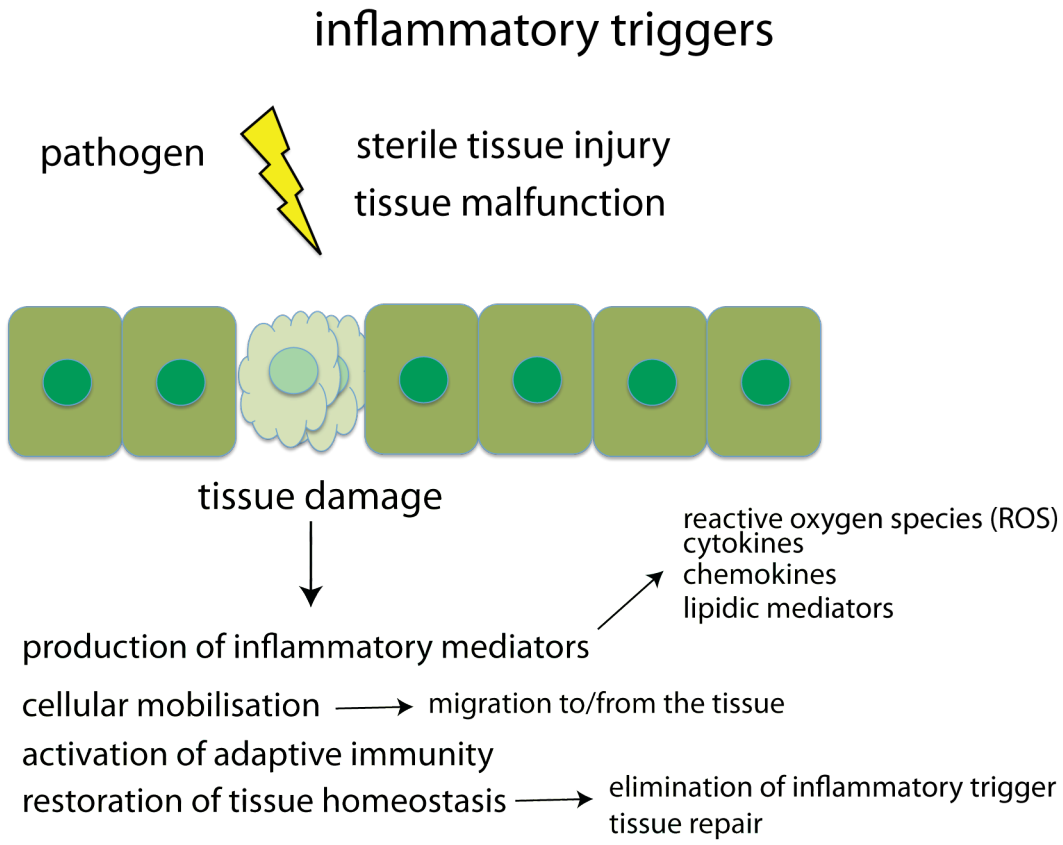

Figure 1.1 Inflammation
Inflammatory triggers commonly leads to tissue damage and initiate inflammation. This process is initiated by the production of several inflammatory mediators, which culminates in cellular mobilisation, activation of adaptive immunity and restoration of tissue homeostasis.

1.2 Detectors of inflammatory triggers

Since pathogens are one of the best characterised inflammatory triggers, it was proposed by Charles Janeway that the innate immune system would express a set of receptors that could recognise conserved features present on pathogens, and absent on endogenous cells, and initiate an inflammatory response that would culminate in the activation of adaptive responses (Janeway 1989). The term pattern recognition receptor (PRR) was proposed to this set of germ-line encoded receptors that would recognise pathogen-associated molecular pattern (PAMP) (Janeway 1989). However as Polly Matzinger pointed out, tissue damage in the absence of infectious agents is capable of activation of innate immune cells (dendritic cells) that leads to the activation of adaptive immunity. Therefore, one of the functions of the innate immune system is to detect tissue damage (Matzinger 1994). Furthermore, Matzinger proposed a model whereby tissue damage would release, expose or induce components that would trigger inflammation (Matzinger 1998). As a matter of fact, it is now clear that the innate immune system expresses receptors that can recognise both PAMPs as well as damage-associated molecular patterns (DAMPs) to initiate the inflammatory response (Seong and Matzinger 2004). Among these innate receptors, there are four families of receptors that stand out from the rest according to their ability to detect and modulate inflammatory responses through regulation of gene expression: the Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), Nod-like receptors (NLRs) and the signalling C-type lectin receptors (CLRs).

The TLRs constitute the first family of receptors identified with a role in detection and initiation of inflammation, and their role in pathogen recognition and regulation of gene expression has been extensively studied. Besides the TLRs involvement in PAMPs recognition, these receptors are also involved in the recognition of several DAMPs (Yu et al. 2010) (see table 1). The TLRs are a family of receptors that contain an extracellular leucin-rich repeat (LRR) domain, a transmembrane domain and an intracellular motif denominated Toll/IL-1 Receptor (TIR) domain. This family of receptors interacts with its

ligands via the LRR and signals to regulate gene expression by recruiting Myd88 and/or TRIF through its TIR domain that results in the activation of several downstream events (Takeda et al. 2003).

Table 1 Toll-like receptors and its ligands

TLR	LOCALISATION	LIGAND	ORIGIN OF THE LIGAND
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus, self
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7	Endolysosome	ssRNA	Virus, bacteria, self
Human TLR8	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa

The RLRs were identified based on its founding member retinoic acid inducible gene I (RIG-I), a helicase that triggers type I interferon and others genes by activating several transcription factors. This receptor family, besides its helicase domain, can express a caspase activation and recruitment domain (CARD) that allows activation of transcription factors by coupling with the adaptor protein mitochondrial antiviral signalling (MAVS). The RLRs are found on the cytoplasm of almost all nucleated cells and signals to gene induction upon recognition of viral nucleic acid (Rehwinkel and Reis e Sousa 2010). Thus it is a family of receptors involved in the detection and initiation of inflammation upon virus infection (table 2).

Table 2 RIG-I-like receptors and its ligands

RLR	LOCALISATION	LIGAND	ORIGIN OF THE LIGAND
RIG-I	Cytoplasm	5' triphosphate RNA short dsRNA,	RNA viruses, DNA virus
MDA5	Cytoplasm	RNA with secondary structure	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses

Another family of innate receptors that is involved in the detection of inflammatory triggers are the NLRs. This family is constituted of proteins that contain a nucleotide binding and oligomerization (NACHT) domain as well as LRR and CARD domains. The LRR is believed to be involved in ligand recognition (as for TLRs) as well as in regulatory functions. The CARD is involved on interaction with the signalling adaptor RIP2 leading to signalling downstream events, including gene induction. NLRs are so called because of its first member: nucleotide-binding oligomerization domain 1 (NOD1). As cytoplasmic proteins expressed by several cell types, they can recognise intracellular bacterial infection and signal for initiation of inflammation (Fritz et al. 2006) (see table 3). Within the NLRs, there is a sub-group of receptors that have a pyrin domain (PYD) in addition to the other domains or replacing the CARD, and can stimulate a protein complex called inflammasome. The inflammasome causes the activation of caspase-1, which cleaves interleukin (IL) 1β and IL-18. The latter are precursors of inflammatory cytokines produced as inactive pro-form proteins. The most studied member of the NLR family that activates the inflammasome is NLRP3. It is activated by stimuli that lead to the production of reactive oxygen species, K^+ efflux and/or lysosomal rupture (Schroder and Tschopp 2010). Thus, NLRP3 is largely considered a sensor of metabolic changes within cells, able to trigger the production of important inflammatory mediators in response to changes in cellular homeostasis (Schroder et al. 2010).

Table 3 NOD-like receptors and its ligands

NLR	LOCALISATION	LIGAND	ORIGIN OF THE LIGAND
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
NRLP3	Cytoplasm	?	?

The CLR family contains some members identified that signal and modulate inflammatory responses, constituting the latest family of innate receptors involved in the detection of inflammatory triggers. This is, in great part, due to the discovery that the β-glucan receptor dectin-1 is able to link the detection of fungi and bacteria to production of inflammatory mediators independently of other receptors (Rogers et al. 2005; Underhill et al. 2005). The CLR family encompasses all proteins that contain at least one C-type lectin domain (CTLD), which is a structural motif that was primarily used to describe carbohydrate-binding proteins, but now is known to be involved in interactions with other molecules as well (Drickamer 1999). The CLRs include a large family of receptors, where only a few are membrane bound proteins with identified signalling domains and functions, thus the usage of the term signalling CLRs. I will describe here some of the features of these signalling CLRs and how they are involved in the modulation of inflammatory responses. On figure 1.2 is depicted how these four distinct families of receptor are located and can signal in a cell.

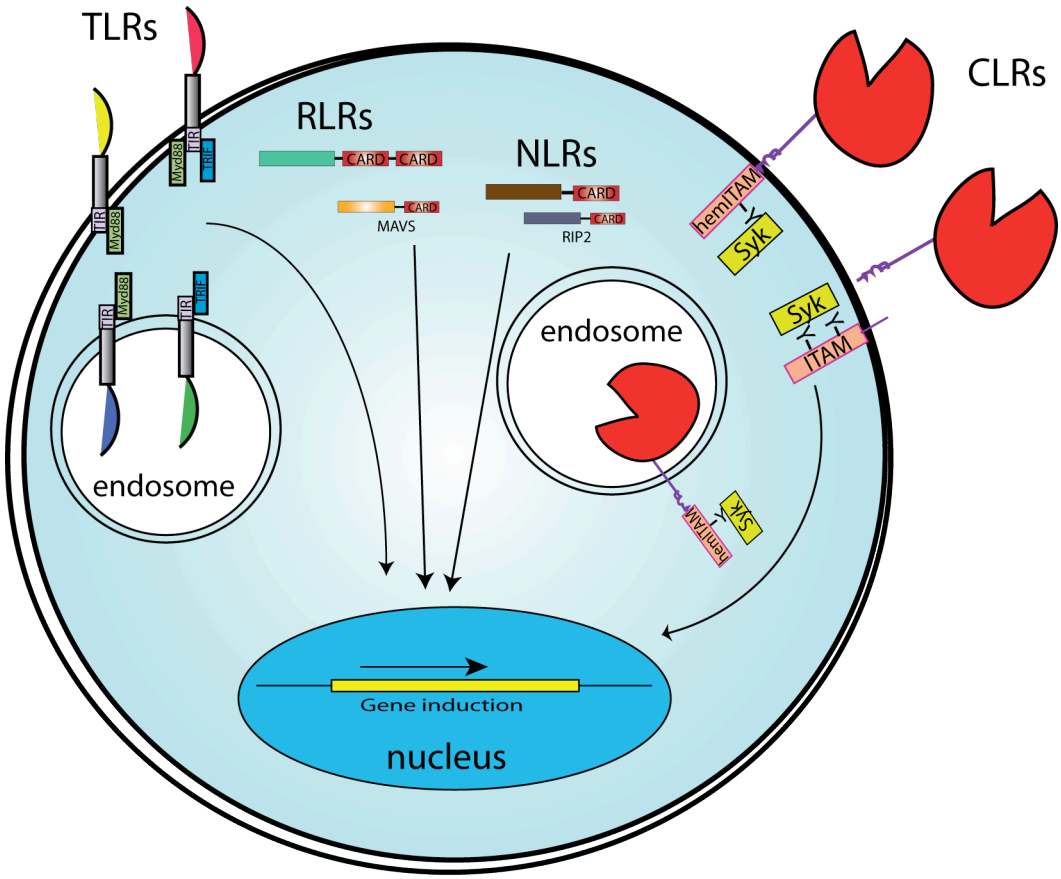

Figure 1.2 Detectors of inflammatory triggers
Innate receptor families that detect inflammatory triggers and can signal to initiation of inflammation by gene induction. TLRs and CLRs are located on plasma membrane and endosomes. NLRs and RLRs are on the cytoplasm. TLRs can signal via its TIR domain by using MyD88 and/or Trif adaptor molecules. CLRs use hemITAM or ITAM-coupled adaptor proteins to signal via Syk. RLRs use its CARD domain to homotypic interact with CARD domain in the signalling molecule MAVS. NLRs also uses its CARD domain to homotypic interacts and activate RIP2 signalling molecule.

1.3 Signalling CLRs

The signalling CLR family comprises membrane-bound proteins that contain a CTLD. Among them are CLRs with identified and unidentified cytoplasmic signalling motifs and proteins with very short cytoplasmic tails that can interact and signal via receptor-associated transmembrane adaptors. The described signalling that originates from these CLRs can be characterised regarding their structural identified components: tyrosine-based and non-tyrosine-based signalling.

1.3.1 Non tyrosine-based signalling

For this group of signalling CLRs there are many proteins that mediate endocytosis of its ligands. Despite these endocytic CLRs, others have been show to modulate gene expression, which have direct implication on the initiation and regulation of inflammatory responses. This is so for DC-SIGN, which was previously implicated in diversion of its several pathogens ligands to non-degradative sub-cellular compartments (Van Kooyk and Geijtenbeek 2003). Recently, this receptor was shown to modulate TLR responses, inducing modulation of NFκB via Raf-1 signalling. In spite of activation of Raf-1 through Ras, no signalling motif on DC-SIGN has been identified to mediate Ras-Raf1 activation (Gringhuis et al. 2007). In addition to DC-SIGN, dectin-1 was also reported to modulate TLR responses via activation of non-canonical NFκB subunits through Raf1. However, as for DC-SIGN, no signalling motif on dectin-1 was identified (Gringhuis et al. 2009).

Among the CLRs that mediate endocytosis there is DEC-205 (CD205) (Mahnke et al. 2000) and mouse DCIR2 (Dudziak et al. 2007). Importantly, these CLRs have been implicated on carrying their ligands to specialized compartments that favour antigen presentation and thus can regulate activation of adaptive immunity. Nevertheless, none of these CLRs have been shown to signal via ligand-dependent triggering or to possess a signalling motif-dependent function. Hence, it is possible that the compartmentalization of the ligand by these CLRs is independent of signalling.

Another receptor that is implicated in endocytosis and antigen presentation the mannose receptor (MR/CD206) (Burgdorf et al. 2007). Besides MR role in endocytosis, the triggering of MR by antibodies or by pathogen-derived molecules suggests that MR can modulate cell activation (Nigou et al. 2001; Chieppa et al. 2003; Zhang et al. 2004; Zhang et al. 2004). Despite that, no signalling motif in MR has been identified and implicated in cellular activation.

1.3.2 Tyrosine-based signalling

The main evidence for signalling downstream of some CLRs came from identification of the signalling mechanism used by dectin-1 (CLEC7A).

Previously, the dectin-1 tail had been shown to increase TLR-induced cytokine production by macrophages and dendritic cells (DCs) stimulated with zymosan, a cell wall extract from *Saccharomyces cerevisiae* (Brown et al. 2003; Gantner et al. 2003). Later, Rogers and colleagues showed that dectin-1, in fact, could couple recognition of PAMPs to induction of cytokines independently of TLRs via activation of spleen tyrosine kinase (Syk) signalling through its intracellular tail (Rogers, Slack et al. 2005). Accordingly, Underhill and colleagues also found that dectin-1 on macrophages could signal via Syk to generate reactive oxygen species (ROS), but not to promote phagocytosis of zymosan (Underhill, Rossnagle et al. 2005).

Subsequently to the identification of Syk as a signalling molecule downstream of innate receptors, many other CLRs have been demonstrated to signal via Syk. The great majority of these signalling CLRs had a very short cytoplasmic tail with no clear signalling motif. However, these CLRs were able to interact with immunoreceptor-tyrosine activatory motif (ITAM) membrane adaptors (FcRγ and DAP12) and to signal to downstream effects via Syk (Underhill and Goodridge 2007). This is the case for DCAR (CLEC4B) that can signal via FcRγ to increase intracellular calcium and induction of tyrosine phosphorylation when cross-linked with antibodies (Kanazawa et al. 2003). Mincle (CLEC4E) has also been demonstrated to couple to Syk signalling via FcRγ, inducing tyrosine phosphorylation, mitogen- activated protein (MAP) kinase, NFAT activation and cytokine production by macrophages (Yamasaki et al. 2008). Interestingly, DAMPs (Yamasaki, Ishikawa et al. 2008) and PAMPs (Wells et al. 2008; Ishikawa et al. 2009; Yamasaki et al. 2009; Yamasaki et al. 2009) can stimulate cells via Mincle. Another CLR that signals via FcRγ and Syk is dectin-2, which was reported to stimulate MAP kinase activation as well as cytokine (Robinson et al. 2009) and leukotriene production (Barrett et al. 2009) upon interaction with fungi and house dust mite, respectively. Likewise, BDCA-2 (CLEC4C/CD303) when cross-linked with antibodies can couple to FcRγ and activate Syk (Röck et al. 2007), tyrosine phosphorylation and calcium signalling (Dzionek et al. 2001). Nevertheless, BDCA-2 signalling was reported to inhibit TLR-induced cytokine production (Dzionek, Sohma et al. 2001; Röck,

Schneider et al. 2007), in contrast with other FcRγ-Syk coupled CLRs, where triggering leads to cytokine production. Another ITAM signalling adaptor used by CLRs is DAP12. MDL-1 (CLEC5A) was shown to use DAP12 to signal, increasing intracellular calcium (Bakker et al. 1999) and cytokine production when stimulated by antibodies or dengue virus (Chen et al. 2008).

In addition to the classical ITAM-based activation of Syk and signalling downstream of CLRs, dectin-1 was shown to use a similar motif on its own intracellular tail. The dectin-1 cytoplasmic domain was named hemITAM because contained a tyrosine and a leucin separated by two aminoacids (YXXL), which resembles half of an ITAM consensus sequence (YXXI/L X_{6-12} YXXI/L), and was able to bind and signal through Syk (Rogers, Slack et al. 2005). In table 4 it is represented the different signalling CLRs and their known ligand(s).

Table 4 Membrane-associated signalling CLRs and its ligands

CLR	SIGNALLING	LIGAND	ORIGIN OF THE LIGAND
DEC-205	Non-tyrosine	?	Self
Mannose receptor	Non-tyrosine	Carbohydrate	Fungi, helminthes
DC-SIGN	Non-tyrosine	Carbohydrate	Virus, bacteria, helminthes, self
Mouse DCIR2	Non-tyrosine	?	?
BDCA-2	Tyrosine (FcγR)	?	?
Dectin-2	Tyrosine (FcγR)	Carbohydrate	Fungi, mite
MINCLE	Tyrosine (FcγR)	proteins, glycolipid	Bacteria, fungi, self
DCAR	Tyrosine (FcγR)	?	?
CLEC5a/MDL-1	Tyrosine (DAP12/DAP10)	?	Virus
DNGR-1/ CLEC9a	Tyrosine (hemITAM)	?	Self
Dectin-1	Tyrosine (hemITAM)	Carbohydrate	Fungi, bacteria, self
CLEC-2	Tyrosine (hemITAM)	Protein	Snake venom, self
DCIR	Tyrosine (ITIM)	?	Self
CLEC12B	Tyrosine (ITIM)	?	?
MICL/DCAL-2/CLEC12A	Tyrosine (ITIM)	?	Self

Therefore, the hemITAM signalling downstream of CLRs constitutes a new mechanism of innate receptor signalling to initiation of inflammatory responses. A computational search for transmembrane proteins that resemble

dectin-1 with respect to its single CTLD domain and intracellular hemITAM motif identified 4 other transmembrane CLRs (clec1b/CLEC-2, clec9a/DNGR-1, clec12a/MICL/DCAL-2 and clec12b) that contain hemITAM motif (see figure 1.2A). Furthermore, all these CLRs are clustered together on human chromosome 12 and on mouse chromosome 6 (figure 1.3B). However, the YxxL motif on clec12a and clec12b is flanked by valine and isoleucine (yellow residues on figure 1.3A), which constitutes an immunoreceptor-tyrosine inhibitory motif (ITIM). In fact, clec12a and clec12b have been shown to recruit the protein tyrosine phosphatase SHP-1 (Hoffmann et al. 2007; Pyż et al. 2008).

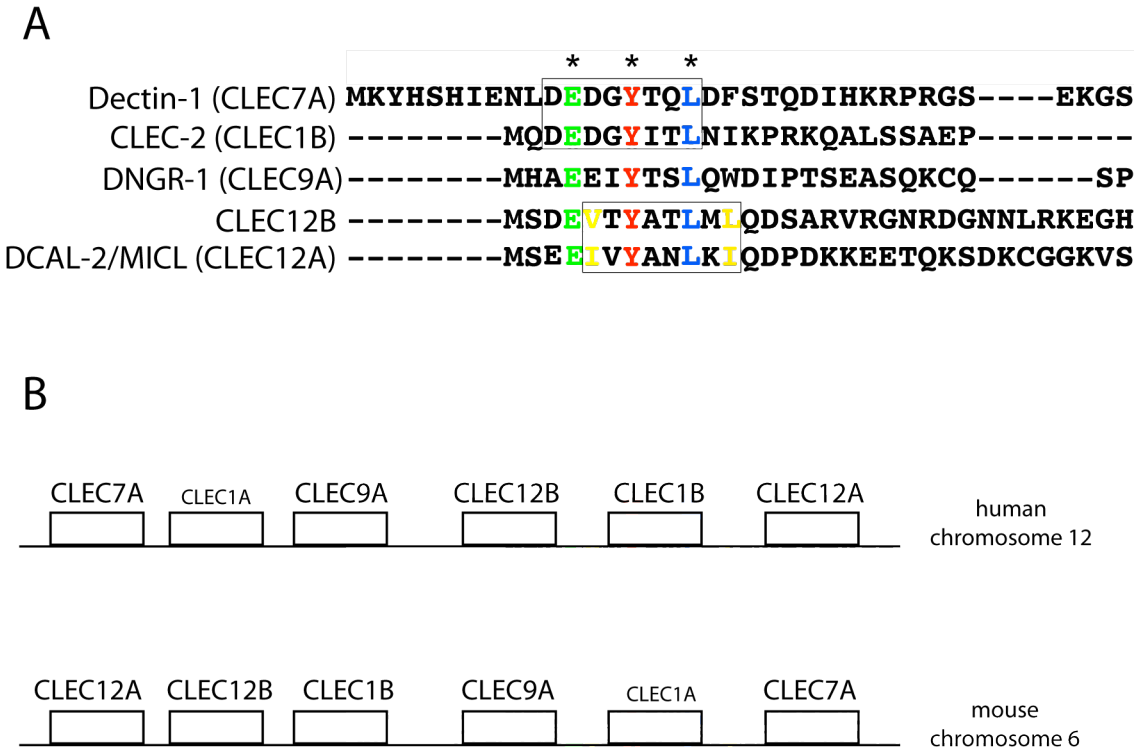

Figure 1.3 Dectin-1-like CLRs
(A) Alignment of dectin-1-like receptors intracellular tails. The * and colours mean conservation of amino acid residue. Boxes show conservation of signalling motifs. (B) Dectin-1-like genes are clustered in a genomic region of human chromosome 12 and mouse chromosome 6.

1.4 HemITAM signalling in the regulation of inflammation

The search for dectin-1-like receptors uncovered 2 other CLRs that contained the hemITAM motif. Furthermore, at the start of this thesis DNGR-1 had not been described yet, whilst CLEC-2 had been described as a mRNA expressed by myeloid cells (Colonna et al. 2000) and a platelet receptor that signals via its hemITAM and Syk (Suzuki-Inoue et al. 2006). The published literature on CLEC-2 indicated a potential role for this receptor on the modulation of myeloid cell function and inflammatory responses via the hemITAM motif, like dectin-1. Therefore, I decided to focus my attention on CLEC-2 as a potential dectin-1 like receptor. In the next section I will describe what is known about dectin-1 function and signalling and how this relates to new findings about CLEC-2.

1.4.1 Dectin-1

Dectin-1 (or DC-associated C-type lectin-1) was first described as an mRNA preferentially expressed on DCs with an unidentified ligand on stimulated T cells (Ariizumi et al. 2000). More specifically, dectin-1 belongs to group V of CLRs, which includes NK-cell receptors and are type II transmembrane receptors containing a stalk region (also termed neck region) and a single CTLD that lacks Ca^{2+} binding capacity and interacts with protein ligands (Zelensky and Gready 2005). Thus, it was surprising that, in a screen for β-glucan receptors, Brown and Gordon demonstrated that dectin-1 could bind to β-glucans present on zymosan. Furthermore, dectin-1 recognition of β-glucans could mediate phagocytosis of *Candida albicans* (Brown and Gordon 2001). A subsequent analysis determined that all myeloid cells expressed dectin-1, and that it is expressed at higher levels on macrophages and neutrophils than on DCs (Taylor et al. 2002).

The identification of dectin-1 as a receptor that binds β-glucans and mediates phagocytosis of fungi had important implications, as dectin-1 deficiency in humans had been implicated on increased susceptibility to mucocutaneous fungal infections (Ferwerda et al. 2009). Due to the complex

composition of zymosan, it is likely that multiple receptors could be involved in responses against it. Dectin-1 blockade greatly impaired zymosan binding indicating that dectin-1 is the key β-glucan receptor on macrophages (Brown et al. 2002). In fact dectin-1 and TLR2 could mediate some of the zymosan effects, with dectin-1 enhancing TLR-mediated tumour necrosis factor (TNF) production (Brown, Herre et al. 2003; Gantner, Simmons et al. 2003). This could mean simply that dectin-1 increased binding of zymosan could be involved in the increased TNF production observed. Nevertheless, it was determined that the dectin-1 intracellular tyrosine was necessary for an increasing cytokine production and NFκB activation (Brown, Herre et al. 2003; Gantner, Simmons et al. 2003). Additionally, zymosan particles induced normal amounts of IL-10 in DCs deficient of TLR signalling (Myd88-/-) (Edwards et al. 2002), suggestive of a TLR-independent and dectin-1-dependent effect.

The discovery that Syk could mediate dectin-1-induced cytokine and ROS production (Rogers, Slack et al. 2005; Underhill, Rossnagle et al. 2005) gave possible insights into the molecular mechanism of dectin-1-mediated functions. Rogers and colleagues formally demonstrated that the dectin-1 single phosphorylated YxxL motif was able to bind Syk. Furthermore, this dectin-1 motif mediated Syk phosphorylation and cytokine production upon zymosan treatment. Also, Syk, but not Myd88, was necessary for induction of IL-2, IL-10 and IL-12/IL-23p40 by DCs stimulated with zymosan (Rogers, Slack et al. 2005). Consistently, Underhill and colleagues showed that src kinases and dectin-1 hemITAM, but not the ITAM adaptors DAP12 and FcRγ, were necessary for zymosan induction of Syk activation. Selective dectin-1 triggering or zymosan stimulation induced ROS production by macrophages, a phenomenon that is src kinase- and Syk-dependent. In addition, both the dectin-1 hemITAM as well as the tri-acidic motif preceding the hemITAM were necessary for dectin-1-mediated zymosan internalization (Underhill, Rossnagle et al. 2005).

Accumulating evidence pointed to the fact that dectin-1 could mediate NFκB activation and induction of cytokine production via a hemITAM motif, src kinases and Syk activation. However, the molecular link of Syk to NFκB

activation was only elucidated upon the identification of CARD9 as a signalling molecule on myeloid cells. CARD9, in a complex with BCL10 and MALT1, was shown to be required to induce NFκB activation following dectin-1 triggering (Gross et al. 2006), similarly to the lymphocyte receptor (BCR and TCR) activation of src kinases, Syk/Zap70 and CARMA1/BCL10/MALT1 leading to NFκB activation (Thome 2004). Of great importance is the fact that CARD-/- mice are highly susceptible to *Candida albicans* infection, probably due to the defect on the production of inflammatory cytokines by myeloid cells, as there is normal lymphocyte activation in the absence of CARD9 (Gross, Gewies et al. 2006). Remarkably, a CARD9 homozygous deficiency in humans is correlated with an increased susceptibility to fungal infections (Glocker et al. 2009). In addition to this activation of NFκB, dectin-1-Syk signalling can activate the NFκB-Inducing Kinase (NIK) complex and induce a canonical NFκB activation. Nevertheless, it is still unclear the requirement for CARD9 to activate this pathway (Gringhuis, den Dunnen et al. 2009). Furthermore, a Syk-independent Raf1-dependent dectin-1 signalling can activate non-canonical NFκB sub-units (Gringhuis, den Dunnen et al. 2009).

Macrophages and DCs respond to zymosan triggering by producing cytokines. The scenario is different when curdlan selectively trigger dectin-1. DCs still produce cytokines, but macrophages do not. This differential response is due to the differential use of CARD9 by DCs and macrophages, where the latter cannot properly use CARD9 (Goodridge et al. 2009). Furthermore, macrophages do not use Syk to phagocyte yeast particles (Herre et al. 2004), whilst dectin-1-mediated phagocytosis in DCs is dependent of Syk (Rogers et al. 2005; Gross, Gewies et al. 2006). This differential use of Syk between macrophages and DCs might reflect different expression of receptors for yeast particles: macrophages might express Syk-independent yeast phagocytic receptor that DCs do not express.

The remarkable similarities between the lymphocyte receptor and dectin-1 signalling were expanded with the demonstration that dectin-1 triggering leads to calcium signalling. DCs stimulated with curdlan (a large β-glucan particle) increased intracellular Ca^{2+} concentrations. This Ca^{2+} increase was dependent

on phopholipase C γ2 (PLCγ2) activation, which was only observed upon src kinases and Syk activation (Xu et al. 2009). In addition to Ca^{2+} signalling downstream of dectin-1 triggering, it was demonstrated that this pathway could lead to activation of the transcription factor, nuclear factor of activated T cells (NFAT). This dectin-1-mediated NFAT activation is important to induce cyclooxygenase 2 (COX-2) and its product prostaglandin E2 (PGE2), in addition to IL-10, IL-2 and IL-12p70, but not TNF or IL-6 (Goodridge et al. 2007).

Furthermore, dectin-1 was shown to localise to lipid rafts upon triggering with zymosan and lipid raft disruption impaired IL-2, IL-10 and TNF production by DCs (Xu et al. 2009). Another study showed that dectin-1 co-localises, on membrane micro domains, with the tetraspanin CD37. Additionally, CD37 deficiency diminished dectin-1 surface expression, while increased IL-6 production by dectin-1 (Meyer-Wentrup et al. 2007). Thus, independent studies indicate that dectin-1 localisation on membrane domains is important for its downstream function of cytokine production. Additionally, the presence of distinct dectin-1 isoforms, with or without the neck, in both mice (Heinsbroek et al. 2006) and humans (Willment et al. 2001) points to different expression and signalling abilities. In this regard, utilisation of β-glucan microparticles or the large β-glucan particle curdlan indicated that the capacity of dectin-1 to internalise its agonist correlates with diminished ability to signal and induce cytokine production (Rosas et al. 2008; Hernanz-Falcón et al. 2009).

Confirmation of the role of dectin-1 in β-glucan recognition of fungi came with the generation of two different dectin-1 deficient mice lines (dectin-1-/-) by two independent groups. Dectin-1 was only partially required for cytokine production on DCs upon zymosan stimulation, while it was a crucial mediator of ROS production on macrophages. This crucial role of dectin-1 on the induction of ROS by macrophages was also observed upon stimulation with the fungi *Candida albicans* or *Pneumocyctis carinii* (Saijo et al. 2007). Moreover, dectin-1-/- macrophages were profoundly impaired in the production of TNF upon *Candida albicans* or *Aspergillus fumigatus* stimulation (Taylor et al. 2007). Although both mice lines showed increased susceptibility to fungi infection, it was against different fungi. Nevertheless, dectin-1-/- mice on a BALB/cA

background were susceptible to *Pneumocyctis carinii* infection. However, these mice were not increasingly susceptible to *Candida albicans* infection, the same phenomenon was also observed with C57BL/6J dectin-1-/- mice (Saijo, Fujikado et al. 2007). On the other hand, Taylor and colleagues reported that dectin-1-/- mice on a mixed 129/Sv C57BL/6 background were highly susceptible to *Candida albicans*, showing increased fungal burden and diminished recruitment of myeloid cells to the inflamed site (Taylor, Tsoni et al. 2007). Overall, dectin-1 has been shown to play an important role in the detection of fungi, mediating production of several inflammatory mediators that are important to resistance to infection in humans (Ferwerda, Ferwerda et al. 2009) and mice (Saijo, Fujikado et al. 2007; Taylor et al. 2007).

Dectin-1 has been clearly demonstrated to be directed involved on the detection and induction of inflammation by recognition of fungi and induction of inflammatory mediators and cellular recruitment. However, in addition to these aspects, dectin-1-induced inflammation has also been shown to control adaptive responses. Stimulation of DCs with curdlan induces increased T cell activation, determined by increased proliferation and production of IL-17 and interferon-γ by these T cells. Intriguingly, the comparison between dectin-1 and TLR9 activation of DCs showed that different T cell responses are obtained; where there is higher IL-17 production stimulated by dectin-1-activated DCs compared to TLR9. This data evidences the role of dectin-1 on the activation of DCs, inducing antigen processing and presentation to activate adaptive immune responses measured by immunoglobulin (Ig) production, T cell production of cytokines and proliferation (LeibundGut-Landmann et al. 2007). Nevertheless, analysis of dectin-1-/- mice in cortisone-treated immunocompromised mice did not show a role for adaptive responses in dectin-1-mediated fungal resistance (Saijo, Fujikado et al. 2007). Furthermore, analysis of cytokine production by C*andida albicans*-restimulated T cells of Candida albicans-infected mice did not reveal any differences in the production of IFN-γ (Th1) or IL-17 (Th17) cytokines (Robinson, Osorio et al. 2009), suggesting that dectin-1 does not affect adaptive responses. Additionally, dectin-1-/- mice did not show any defect in T cell and antibodies responses (Saijo, Fujikado et al. 2007; Taylor, Tsoni et al.

2007) despite dectin-1 was implicated in recognition of a T cell ligand and increasing T cell proliferation (Ariizumi, Shen et al. 2000)

In summary, dectin-1 triggering by β-glucans induces ROS, inflammatory cytokines, antigen processing and presentation in a signalling pathway that resembles the antigen receptor signalling (fig.1.4). The identification of this signalling pathway and its implications on myeloid cell function are of great value for the understanding of qualitative differences between different innate receptors.

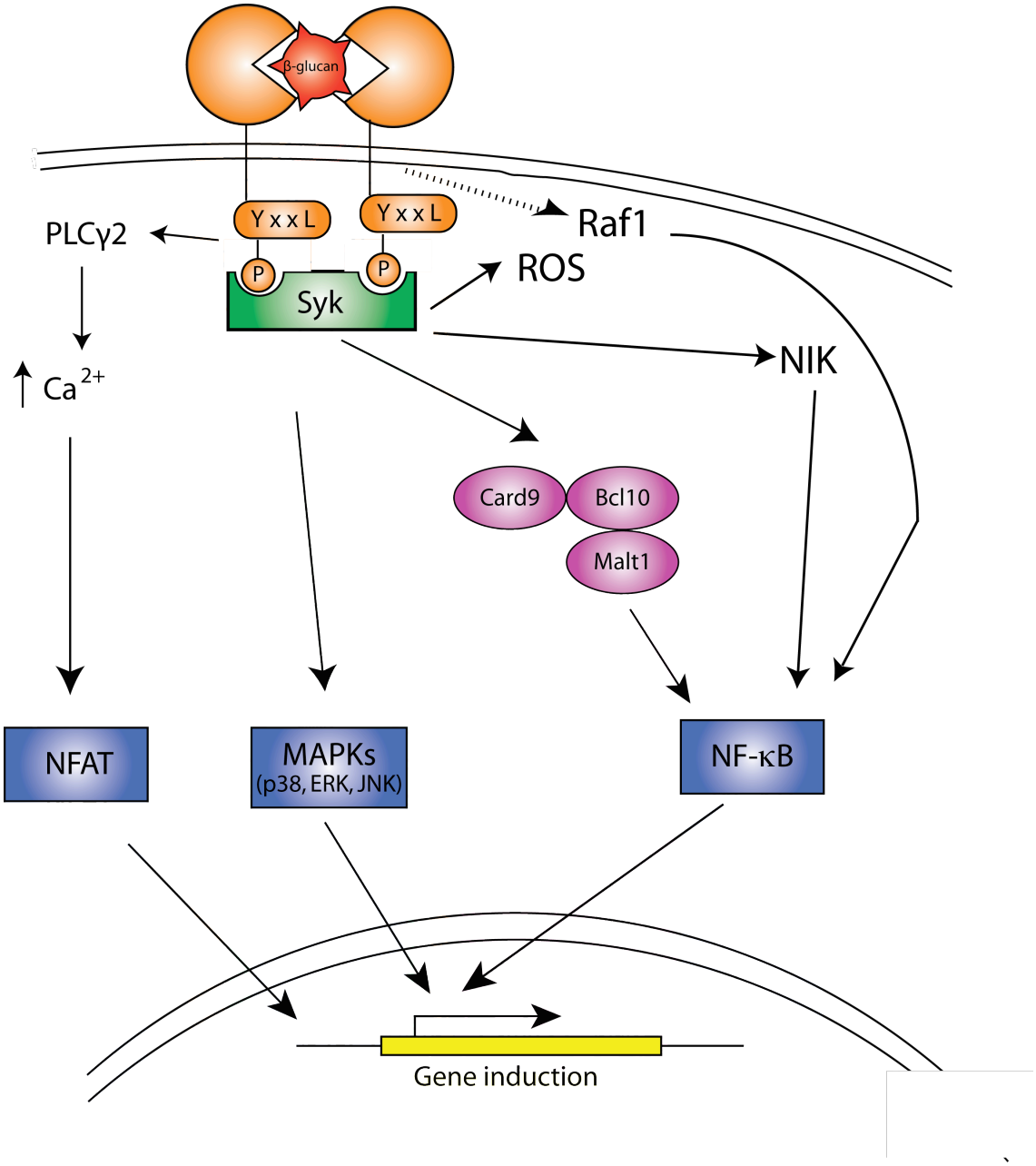

Figure 1.4 Dectin-1 signalling to downstream function
Dectin-1 CTLD interacts with β-glucan particles, which leads to phosphorylation of its hemITAM motif allowing Raf1 signalling and Syk recruitment and activation. Syk signalling downstream of dectin-1 leads to Ca^{2+} increase, MAPK, NFAT and NFκB activation. Dectin-1 signalling via Syk leads to NFκB-Inducing Kinases (NIK) and Card9-Bcl10-Malt1 complex to activate NFκB. Raf-1 signalling downstream of dectin-1 is independent of Syk and leads to non-canonical NFκB activation.

1.4.2 CLEC-2

CLEC-2 was identified in a screen for receptors homologous to the natural killer (NK) cell receptors CD94, NKG2A and NKG2D (Colonna, Samaridis et al. 2000). A report showed that in ectopic expression assays, CLEC-2 was on the surface of cells, and mRNA was found on human samples of bone marrow, granulocytes, DCs, NK cell clones and peripheral blood mononuclear cells (Colonna, Samaridis et al. 2000).

Prior to the start of this thesis Suzuki-Inoue and colleagues demonstrated that rhodocytin, a platelet aggregation-inducing snake venom toxin, interacted with a 32KDa protein identified as CLEC-2. Further analysis of CLEC-2 expressed on platelets revealed it as a three protein bands ranging from 32 KDa to 40 KDa. These different sizes might be related to differential glycosylation of CLEC-2, as there are 3 potential N-glycosylation sites, and n-glycosidase treatment of CLEC-2 collapses all the bands into one 26 KDa band, which correspond to the predicted size of the polypeptide chain (Suzuki-Inoue, Fuller et al. 2006). Furthermore, mouse and human *clec1b* gene (CLEC-2) show a similar genomic structure with 6 exons. Exon 1 and part of exon 2 encode the intracellular domain, while the majority of exon 2 encodes the transmembrane domain, exon 3 with a part of exon 4 encodes the neck and exons 4, 5 and 6 encodes the CTLD (fig.1.5A and 1.5B). Additionally, mouse CLEC-2 mRNA transcripts have three alternative transcripts (isoforms CLEC-2A, CLEC-2B and CLEC-2C) that differs in the presence of the transmembrane domain and part of the CTLD (Xie et al. 2008) (see figure 1.5C). Isoform A, the full length transcript, is highly expressed on the liver and spleen, while the isoforms B and C transcripts are found on the kidney and lung. However, isoforms B and C are not expressed on the membrane nor are they secreted, but instead are found on the cytoplasm of cells. In addition the full length mouse CLEC-2 can be cleaved from the plasma membrane and made soluble. This cleaved mouse CLEC-2 forms a dimer, dependent on disulfide bridges within cysteine residues in the neck region (Xie, Wu et al. 2008). In addition, human

CLEC-2 is also described as a dimer, although this was proposed as independent of disulfide bridges (Watson et al. 2009).

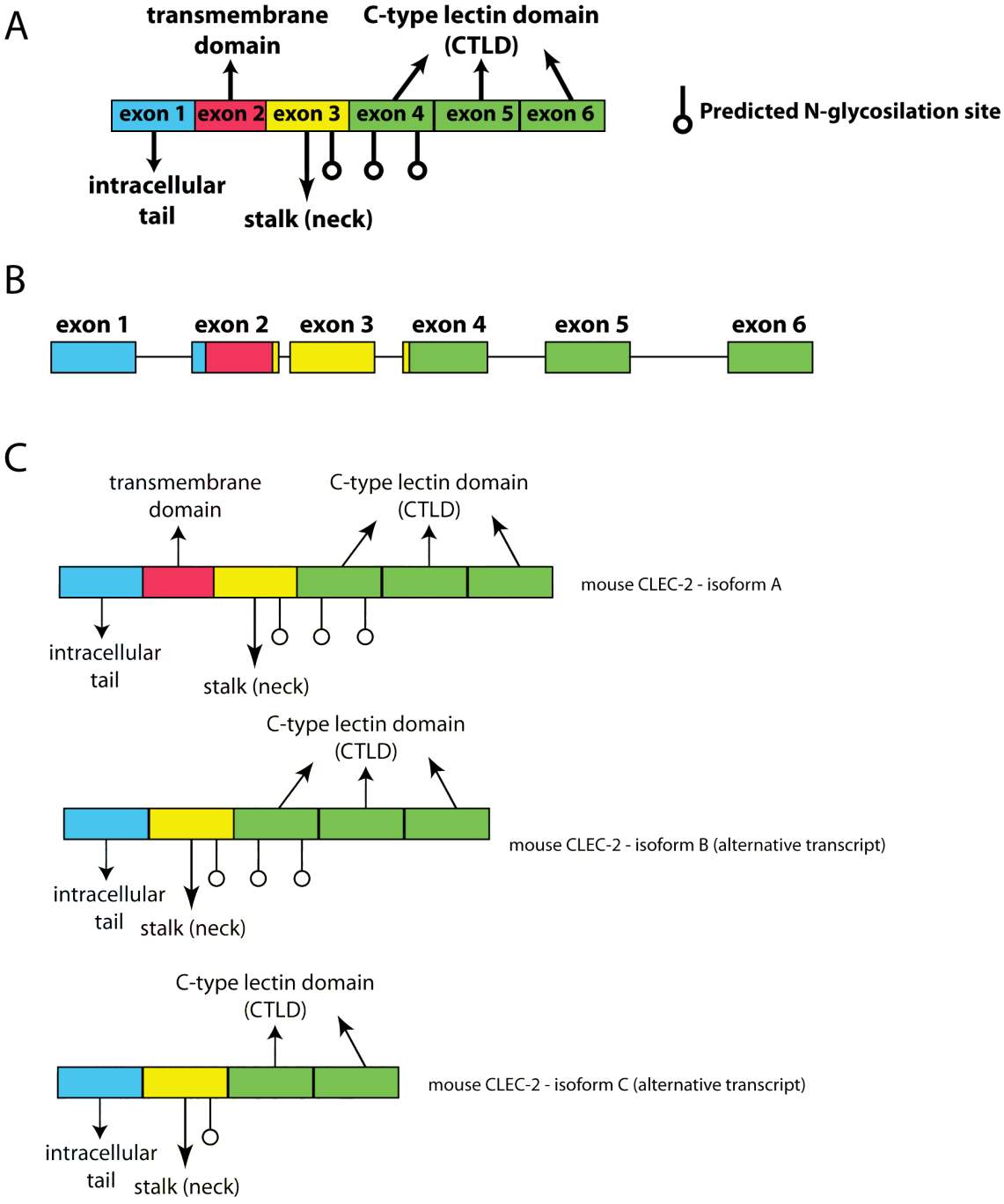

Figure 1.5 CLEC-2 gene and protein structure
(A) Mouse and human CLEC-2 gene are made of six exons. Colours correlate with (B) the structural domains of mouse CLEC-2. (C) Mouse CLEC-2 alternative transcripts differs on the presence transmembrane domain and part of CTLD

As mentioned above, CLEC-2 was identified as a platelet receptor that mediates aggregation upon interaction with rhodocytin. The endogenous protein podoplanin (also known as gp38, aggrus, OTS-8 and T1α) can also bind to CLEC-2 and mediate platelet aggregation (Suzuki-Inoue et al. 2007). Podoplanin (PDPN) is an O-glycosylated protein described on human kidney podocytes (Breiteneder-Geleff et al. 1997), rat lung type I cells (Rishi et al. 1995), lymphatic endothelial cells and some tumour cells (Breiteneder-Geleff et al. 1999). CLEC-2 on platelets was also shown to bind human immunodeficiency virus (HIV). This was independent of viral protein, which suggests that HIV particles budding from cells carry with them an endogenous CLEC-2 ligand on the viral envelope (Chaipan et al. 2006). In fact, the cell lines used to produce HIV were demonstrated to express PDPN (Christou et al. 2008). The interaction of CLEC-2 with rhodocytin or PDPN does not depend on the glycosylation of CLEC-2, as recombinant non-glycosylated CLEC-2 produced in bacteria can bind to rhodocytin (Watson et al. 2007) and to PDPN as efficiently as glycosylated CLEC-2 or N-glycosidase treated CLEC-2 (Christou, Pearce et al. 2008).

Several studies have focused on the signalling and function of CLEC-2 on platelets. The interaction of CLEC-2 with rhodocytin, PDPN or a polyclonal anti-CLEC-2 antibody induces a downstream signal that culminates in platelet-aggregation in a Syk-dependent manner (Suzuki-Inoue, Fuller et al. 2006; Suzuki-Inoue, Kato et al. 2007). The proposed ITAM-based Syk signalling is a classical pathway that has been extensively studied downstream of lymphocyte receptors (TCR and BCR) and also Fc receptors. The current ITAM signalling model proposes that src family kinases phosphorylate the two tyrosines residues on ITAM-associated receptors, allowing Syk double src homology 2 (SH2) domains to interact with it. Sequentially, Syk is phosphorylated either by itself or by src kinase family members becoming activated resulting in the phosphorylation of downstream tyrosine kinases (Mócsai et al. 2010). The presence of a single tyrosine on hemITAM-bearing receptors raised the question of whether Syk can get activated through recruitment of a single SH2

domain. Nevertheless, a study with point mutations on Syk SH2 domains indicated that CLEC-2 signalling required both Syk SH2 domains to transduce signal (Fuller et al. 2007), which can be explained by the fact that CLEC-2 may act as dimer and activate Syk after dimerisation upon stimulation (Hughes et al. 2010).

CLEC-2 becomes tyrosine phosphorylated following stimulation with rhodocytin or anti-CLEC-2 (Suzuki-Inoue, Fuller et al. 2006). These stimuli also lead to phosphorylation of Syk, linker of activated T cells (LAT), PLCγ2 and SH2 domain containing leukocyte protein of 76 KDa (SLP-76) (Suzuki-Inoue, Fuller et al. 2006; Spalton et al. 2009). CLEC-2 hemITAM phosphorylation following activation with rhodocytin is dependent on both Syk and src kinases, with src kinases being proposed to contribute to Syk activation (Spalton, Mori et al. 2009). Rac1, another known Syk target, is also involved in rhodocytin-induced platelet aggregation, suggesting a role for CLEC-2 in rac1 activation (Pleines et al. 2009).

Similarly to dectin-1, CLEC-2 was shown to localise to lipid raft upon stimulation, where it is phosphorylated by a mechanism dependent on rac1, Syk and src kinases. Interestingly, translocation of CLEC-2 to lipid rafts is independent of actin polymerisation and src kinases (Pollitt et al. 2010). This model proposes that CLEC-2 stimulation with rhodocytin results in CLEC-2 translocation to lipid rafts where there are several src kinases members that contribute to CLEC-2 phosphorylation together with rac1 signalling and actin polymerisation.

Besides CLEC-2 studies on platelets, other groups have reported CLEC-2 expression on myeloid cells, such as neutrophils and monocytes (Kerrigan et al. 2009) (Kerrigan et al. 2009)and Kupffer cells on the liver (Tang et al. 2010). In addition, CLEC-2 function was studied on a chimeric receptor that had CLEC-2 CTLD swapped by dectin-1 CTLD. Therefore, B-glucan binding to this chimeric receptor leads to CLEC-2 signalling. On this system CLEC-2 intracellular tail could signal to phagocytosis and production of TNF (Kerrigan, Dennehy et al. 2009), which resembles the functions of dectin-1. However, despite CLEC-2 and dectin-1 sharing very similar signalling motifs (as depicted on figure 1.3),

CLEC-2 signalling is unable to induce ROS production (Kerrigan, Dennehy et al. 2009).

The first demonstration of a CLEC-2-mediated effect *in vivo* came from the report of May and colleagues. They showed that depletion of CLEC-2 from the surface of platelets using anti-CLEC-2 antibodies is associated with longer bleeding times and reduced thrombus formation (May et al. 2009). Nevertheless, injection of a soluble form of CLEC-2 did not significantly affect the bleeding time of mice (Tang et al. 2010). The recent generation of CLEC-2 deficient mice by three independent groups revealed a crucial role for CLEC-2 during vascular development. These groups reported an increased lethality of embryos, which was associated with increased haemorrhage and defect vessels formation (Bertozzi et al. 2010; Suzuki-Inoue et al. 2010; Tang, Li et al. 2010). Remarkably, the phenotype of CLEC-2 deficient mice resembles the phenotype of mice deficient in Syk and Slp-76 (Turner et al. 1995; Abtahian et al. 2003), two molecules involved in CLEC-2 signalling.

In summary, CLEC-2 induces signalling by translocation to lipid rafts that leads to activation of its hemITAM motif culminating in Syk activation. CLEC-2 has been largely characterised as a platelet receptor that possess two distinct ligands that induces platelet aggregation. Furthermore, CLEC-2 function *in vivo* had been linked to normal clotting and vascular development.

1.5 Thesis hypothesis

CLEC-2 and dectin-1 share many structural and signalling features. Both receptors show similarities on their CTLD structure (Zelensky and Gready 2005) and are clustered together in the same gene locus in mouse and humans (figure 1.3). Furthermore, CLEC-2 and dectin-1 have similar intracellular motifs and use the hemITAM to couple to Syk signalling. At the start of this thesis CLEC-2 had been identified as a platelet Syk-coupled receptor targeted by a snake venom toxin. Moreover, CLEC-2 transcripts had been found in human myeloid cells but its functions had not been explored. In light of these findings, I hypothesized that CLEC-2 and dectin-1 similarities were related to similar functions. Therefore, I sought to characterise CLEC-2 as an innate receptor in myeloid cells involved in inflammatory responses. More specifically I wanted to:

- Identify CLEC-2 ligand(s)
- Determine CLEC-2 expression in the immune system
- Study CLEC-2 function and signalling in myeloid cells
- Develop mouse models to study CLEC-2 function *in vivo*

Chapter 2. Materials and Methods

2.1 Reagents

2.1.1 Buffers, solutions and media

PBS (GIBCO-BRL, Gaithersburg, MD)

PBS-EDTA: PBS containing 5mM EDTA (Sigma, Poole, UK)

Foetal calf serum (FCS) (batch 154-161457: Autogen Bioclear, Mile Elm Calne, UK)

MACS-buffer: PBS-EDTA and 1% FCS heat-inactivated for 30 minutes at 56^{o}C .

FACS-buffer: MACS-buffer containing sodium azide (NaN_3) 0.05%

RPMI 1640 medium (GIBCO-BRL)

R10: RPMI 1640 medium containing 10% FCS, L-glutamine 0.3μg/ml, penicillin 100U/ml, streptomycin 100U/ml (GIBCO-BRL) and 50μM β-mercaptoethanol.

Dulbeccos Modified Eagle Medium (DMEM) (GIBCO-BRL)

D10: DMEM containing 10% FCS, L-glutamine 0.3μg/ml, penicillin 100U/ml, streptomycin 100U/ml (GIBCO-BRL)

Wash buffer: PBS with 0.05% tween-20 (Sigma)

Elisa block: PBS with 2.5% FCS and sodium azide 0.05%

Western block buffer: wash buffer with 3% dried skimmed milk (Marvel)

TAE buffer: 0.04M tris-acetate (Sigma) and 0.002M EDTA

2.1.2 Innate receptors agonists

LPS from *Salmonella abortus* was purchased from Alexis Biochemicals (San Diego, CA) and zymosan and Pam3CSK4 were purchased from InvivoGen. Curdlan was purchased from Wako Pure Chemicals Industries Ltd (Osaka, Japan). Rhodocytin was a kind gift from Stephen Watson (University of Birmingham). Fixed *Staphylococcus aureus* and *Mycobacterium tuberculosis* were commercially purchased. The *Schistosoma* egg antigen (SEA) and the *Toxoplasma gondii* extract soluble tachyzoite antigen (STAg) were a gift from Alan Sher (National Institute of Health – USA).

2.2 Molecular biology and cloning

2.2.1 Transformation of competent bacteria

The competent bacteria used throughout my studies were the *Escherichia coli* strain TOP10 (Invitrogen). One vial of TOP10 was incubated with a plasmid for 20 minutes on ice and then transferred to a 42^{o}C water bath for 45 seconds. Next the vial was returned to ice for additional 2 minutes before the addition of 500µl of SOC medium (Invitrogen) and 37^{o}C 900 RPM shaking for 60 minutes. After shaking, the bacteria were plated on LB agar plates (Cancer Research UK media services) with appropriate antibiotic resistance.

2.2.2 Plasmid isolation

Single resistant clones were picked with sterile pipette tip and transferred to 200mL of LB medium (Cancer Research UK media services) containing the appropriate antibiotic. Then it was incubated overnight at 37^{o}C shaking before applying the maxiprep plasmid isolation kit (QIAGEN). For small-scale plasmid isolation, single colonies were incubated in 5mL of LB medium and the purification was performed by Cancer Research UK miniprep service.

2.2.3 PCR and PCR product isolation

All PCR were performed using Taq polymerase manufacturer's instructions. I used PWO (Roche) and High-fidelity system (Roche) Taq polymerase enzymes. After the completion of cycles of denaturing, annealing and amplification, the DNA loading dye buffer (Sigma) was added to the final

PCR product and loaded into an agarose gel. After applying 100V voltage to the gel I excised the band of the corrected size and purified it by Gel extraction kit (QIAGEN).

2.2.4 Restriction enzyme digestion and ligation

The restriction enzyme digestion was performed on PCR products and plasmid to generate complementary ends for DNA ligation. The digestions were performed by incubating the desired DNA with an appropriate buffer, enzyme(s) and, when required, BSA following manufacturer's instructions. All the restriction enzymes were acquired from New England Biolabs.

The DNA ligation was done by incubating the PCR product with the plasmid (20-50ng) at a molar ratio of 3:1, respectively. The usage of the buffer and the T4 DNA ligase enzyme was done accordingly to the instructions manual (PROMEGA).

2.2.5 Cloning of CD3ζ.mouse CLEC-2-HA, CD3ζ.human CLEC-2 and mouse CLEC-2-Fc

Matthew Robinson (Immunobiology lab- CRUK/LRI) performed the mouse CLEC-2 cloning from GMCSF BMDC cDNA library and added the mouse CLEC-2 Y7F mutation. The cloning of mouse and human CLEC-2 performed by me followed his initial cloning and is described below.

I sub-cloned mouse CLEC-2 WT and mouse CLEC-2 Y7F into the pFB retroviral vector using the following primers: C1b F MR1 and C2CTLDNotIRVSTOP. Underlined are the mouse CLEC-2 specific sequences and on small caps are the restriction sites.

A PMSCV2.2-CMV-IRES-GFP (pMCIG) plasmid containing CD3ζ intracellular and NKR-P1B was obtained from James Carlyle (University of Toronto). I added mouse CLEC-2 extracellular portion tagged with HA to this plasmid using Xho I and Not I sites with the following primers: C1B F MRZ1 (Small caps is depicting XhoI site and underlined is the CLEC-2 sequence) and C1B R MR HA Stop (Small caps is depicting Not I site, underlined is the CLEC-2 sequence and in red is the HA sequence).

I chose to add a part of Exon 2 (predicted to encode the transmembrane domain) to the construct to make sure that I was choosing all the extracellular portion of CLEC-2 (encoded by Exons 3 to 6)

Sequencing using C1b R1 primer checked the correct in frame cloning.

To clone human CLEC-2 into the CD3ζ/NKR-P1B plasmid I firstly cloned human CLEC-2 from the human DC library of the Immunobiology lab. I used the following primers and cloned it on the PGEM-T vector (PROMEGA): FW hc2 full and Rv hc2 full. Underlined is the human CLEC-2 sequence and small caps is the restriction sites.

After this cloning I used the following primers to clone the extracellular portion of human CLEC-2 into CD3ζ/NKR-P1B. The primers were: fw hc2 ec z and Rv hc2 ec z. Underlined are the human CLEC-2 specific sequences and on small caps are the restriction sites.

The following primers were used to check the sequence of human CLEC-2: hCLEC-2 seq Fw and hCLEC-2 seq Rv.

To generate mouse CLEC-2 CTLD soluble, I used a plasmid (mutFc pSecTag2(C)) kindly donated by Philip Taylor (University of Cardiff). This plasmid is a pSecTag2(C) vector (Invitrogen) containing a human IgG1 Fc mutated on 4 residues for less Fc receptor binding and complement activation (McGreal et al. 2006). I used the following primers for this cloning: C2CTLD EcoRI FW and C2CTLD NotI RV. Underlined are the CLEC-2 specific sequences and on small caps are the restriction sites.

Mouse CLEC-2:

ATG CAG GAT GAA GAT GGG TAT ATC ACT TTA AAC ATC AAG CCC CGG
AAA CAA GCT CTC AGC TCA GCG GAA CCT GCC TCT TCT TGG TGG CGT
GTG ATG GCT TTA GTT CTG CTG ATC TCA TCC ATG GGG CTG GTT GTT
GGA CTC GTG GCT CTG GGG ATC ATG TCG GTC ACA CAG CAA AAG TAT
CTA CTG GCG GAG AAG GAA AAT CTC TCA GCG ACT CTG CAA CAA TTG
GCC AAG AAA TTC TGC CAA GAG TTG ATT AGA CAA TCA GAA ATT AAG
ACA AAG AGC ACT TTT GAG CAC AAG TGC AGC CCC TGC GCC ACG AAG
TGG AGA TAC CAT GGA GAT AGT TGC TAC GGG TTC TTC AGG CGT AAC
CTA ACA TGG GAA GAG AGC AAG CAG TAT TGC ACT GAG CAG AAT GCA
ACA CTT GTG AAG ACT GCC AGC CAG AGC ACC CTG GAC TAC ATT GCA
GAA AGG ATT ACT TCA GTC CGT TGG ATT GGA TTA TCA CGC CAG AAC
TCT AAG AAA GAC TGG ATG TGG GAG GAT AGC TCA GTT CTT CGC AAG
AAC GG G ATT AAT CTT TCT GGG AAT ACA GAA GAA AAC ATG AAT TGT
GCT TAT CTT CAT AAT GGA AAA ATC CAT CCA GCT TCC TGT AAA GAG
AGA CAT TAC TTA ATA TGT GAG AGA AAT GCT GGC ATG ACA AGA GTG
GAC CAA CTG CTT TAA

Exon1 Exon2 Exon3 Exon4 Exon5 Exon6

Figure 2.1 Primer design and strategy to sub-clone mouse CLEC-2
Underlined are the primers used to fuse the extracellular portion of mouse CLEC-2 with CD3ζ/NKR-P1B and in shaded gray is the portion of CLEC-2 used to fuse to the human IgG1 Fc and generate CLEC-2.Fc chimera.

2.2.6 Cloning of PDPN, PDPN T34A and Fc chimeric protein

To obtain mouse PDPN DNA I generated colo 26 cDNA library by growing colo 26 cells (obtained from Cancer Research UK cell services) and extracting RNA by RNeasy kit (QIAGEN). The RNA was subjected to reverse transcription using Superscript II (Invitrogen) standard protocol. Next I used the following primers to clone mouse PDPN into the pFB-NEO vector (Stratagene): mPDPN FW and mPDPN Rv. Underlined are the mouse PDPN specific sequences and in small caps are the restriction enzymes sites used.

After cloning it I generated a point mutation on the amino acid residue 34 (T34A) by QuikChange Site-Directed mutagenesis kit (Stratagene) standard protocol. For this I used the following primers: mPDPN T34A Fw and mPDPN T34A Rv. In bold is depicted the nucleotide change inserted: from A to a G.

To generate PDPN soluble I used primers described by Suzuki-Inoue and colleagues that showed a soluble PDPN able to bind to CLEC-2 (Suzuki-Inoue, Kato et al. 2007). After amplification of this PDPN region I cloned on the mutFc pSecTag2(C) as described for mouse CLEC-2. The primers are as follow: PDPN EC EcoRI Fw and PDPN EC NotI RV. Underlined are the mouse PDPN specific sequences and on small caps are the restriction enzyme sites.

Table 5 Primers

Primer name	Primer sequence
C1b F MR1	GcacctcgagggtacccccgccaccATGCAGGATGAAGATG G
C2CTLDNotIRVSTOP	GGGAAAgcggccgcttaaagCAGTTGGTCCACTCTTG
C1B F MRZ1	ATGCctcgagGGGATCATGTCGGTCACACAGC
C1B R MR HA STOP	ATGCgcggccgcTCAGGCGTAATCGGGCACATCGTAA GGGTAAAGCAGTTGGTCCACTCTTGTC
C1b R1	AAGCAGTTGGTCCACTCTTG
FW hc2 full	TATATAgtcgacCCCGCCACCATGCAGGATGAAGATG GATAC
Rv hc2 full	ATATAgaattcTTAAGGTAGTTGGTCCACCTTG
fw hc2 ec z	TATATActcgagAATTACCTACAAGATGAGA
Rv hc2 ec z	TATATAgcggccgcTTAAGGTAGTTGGTCCACCT
hCLEC-2 seq Fw	AGCTGCTATGGGTTCTTCAG
hCLEC-2 seq Rv	TCTCTTCCCATGTTAAGTTG
C2CTLD EcoRI FW	GGGAAAgaattcACAAAGAGCACTTTTGAGCAC
C2CTLD NotI RV	GGGAAAgcggccgcAGCAGTTGGTCCACTCTTG
mPDPN FW	TATATAgaattcCCACCATGTGGACCGTGCCAGTGT
mPDPN Rv	TATATActcgagTTAGGGCGAGAACCTTCCAG
mPDPN T34A Fw	gtgaatgaagatgatattgtg**G**ccccaggtacaggagacggcatg
mPDPN T34A Rv	catgccgtctcctgtacctgggg**C**cacaatatcatcttcattcac
PDPN EC EcoRI Fw	GGGAAAgaattcGGGAGCGTTTGGTTCTG

PDPN EC NotI RV	GGGAAAgcggccgCGGCGAGAACCTTCCAGAAA
Clec1b BAC Fwd	TATTACCTGATGCTGTTACATCTCAGCTCTGCAGTA TTTAGCCACCTTAGAGTTCCTAGCTGCTGACTCTGG GTACCGAGCTCGAATTCTACCG
Clec1b BAC Rev	CTGGGTTCTTTCCAGCTTCTGGCTATTATAAATAAG GCTGTTATGAACATAGTGGAGCATGTGTCCTTCTTG CGGCCGCCACCGCGGTGGAGCTCCA
1st loxP FW	AAAACCCAAAACCAAAAAACCAAAACCAACAACAAA ACAAAAAAACAGATAATTAACCCTCACTAAAGGGCG
1st loxP RV	ACTTATTCTCTGTCCATTCTAACATATAACTGGCTAC CAAGGCCACGTGtTAATACGACTCACTATAGGGCTC
FW Rb2	TCCCATGTCAAGCATTTTGGAATGCTGAGGGGAAA CATTGAAATGCTGTTAATTAACCCTCACTAAAGGGC
Rv Rb2	TCTCAGAGGAGCACACAGTGCAAACCATTAAGAAA CACATGAAAAGGAAATAATACGACTCACTATAGGG CTCG
Fwd seq 5'flxNeo	agggatgcaaaaacaaaaacaaaac
Rv seq 3'flxNeo	tttataccacccatccattgccact
Seq FRT FW Rb2	ggtaactggagagaggtcca
Seq Rv FRT	agcatccctagcagactcat
Seq Neo Fw	ATTCGGTACCAATAAAAGAG
Seq PGK RV	CTAGGGGAGGAGTAGAAGGT

2.3 Cell culture

2.3.1 Stable cell line generation

Stable cell lines were generated by using retroviral transduction. Firstly, I transfect GP2-293 cells (Clontech) with the retroviral vector (I used the pMSCV and pFB) containing the desired protein and the pVSV-G plasmid that contains the virus structural proteins. This transfection is performed using Lipofectamine 2000 (Invitrogen) according to protocol in manufacturer's manual instructions.

Next, 24, 48 and 72 hours after transfection I harvest the supernatant of these transfected cells and filtered it on a 0.45μM Millex-HV PVDF filter (Millipore) and added 5μg/ml of polybrene (Sigma). This virus-containing supernatant is used to replace the medium of Phoenix cells, and then this is centrifuged at 2600 RPM at 26^{o}C for 90 minutes. These Phoenix cells will stably express the desired protein as well as produce virus to infect other cells. I used the virus-containing supernatant of Phoenix cells to induce ectopic expression of the desired protein in other cells.

2.3.2 Cells

B3Z cells: Mouse CD8 T cell hybridoma B3Z cells expressing the NFAT-LacZ reporter (referred as B3Z cells throughout the document) were a gift from Nilabh Shastri (University of California - Berkeley). B3Z cells expressing ζdectin-1-HA were generated by David Sancho (Immunobiology lab – CRUK/LRI). These cells were cultured in R10.

B3Z.ζCLEC-2-HA and B3Z.ζhumanCLEC-2: Stable cell line obtained by FACS sorting (CRUK FACS Lab) and cultured in R10.

B3Z.Syk cells: B3Z cells expressing ectopically expressing Syk generated by Patricia Hernanz-Falcon (Immunobiology lab – CRUK/LRI). These cells were cultured in R10.

B3Z.Syk CLEC-2 WT and B3Z.Syk CLEC-2 Y7F: Generated by stably transfecting B3Z.Syk cells. Cell population was enriched by MACS (described below)

LK: mouse B cell line.

LK CLEC-2 WT and LK CLEC-2 Y7F: LK cells stably expressing CLEC-2 WT and Y7F. Cell population was purified by FACS sorting.

3T3: mouse fibroblast cell line obtained from CRUK cell services cultured in D10

293.T: human kidney fibroblast cell line cultured in D10

RBL: RBL-2H3 is a rat basophilic leukaemia cell line cultured in R10

RBL ζCLEC-2-HA: RBL cells stably expressing stably expressing ζ.CLEC-2-HA

RMA-S: mouse thymoma cell line cultured in R10

CHO K1 and CHO mutants: hamster ovarian fibroblast cell line. The parental cell line CHO-K1 was obtained from CRUK cell services. The mutant CHO pgsD-677 was obtained from Holger Gerhardt lab (Vascular Biology laboratory - CRUK/LRI). The mutant CHO H661 was from Jeffrey Esko (UCSD). The mutant pgsF-17 was from Nancy Hogg lab (Leukocyte adhesion lab – CRUK/LRI). CHO H661 Ext1 was stably transfected with human Ext1-pcDNA3 (Gift from Holger Gerhardt lab) and selected with 100µg/ml of G418 (Gibco). All cells were cultured on D10.

2.3.3 B3Z assay for ligand screening

B3Z cell clones were cultured at $1x10^5$ cells on a 96-well flat-bottom plate and the final volume of the assay was always 200µl. After 18 hours of culture the supernatants were discarded and the cells were resuspended in 100µL of CPRG buffer for measurement of enzymatic activity. CPRG buffer contains 300nM of CPRG (Roche), 0.1% of NP-40 and 10µM of $MgCl_2$. CPRG changes colour from yellow to red according to LacZ activity. This colour change can be detected on different time points by spectrophotometer readings of red at 595nm wavelength subtracted by unspecific readings at 650nm. Commonly monoclonal anti-HA antibody (clone HA-7 ascites fluid – Sigma) was used at 1:400 dilution as positive control. Also, ionomycin was used at 0.5µg/ml as positive control for LacZ induction on B3Z clones.

3T3 cells were used as a source of CLEC-2 ligand and for studies of CLEC-2 ligand biology. For inhibition assays, 3T3 cells were treated for 18 hours with tunicamycin, anysomyciyn and actinomycin D (all from Sigma). Next the cells were washed in R10 before being cultured with B3Z cell clones. For the transwell assay, a 24-well plate was used with a corning transwell insert with a membrane pore size of 8µm. On this assay $4x10^5$ B3Z cells were cultured on the bottom part of the well with $1x10^5$ 3T3 cells separated or not by the transwell insert. Cells were resuspended on 500µl of CPRG buffer, transferred to a 96-well plate and spectrophotometer readings were performed.

2.3.4 Generation, production and staining with Fc chimeric proteins.

Vectors carrying PDPN.Fc, PDPN T34A.Fc and dectin-1.Fc were transfected into CHO K1 cells and CLEC-2.Fc was transfected into CHO H661 cells. All transfections were performed using Lipofectamine 2000 (Invitrogen). The cells were plated at limiting dilutions and selected with 100μg/ml of G418 until confluent growing in 96-well plates. The supernatants of culture were screened for binding to ligands. I incubated 50μl of supernatants for 20 minutes with zymosan, B3Z.ζCLEC-2-HA or LK PDPN cells. This was washed by adding 200μl of FACS buffer and centrifuging for 5 minutes at 1500 RPM. Next, I stained with F(Ab')2 goat anti-human IgG (H+L) Cy5 (Jackson Immunoresearch Laboratories) for another 20 minutes. This was washed as before and analysed on the FACScalibur.

Selected clones were grown on two compartments Bioreactor (CELLine CL 1000 – Integra Bioscience) and cells were changed and concentrated supernatants collected every week.

2.3.5 Western blot and immunoprecipitation

Protein extracts were mixed with SDS loading buffer and loaded into a 4-20% Tris-glycine gel (Invitrogen) run at 100V on a Tris-glycine-SDS buffer (Biorad). Next, the separated proteins were transferred to a PVDF membrane by applying a constant current of 250mA on a wet chamber containing a tris-glycine buffer with 20% methanol. The membrane was blocked for 30 minutes with 3% dried skimmed milk (Marvel) in wash buffer followed by an overnight incubation of the blocked membrane with specific antibodies. The antibody-probed membrane is washed 4 times with wash buffer and then a secondary reagent coupled with the peroxidase enzyme is added for 60 minutes. After this incubation, the membrane is washed again and developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

For detection and immunoprecipitation of mouse CLEC-2, B3Z and B3Z cells expressing CLEC-2 Y7F were lysed on RIPA lysis and extraction buffer (Pierce). Half of each lysate was added SDS loading buffer and the other half

was incubated with 2µg of anti-CLEC-2 mAb for 18 hours. Next, there was 60 minutes incubation with GammaBind Plus Sepharose (GE Healthcare. After 3 rounds of centrifugation and washes on RIPA Buffer, the precipitated was resuspended on SDS loading buffer. After running the gel, the membrane was probed with 2µg/ml biotinylated anti-CLEC-2 mAb on wash buffer. The secondary reagent used was ExtrAvidin-peroxidase (Sigma) diluted 1:5000 in wash buffer.

For the anti-phosphorylated Tyrosine it was used the mouse monoclonal antibody clone 4G10 (Milipore) followed by a secondary rabbit anti-mouse-HRP (1:5000). For the anti-phosphorylated Syk (Tyr352) it was used a rabbit polyclonal (Cell Signaling, cat. 2701) and for anti-Syk blots it was used a rabbit polyclonal (2131, gift from Victor Tybulewicz - NIMR). The secondary reagent used was a goat anti-rabbit-HRP (Santa Cruz) 1:5000 dilution.

2.3.6 Generation of FLT3L and GMCSF BMDCs

Bone marrow was obtained from femur and tibias by flushing the bones with R10. For FLT3L BMDC the cells were centrifuged at 15000 RPM for 5 minutes and then resuspended in 2ml of red blood cell lysis buffer (Sigma) and incubated for 2 minutes in ice. Next, I added 25ml of R10 and centrifuged cells at 1500RPM for 5 minutes. After centrifugation cells were resuspended in R10 containing 100ng/ml of mouse FLT3L (R&D Systems) at $1.5x10^6$ cells/ml and cultured in 4ml in a 6-well culture plate for 9 days.

For GMCSF BMDC I cultured 4ml of R10 containing 1:2000 dilution of GM4 (GMCSF-from Cancer Research UK services) and $1.5x10^6$ cells/ml in a 6-well culture plate. Two days later 2ml of the culture medium was taken off and replenished with fresh 2.5ml R10 containing 1:10000 GM4. On the next day all culture medium was replenished with fresh 4ml of R10 and 1:20000 GM4. Two days later the cells were harvested and enriched for CD11c by MACS.

2.3.7 MACS enrichment

For GMCSF BMDC enrichment I harvest the GMCSF culture and centrifuged the cells at 1500 RPM for 5 minutes. The cells were then

resuspended in 140µl MACS buffer containing 60µl of CD11c MicroBeads (Miltenyi Biotec) and incubated for 15 minutes on ice. After the incubation I added 20ml of MACS buffer and centrifuged cells at 1500RPM for 5 minutes. The cells were resuspended in 1ml of MACS buffer and passed through a 70µm cell strainer (BD). The cells were then loaded into LS column (Miltenyi Biotec) that had been equilibrated with 5ml of MACS buffer and attached to a MidiMACS Separator on a MACS MultiStand (both from Miltenyi Biotec). Next I washed the column with 3mls of MACS buffer to and waited for all the liquid to drain, allowing the unstained cells to drain off the column. This wash was repeated two more times. After the third wash I loaded 5 ml of R10 and flushed the cells into a tube by applying force with the column plunger.

For CD11c enrichment from splenocytes, the spleen were obtained from C57BL/6J mouse and treated for 20 minutes with Collagenase II (Roche) and DNase (Roche) and passed through a 45µm cell strainer (BD Pharmingen). I added 20 ml of MACS buffer and centrifuged cells at 1500 RPM for 5 minutes. Next cells were resuspended in 190µl of MACS buffer and 10µl of CD11c MicroBeads (Miltenyi Biotec). In the sequence I followed the same procedures as described above.

For B3Z.Syk CLEC-2 enrichment, I resuspended harvested cells into 200µl of MACS buffer containing 10µg/ml of anti-CLEC-2 biotinylated. After 15 minutes incubation I added 20 ml of MACS buffer and resuspended the cells in 180µl of MACS buffer and 20µl of Streptavidin MicroBeads (Miltenyi Biotec). Next I followed the same procedures described above.

2.3.8 CLEC-2 triggering

The indicated cells were cultured in R10 with the indicated concentration of LPS or Pam3Cys. For PDPN.Fc triggering, the cells were cultured on 240 mJ/cm^2 UV-irradiated ELISA plates (Nunc) that had been coated overnight with 50µl PBS containing 40µg/ml of F(Ab')2 donkey anti-human IgG (Jackson Immunoresearch Laboratories). After overnight incubation the coated wells were washed by adding and discarding 200µl of PBS for four times. The wells

where then coated with supernatants from Bioreactors of PDPN.Fc, PDPN T34A.Fc or dectin-1.Fc (control Fc).

For triggering with anti-CLEC-2, the cells were cultured on 240 mJ/cm^2 UV-irradiated ELISA plates (Nunc) that had been coated overnight with 50µl PBS containing 40µg/ml of anti-CLEC-2 or isotype mAb. For triggering with Fabs, 240 mJ/cm^2 UV-irradiated ELISA plates (Nunc) had been coated overnight with 50µl PBS containing 40µg/ml of F(Ab')2 goat anti-rat IgG (Jackson Immunoresearch Laboratories). After incubation the coated wells were washed by adding and discarding 200µl of PBS for four times. The wells where then coated with 20µg/ml of anti-CLEC-2 or control Fab.

For CLEC-2 triggering and western blot analysis, 1x10^5 cell/well in 200µl were centrifuged at 1000RPM for 30 seconds into wells coated with anti-CLEC-2 or isotype mAbs (4 wells per treatment). The medium was discarded at the indicated time point and the cells were lysed on in 30µl RIPA buffer containing 100mM NaF and 1µM of Na_3VO_4. The corresponding wells were pooled together and analysed by western blot as described above.

2.3.9 Quantitative PCR (qPCR)

GMCSF BMDCs were treated as described above and the RNA was extracted at the indicated time point by RNeasy Midi Kit (Qiagen) instructions. The extracted RNA was subjected to reverse transcription (RT) by following instructions of Superscript II (Invitrogen). I used 1µg of RNA and random primers to in a 20µl reaction to generate cDNA. Next, I added 80µl of 0.1µg/ml of glycogen (Aqueous solution from Blue Mussels) (Sigma).

For qPCR using sybr green as dye, 3µl of cDNA was added to wells on 96-well plates containing 10µl of 2X SYBR Advantage qPCR Premix (Clontech), 6.8µl of water and 0.2µl of 10µM primer mix. The primers used were designed by past members of the Immunobiology Lab.

For qPCR using Taqman system, 3µl of cDNA was added to wells on 96-well plates containing 10µl of 2X Taqman Universal PCR Master Mix (Applied Biosystems), 6.8µl of water and 1µl of primer mix. The primers were acquired with FAM dye from Applied Biosystems.

On both qPCR applications I obtained CT values an used the ∆ct method to analyse the data.

2.3.10 ELISA

ELISA was performed by overnight incubation of primary coating antibody in 50µl PBS per well in 96 well Maxisorb immunoplates (Nunc, Denmark). Next, wells wee washed four times with wash buffer and incubated with blocking buffer for a minimum of 60 minutes. Wells were then incubated for 4 hours with supernatants and standard concentrations of recombinant cytokines diluted in culture medium. After the incubation, wells were washed five times and incubated with secondary detection antibody diluted in blocking buffer for 1 hour followed by five washes. Next, wells were incubated with Streptavidin – Alkaline phosphatase (Sigma) diluted 1:5000 in blocking buffer for 60 minutes. The next step was to washed the wells for six times with wash buffer and proceed with developing with SIGMA*FAST*™ p-Nitrophenyl phosphate Tablets tablet (Sigma). Readings were made on spectrophotometer at 405nm.

The following antibodies were used:

Table 6 Antibodies used for ELISA

Antibodies	Capture	Detection
IL2	JES6-1A12 (4µg/ml) (BD Pharmingen)	JES6-5H4-Biotin (1µg/ml) (BD Pharmingen)
IL12/IL23p40	C15.6 (5µg/ml) (BD Pharmingen)	C17.8-Biotin (1µg/ml) (BD Pharmingen)
IL10	JES5-2A5 (4ug/ml) (BD Pharmingen)	SXC-1- Biotin (1.5ug/ml) (BD Pharmingen)
TNF	AF-410-NA (2µg/ml) (R&D)	BAF410-Biotin (250ng/ml) (R&D)

2.3.11 Cytometric Bead Array (CBA)

CBA was performed following manufacturer's instructions. All CBA were performed by using mouse flex set (BD Pharmingen) for measuring the indicated inflammatory mediators. The assay was performed on a FACScalibur and analysed by FlowJo (TreeStar)

2.4 Generation of monoclonal antibody against mouse CLEC-2

2.4.1 Immunisation and serum testing

I immunised Wistar rat with 240 mJ/cm^2 UV-irradiated 10x10^6 RBL-ζ.CLEC-2-HA and 1μg/ml of LPS via intraperitoneal injection. This procedure was repeated another two times with 10 days interval. Two days after the last immunisation I obtained blood sample from the tail vein of rat and generated serum. The serum was tested for the presence anti-CLEC-2 antibodies. For this, I incubated B3Z. ζ.CLEC-2-HA-GFP mixed to B3Z cells with or without several serum dilutions (in FACS buffer) for 20 minutes. After the incubation, cells were washed in FACS buffer and incubated for another 20 minutes with 10μg/ml of F(Ab')2 goat anti-rat-Cy5 (Jackson Immunoresearch laboratories) (fig.2.2).

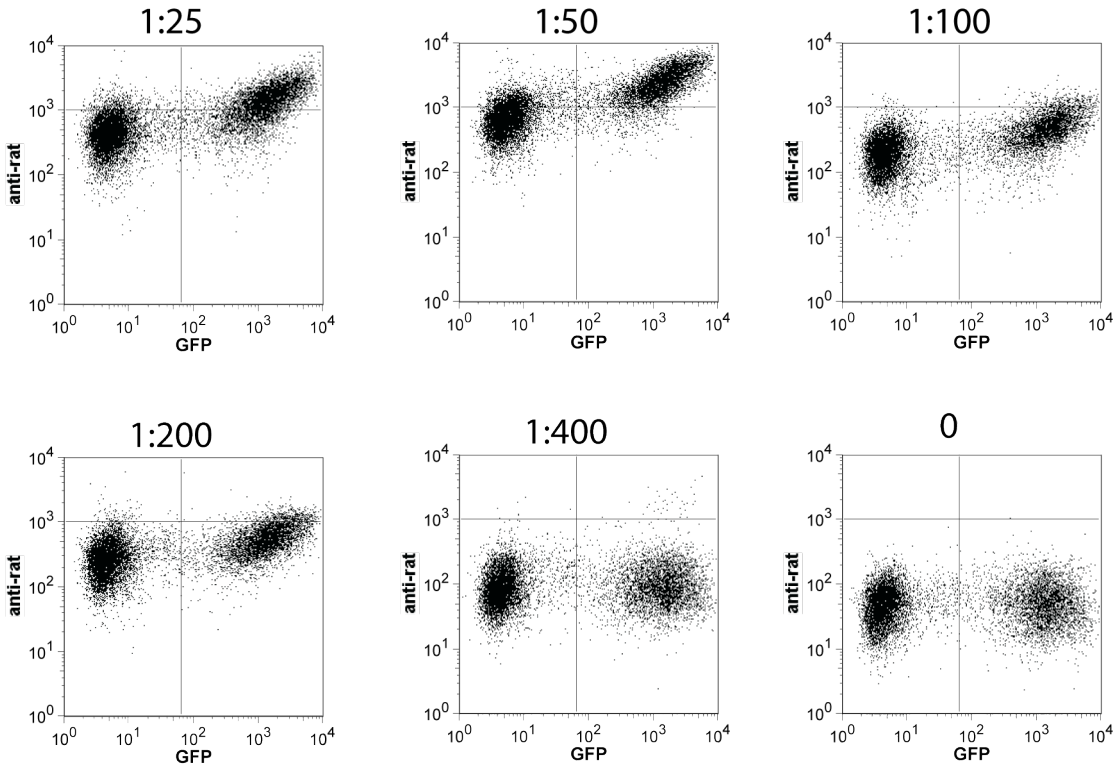

Figure 2.2 Serum from immunised rat contains anti-CLEC-2 antibodies
B3Z. ζ.CLEC-2-HA-GFP and B3Z cells mixed at 1:1 ratio were incubated with or without (0) the indicated dilution of serum from immunised rat. After 20 minutes the cells were washed and incubated for 20 minutes with 10µg/ml of F(Ab')2 goat anti-rat-Cy5. Cells were analysed by FACS for GFP and anti-rat staining.

2.4.2 Hybridoma fusion

Two days after the test bleed I sacrificed the rat and obtained the spleen. The Splenocytes were obtained by flushing the spleen with injections of DMEM. After several flushings the spleen was mashed and passed through a 70µm cell strainer (BD Europe). The cell suspension was centrifuged at 1400 RPM for 5 minutes. Cells were resuspended on 10ml of Red Blood Cells Lysis Buffer (Sigma) for 5 minutes to lyse erythrocytes. Cells were washed twice on DMEM by centrifugation at 1400 RPM for 5 minutes. Rat Y3 myeloma cells were used to fuse with rat splenocytes. These cells were grown on D10 and washed twice on DMEM by centrifugation at 1400 RPM for 5 minutes.

I mixed the splenocytes and Y3 cells at a ratio of 2:1 (splenocytes:Y3), using all splenocytes. The mixture was centrifuged and resuspended in 1.2ml of

DMEM. The fusion was performed with continuous stirring on 12 minutes on a 37^{o}C water bath by the following procedures:

0-1min – addition of 1.2ml of PEG1500 (Boehringer)

1-2min – stirring

2-3 min – addition of 1ml of DMEM

3-4min - addition of 4ml of DMEM

4-5mim - addition of 5ml of DMEM

5-7min - addition of 10ml of DMEM

7-12min - stirring

Next, cells were centrifuged at 1400 RPM for 5 minutes 25^{o}C with low break and resuspended in 240ml of DMEM supplemented with 20%FCS, 2ng/ml of rat IL-6 (hybridoma medium). Cells were plated in 8 U-bottom 96-well plates in 200µl. Next I added another 80ml of hybridoma medium and plated the rest of cells in 200µl in U-bottom 96-well plates.

The culture medium was changed every 2 days by removing 120µl and adding 150µl of hybridoma medium. This procedure was repeated until confluent grow of cells in most of wells.

2.4.3 Screen and cloning

The screen for anti-CLEC-2-producing hybridomas was performed by capturing rat antibodies from supernatants of hybridoma culture medium. This was done by adding 120µl supernatants to flat bottom 96-well plates coated overnight with 50µl of PBS containing 20µg/ml of goat anti-rat IgG (H+L) (Jackson Immunoresearch laboratories). The supernatant was incubated for 4 hours followed by three washes with 200µl of PBS. On each plate two wells were incubated only with mouse monoclonal anti-HA diluted 1:400 (ascites fluid from Sigma) for use as a positive control. Next, a B3Z assay for ligand screen was performed (as described previously and shown below on figure 2.3).

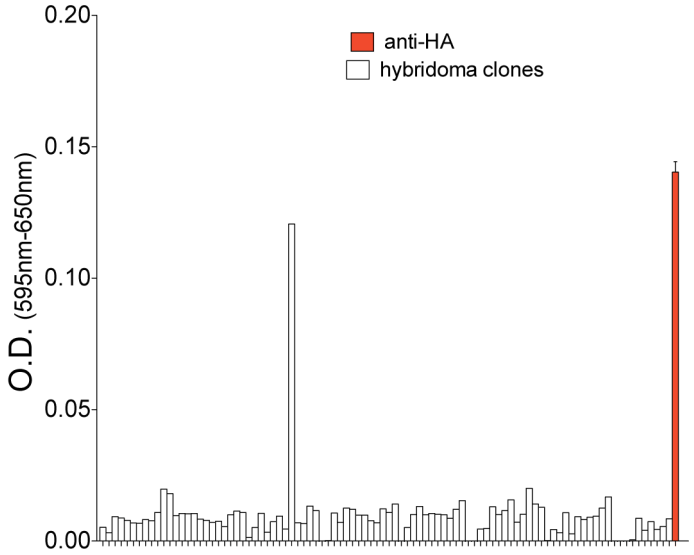

Figure 2.3 B3Z assay for screening for anti-CLEC-2 antibody
B3Z. ζ.CLEC-2-HA cells were cultured for 18 hours on wells coated with anti-HA or anti-rat IgG and hybridoma supernatant. High LacZ activity indicates wells containing anti-CLEC-2 antibodies.

All wells tested positive on the assay had the cells collected and expanded for further analysis. Supernatant of these cells were tested for specific staining of B3Z.ζ.CLEC-2-HA in a mix with B3Z cells (as described on the previous section, figure 2.1).

Next, the supernatants were tested for specific staining of CLEC-2 and not the HA tag. This was done by comparing staining of B3Z.ζ.CLEC-2-HA with B3Z.ζ.dectin-1-HA. Consequently, I selected two cell populations (5F11 and 17D9) that selectively stained CLEC-2-expressing cells.

These two cell populations were plated at one cell per well in five different U-bottom 96-well plates until grow to 90% confluence. Each clone was tested for selectively CLEC-2 staining. This process was repeated three times, until all the clones tested positive for CLEC-2 staining. Finally the cells were considered derived from the same unique clones.

2.4.4 Antibody purification and Fab generation

Hybridoma clones were cultured on two-compartments disposable bioreactor (Integra bioscience) according to manufacturer instructions. The cells were cultured on R10 and the medium was changed every three days. Concentrated supernatant was collected by centrifuging cells at 2000 RPM for 5

minutes and filtering through 0.45µm Millex-HV PVDF filter (Millipore). Both rat anti-mouse CLEC-2 (IgG2b) monoclonal antibody (mAb) and a rat IgG2b mAb (isotype with irrelevant specificity) were purified using GammaBind Sepharose Plus (GE Healthcare) following manufacturer's instructions. Purity was tested on a 4-20% Tris-glycine gel followed by a coomassie blue staining (fig.2.3).

For generation of anti-CLEC-2 or control Fab I used the Pierce Fab preparation kit (Pierce-Thermo Scientific) following manufacturer's instructions. The purity was tested by separation on a 4-20% Tris-glycine gel followed by a coomassie blue staining (fig.2.3).

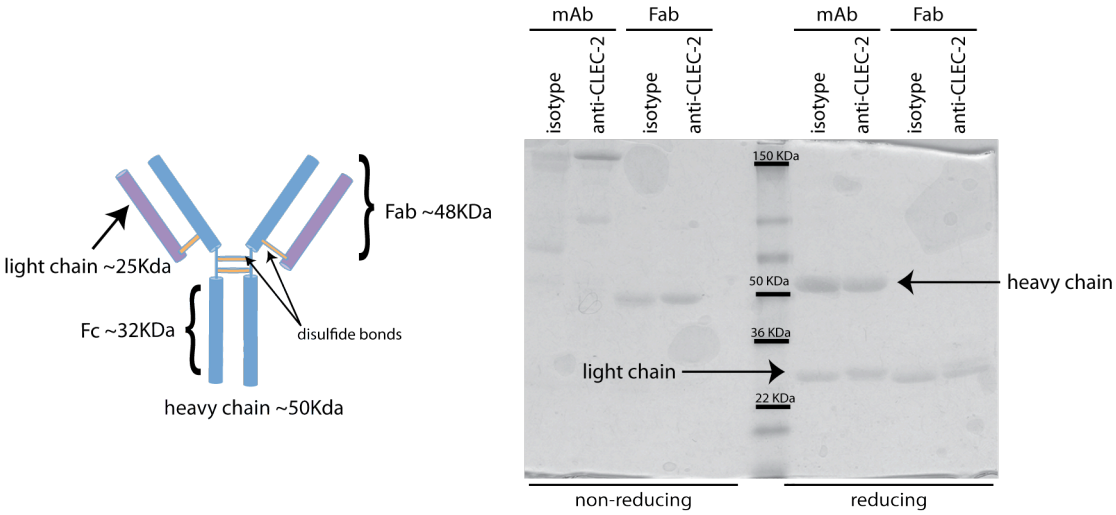

Figure 2.4 Analysis of Fab and mAb purification
1 µg of mAb or Fab were loaded into a 4-20%Tris-Glycine gel with (reducing) or without (non-reducing) 10mM β-mercaptoethanol. Gel was run at 100V, stained with coomassie blue solution (water 0.01%coomassie, 40%methanol, 10%glacial acetic acid) for 20 minutes and washed on destaining solution (water 10% glacial acetic acid 20%methanol).

2.4.5 Anti-CLEC-2 modifications

I generated anti-CLEC-2 biotinylated by following instructions on DSB-X Biotin Protein Labelling Kit (Molecular Probes-Invitrogen). For anti-CLEC-2 coupling to Alexa 647 I followed instruction on Alexa Fluor 647 Protein Labelling Kit (Molecular Probes – Invitrogen).

2.5 Microarray

GMCSF BMDCs were cultured with 10ng/ml of LPS on wells coated with control or anti-CLEC-2 Fab, as described above. After 3 or 6 hours, corresponding wells were pooled together and RNA was extracted using RNeasy Midi Kit (Qiagen). This assay was performed twice and extracted RNA was pooled together and sent to microarray analysis at Cancer Research UK Paterson Institute Microarray Service. In summary, I performed genome-wide analysis of genes derived from RNA samples of LPS-stimulated GMCSF BMDC treated either with anti-CLEC-2 Fab for 3 and 6 hours or control Fab treated for 3 or 6 hours. The RNA is a product of 2 independent assays.

2.5.1 Microarray Analysis

Gavin Kelly and Probir Chakravarty (Bioinformatics and Biostatistics Services – CRUK/LRI) performed all microarray analysis. The Affymetrix GeneChip Mouse Genome MOE430_2 was used to define gene expression profiles in each sample. Probe synthesis and microarray hybridization were performed according to standard Affymetrix (Santa Clara, CA) protocols.

Data was analysed using Bioconductor 1.9 running on R 2.6.0. Normalised probe-set expression measures were calculated using the 'affy' package's Robust Multichip Average (RMA) default method. Differential gene expression was assessed between anti-CLEC-2 vs control at each time point and different response between time-points using two-way ANOVA, with empirical Bayes (limma package) to help estimate variance; p values were adjusted for multiple testing using the Benjamini-Hocberg method. Any probe-sets that exhibited an adjusted p value of 0.05 were called differentially expressed. There was total of 274 probe-sets, which showed differential expression between 3 hours and 6 hours time points.

2.5.2 Gene Set Enrichment Analysis

Differentially expressed probes were used to determine enrichment using Genego pathways and processes within Metacore pathway tool (Genego Inc, St.Joseph, MI). The analysis employs a hyper-geometric distribution to determine the most enriched gene-set. A number of processes and pathways

appeared to be enriched when using the 274 probe-set list. Metacore was used to determine a list of experimentally derived target genes for the transcription factor NFAT1.

2.5.3 Heatmaps

Two-dimensional (2D) hierarchical clustering of expression data using differentially expressed genes that occurred in Immune response - NFAT signalling biological processes or NFAT1 target genes was performed. Genes were clustered using a Euclidean distance matrix and average linkage clustering using Cluster and visualised using Java TreeView. Samples were not subjected to any clustering methods.

2.6 FACS acquisition

All staining were done on V-bottom 96-well plates in 100µl. The cells were acquired either at FACScalibur (BD) or LSR II (BD). Samples were analysed by FlowJo (TreeStar).

2.6.1 Anti-CLEC-2 staining on splenocytes and lymph node cells

Four C57BL/6J mice were injected intravenously with 5µg of LPS (2 mice) or PBS (2 mice) and spleen and lymph nodes were obtained 18 hours later. The organs were treated for 20 minutes with collagenase II (Roche) and DNase (Roche) and then passed through a 40µm cell strainer (BD Pharmingen), added 10 ml of FACS buffer and centrifuged at 1500 RPM for 5 minutes. Splenocytes were resuspended in 5ml red blood cell lysis buffer (Sigma), incubated for 5 minutes, added 20 ml of FACS buffer and centrifuged as before. Cells were plated at $1x10^6$ cells/well in FACS buffer containing 10µg/ml of purified rat anti-mouse CD16/CD32 (BD Pharmingen) and incubated for 15 minutes. Next cells were washed with 200µl of FACS buffer. Cells were than stained for 20 minutes with 10µg/ml anti-mouse CLEC-2 biotinylated or rat IgG2b biotinylated (BD Pharmingen) and different combinations of 5µg/ml antibodies:

- Hamster anti-mouse CD11c-APC, rat anti-mouse CD45R/B220-PE, rat anti-mouse CD41-FITC
- Rat anti-mouse CD11b-Percp, rat anti-mouse CD41-FITC, rat anti-mouse Gr1 (Ly6G and Ly6C)-PE

- Rat anti-mouse CD41-FITC, rat anti-mouse NK1.1-PE, rat anti-mouse CD3-APC

After incubation cells were washed as before and incubated for 20 minutes with 2µg/ml of Streptavidin-PE-Cy7. Next cells were washed as before and 5µg/ml of DAPI was added to FACS acquisition tubes prior to acquisition.

Data represented in the thesis correspond to DAPI negative (dead cells) and SSC-W high negative (correspondent of doublets).

2.6.2 Calcium flux

B3Z.Syk clones were harvested form culture plates and centrifuged and resuspended in RPMI with 1mg/ml of BSA (Cell-loading buffer – CLM). Next, $1x10^6$ cells were resuspended in 1ml CLM with 2µM of Indo-1 (Molecular Probes – Invitrogen) for 30 minutes at 37^oC in a water bath. After, I washed cells by adding 14ml of RPMI and centrifuging cells at 1500RPM for 5 minutes. After wash I stained the cells with anti-CLEC-2 biotinylated or rat IgG2b biotinylated (BD Pharmingen) in PBS for 20 minutes in ice. In the sequence, cells were washed in PBS and resuspended in 500µl of RPMI and acquired on the LSR II. Calcium flux was induced by addition of 2µg of Streptavidin-PE (BD Pharmingen) or 0.5µg of ionomycin. Data was analysed by FlowJo.

2.7 Mouse model of LPS responses

C57BL/6J mice were injected intraperitoneally with 20µg of LPS and the indicated amount of anti-CLEC-2 or isotype mAb. Mice were sacrificed and blood was obtained by cardiac puncture. Sera were generated by incubating blood at 37^oC for 30 minute, followed by centrifugation at 2000 RPM for 5 minutes and collection of supernatants.

2.7.1 Phagocytes depletion

C57BL/6J mice were injected intraperitoneally with 300µl of clodronate-loaded liposomes or PBS-loaded liposomes. Four days later mice were treated as described in “Mouse model of LPS responses” above. Depletion of myeloid cells were analysed by FACS.

Nico Van Rooijen provided loaded liposomes (Van Rooijen and Sanders 1994; van Rooijen and van Kesteren-Hendrikx 2003) and Roche Diagnostics provided clodronate.

2.7.2 Dendritic cell depletion

C57BL/6J (WT) mice were irradiated with a total of 1100 Rad, given in 2 doses of 550 Rad. On the next day I intravenously injected $5x10^6$ bone marrow cells from CD11c-DTR-GFP (Jung et al. 2002) or WT mice. Chimerism was confirmed by analysing GFP expression on CD11c cells on the spleen. Ten weeks later mice were injected intraperitoneally with 100ng of diphtheria toxin (Sigma). On the next day (16-18 hours later) mice were treated as described in "Mouse model of LPS responses" above.

Depletion of DCs was confirmed after the endotoxic shock model by analysing GFP expression and staining splenocytes with hamster anti-mouse CD11c-APC (BD Pharmingen).

Bone marrows from CD11c-DTR-GFP were kindly donated by Clare Bennett (UCL).

2.8 Statistical analysis

The statistical analyses were performed using Graphpad Prism 5 for Mac. The tests and the significance comparison used are stated on the legend of figures.

2.9 CLEC-2 conditional knockout mouse generation

The BAC clone R248K14 (NCBI nomenclature, purchased from Invitrogen) was used as a source of C57BL/6J genomic DNA containing the CLEC-2 gene (*clec1b*). The targeting vector region was obtained from the BAC clone by using Quick and Easy BAC modification Kit (Gene Bridges, Dresden – Germany) instructions. Briefly, the method by which the system works is based on *E. coli* expressing specific exonucleases and annealing proteins. The expression of homologous DNA regions on these bacteria allows homologous recombination between different DNA vectors.

Therefore, a PCR product containing homologous regions to the BAC can be recombined by expression of the linearized vector pFloxRI+TK (from

transgenic lab – Cancer Research UK) with a stretch of DNA (on each end) homologous to BAC regions. This allows homologous recombination between the two DNA pieces and the generation of pFloxRI+TK – Clec1b. The primers used for this were: Clec1b BAC Fwd and Clec1b BAC Rev. The regions in red represent the homology to the pFloxRI+TK vector. Neil Rogers (Immunobiology Lab – CRUK/LRI) cloned the targeting region in pFloxRI+TK.

Next, I started modifying the pFloxRI+TK-Clec1b (targeting region) to express DNA sequences to be inserted into the mouse genome. I used the following selection cassette loxP-gb2-pgk-NEO-loxP (Gene Bridges, Dresden – Germany) to insert between exons 1 and 2 of the targeting region by the method described above (fig.1.5 – step 1). The primers used were 1st loxP FW and 1st loxP RV. The regions in red represent the homology to the selection cassette.

The next step that I performed was to delete this selection cassette by expressing the vector on EL350 (a CRE-expressing *E. coli*). This induced the deletion of the cassette, leaving one loxP in the desired position (fig.1.5 – step 2).

The final step for generation of the targeting vector was to insert a selection cassette and a second loxP site. I used the FRT-gb2-pgk-frt-loxp selection cassette (Gene Bridges, Dresden – Germany) and the following primers to induce homologous recombination in bacteria (fig.1.5 – step 3) (as described above): FW Rb2 and Rv Rb2.

The regions in red represent the homology to the selection cassette.

All insertions and deletion were confirmed by selection of *E. coli* with appropriate antibiotic and by sequencing with the following primers:

Designed for sequencing the insertion and deletion of loxP-PGK-gb2-Neo-loxP cassette: Fwd seq 5'flxNeo and Rv seq 3'flxNeo

Designed for sequencing the insertion of FRT-PGK-gb2-Neo-FRT-loxP cassette: Seq FRT FW Rb2, Seq Rv FRT, Seq Neo Fw and Seq PGK RV.

Finally, the targeting vector was obtained. A maxiprep was performed and the targeting vector was linearized by digestion with SfiI restriction enzyme (New England Biolabs).

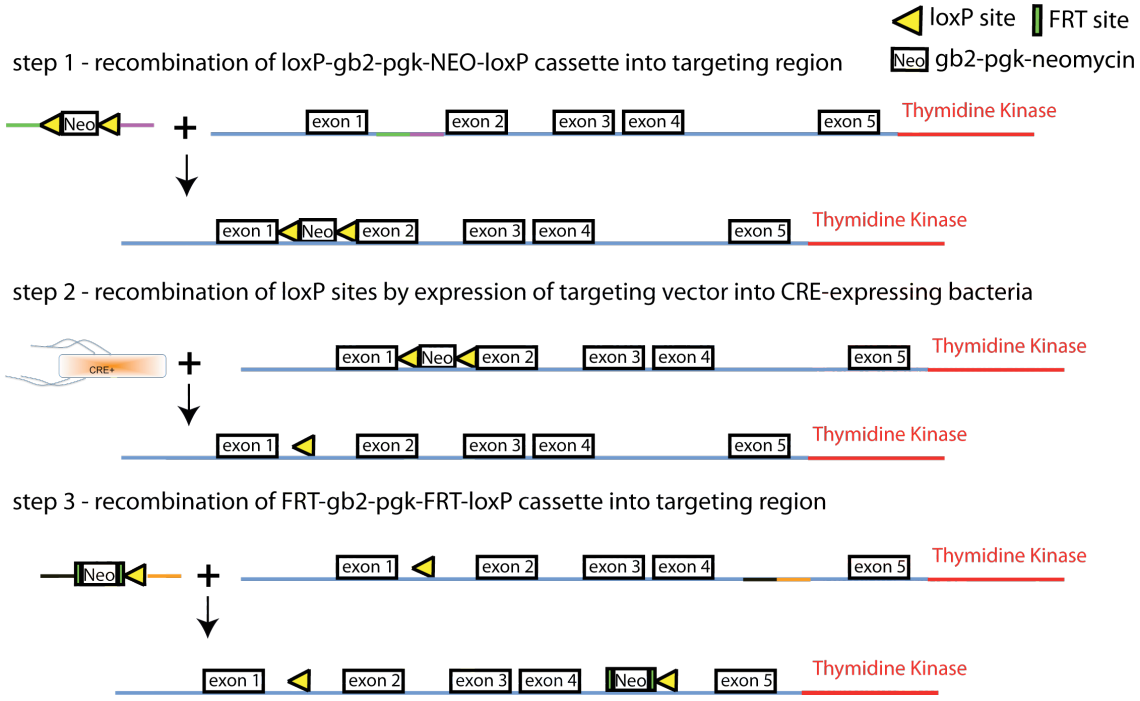

Figure 2.5 Targeting vector construction
The targeting region of CLEC-2 (*clec1b*) gene was cloned into pFLOXRI+TK vector that contains thymidine kinase gene controlled by pgk promoter. CLEC-2 regions (green and purple between exons 1 and 2) in this plasmid were chosen to accommodate loxP-NEO-loxP cassette generated with the homolog regions. The recombination in bacteria generated a plasmid containing loxP-NEO-loxP cassette in the desired region (Step 1). Next this plasmid was expressed in CRE-expressing bacteria to induce recombination loxP sites, generating a plasmid containing a single loxP in the desired region (Step 2). In the final step the FRT-NEO-FRT-loxP cassette was generated containing regions homolog to CLEC-2 regions desired for recombination (black and orange regions between exons 4 and 5). This cassette was recombined with loxP-CLEC-2 plasmid generated in step 2, generating the final targeting construct that contains a loxP site between exons 1 and 2 and FRT-NEO-FRT-loxP between exons 4 and 5.

The targeting vector was transfected into C57BL/6N embryonic stem (ES) cells PRX-B6N (Primogenix Inc, Missouri – US), cloned and selected by the Transgenic Lab – Cancer Research UK. The Transgenic lab provided 96-well plates with ES cell lysed in 50µl of cell lysis buffer (10mM TRIS pH7.5, 10mM EDTA, 10mM NaCl, 0.5% sarcosyl, 2mg/ml proteinase K).

I obtained ES cell DNA from 96-well plates by precipitating by addition of 50µl of isopropanol and 30 minutes incubation. Next I centrifuged the plates at 1200g for 5 minutes. Wells were washed three times with 200µl of 70% ethanol followed by centrifugation as before. Plates were emptied and air-dried for 20 minutes at room temperature.

Wells were resuspended in 50µl of water and DNA was screened by PCR (primers locations are depicted on figure 2.4)

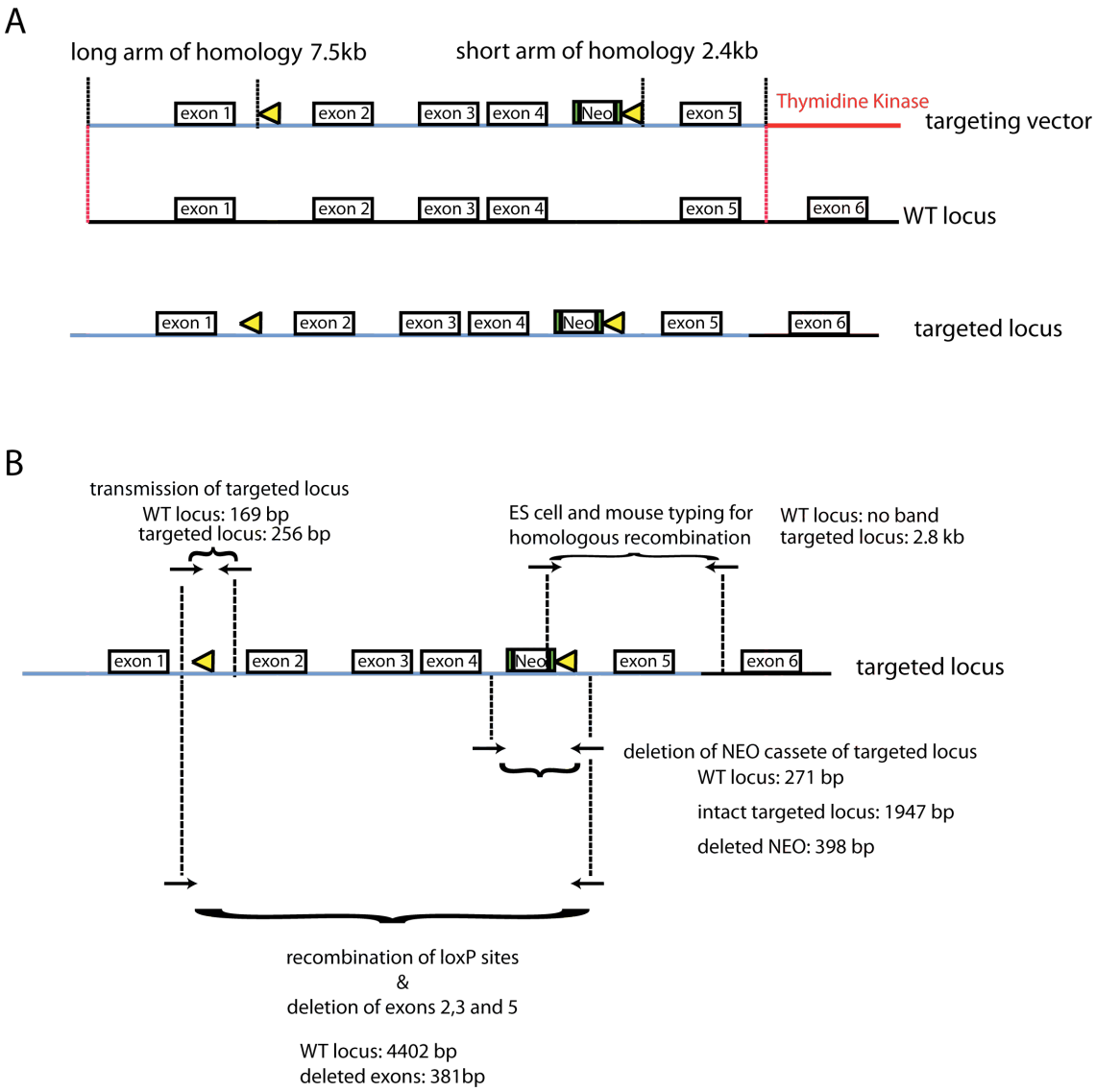

Figure 2.6 Targeting and typing strategy
(A)ES cells were transfected with the targeting vector and selected for resistance to G418 and gancyclovir. (B) ES cells and chimeric mice were screened for the presence of a PCR product of a NEO primer with a primer close to exon 6 (outside the targeting vector). Transmission of targeted allele was confirmed by PCR product of loxP site. Arrows represent primers.

Chapter 3. CLEC-2 ligands and CLEC-2 cellular distribution

3.1 Introduction

The CLR CLEC-2 belongs to the group V of CLR, which contain CTLD that interact with proteins and carbohydrate ligands (Zelensky and Gready 2005). Besides, CLEC-2 and dectin-1 share similar CTLD and signalling motifs, suggestive of related functions. Nevertheless, there is a lack of information regarding CLEC-2 ligands and CLEC-2 expression in the immune system.

At the beginning of this thesis, CLEC-2 had been identified as mRNA expressed on human dendritic cells, monocytes, granulocytes and some NK cell clones (Colonna, Samaridis et al. 2000). Moreover, CLEC-2 was identified as a platelet receptor targeted by the snake venom toxin rhodocytin (Suzuki-Inoue, Fuller et al. 2006).

Collectively, several reports pointed out that CLEC-2 is expressed on different cell types, including myeloid cells. Furthermore, CLEC-2 ligand was identified to be a snake venom toxin that exploits CLEC-2 expression on platelets to induce toxicity, suggesting that CLEC-2 might recognise a different ligand.

Herein I will describe a screen to identify new CLEC-2 ligands. Moreover, I generated rat anti-mouse CLEC-2 monoclonal antibodies and I will use it to analyse CLEC-2 expression on myeloid cells and other leucocytes on normal and inflammatory conditions.

3.2 CLEC-2 ligand screening assay

To screen for CLEC-2 ligands I decided to use a method described by Carlyle and colleagues (Carlyle et al. 2004) used for finding NK cell receptor ligands. This method involves the generation of a chimeric protein, ζCLEC2-HA,

which is a fusion of the intracellular domain of CD3ζ (mentioned as ζ throughout the document), the transmembrane domain of NKR-P1B and the HA-tagged extracellular portion of mouse CLEC-2. This chimeric ζCLEC-2-HA allows signalling by CD3ζ whenever there is interaction between CLEC2 and a ligand. The expression of ζCLEC2-HA on B3Z cells, an NFAT-LacZ reporter T cell line (Karttunen et al. 1992), allows for measuring NFAT activation as readout for CLEC-2 interaction with a ligand (see figure 3.1A). Because of chimeric ζCLEC-2-HA the activation of NFAT-LacZ will indicate binding/clustering of the receptor. As there is no CLEC-2 signalling motif expressed, the assay does not indicate actual agonist of CLEC-2. The quantification of LacZ induction by a colorimetric assay gives a rapid observation of the interaction of CLEC-2 and its putative ligands.

As the ζCLEC-2-HA expression vector contains an IRES-GFP, both the ζCLEC-2-HA and the GFP are made as a single mRNA allowing correlation of the expression of both proteins. To validate the expression of on the surface of B3Z cells, I analysed the anti-HA staining and the expression of GFP on these cells before enriching these cells by sorting. By doing this I will have two populations of cells, a GFP negative and HA- negative and a population that will be GFP- and HA- positive. The anti-HA staining indicates that the extracellular portion of CLEC-2 is expressed on the surface of cells, while GFP negative cells do not show HA staining (fig.3.1B). Making use of the HA tag on ζCLEC-2-HA, I used the anti-HA antibody to induce cross-linking and signalling downstream of the chimeric receptor. As observed on figure 3.1C, anti-HA antibody treatment of B3Z ζCLEC-2-HA, but not B3Z cells, induces NFAT activation on a level similar to ionomycin. Ionomycin had been broadly used as an inductor of intracellular calcium increase because of its capacity to bind and transport calcium to the cytoplasm (Liu and Hermann 1978), which induces NFAT activation.

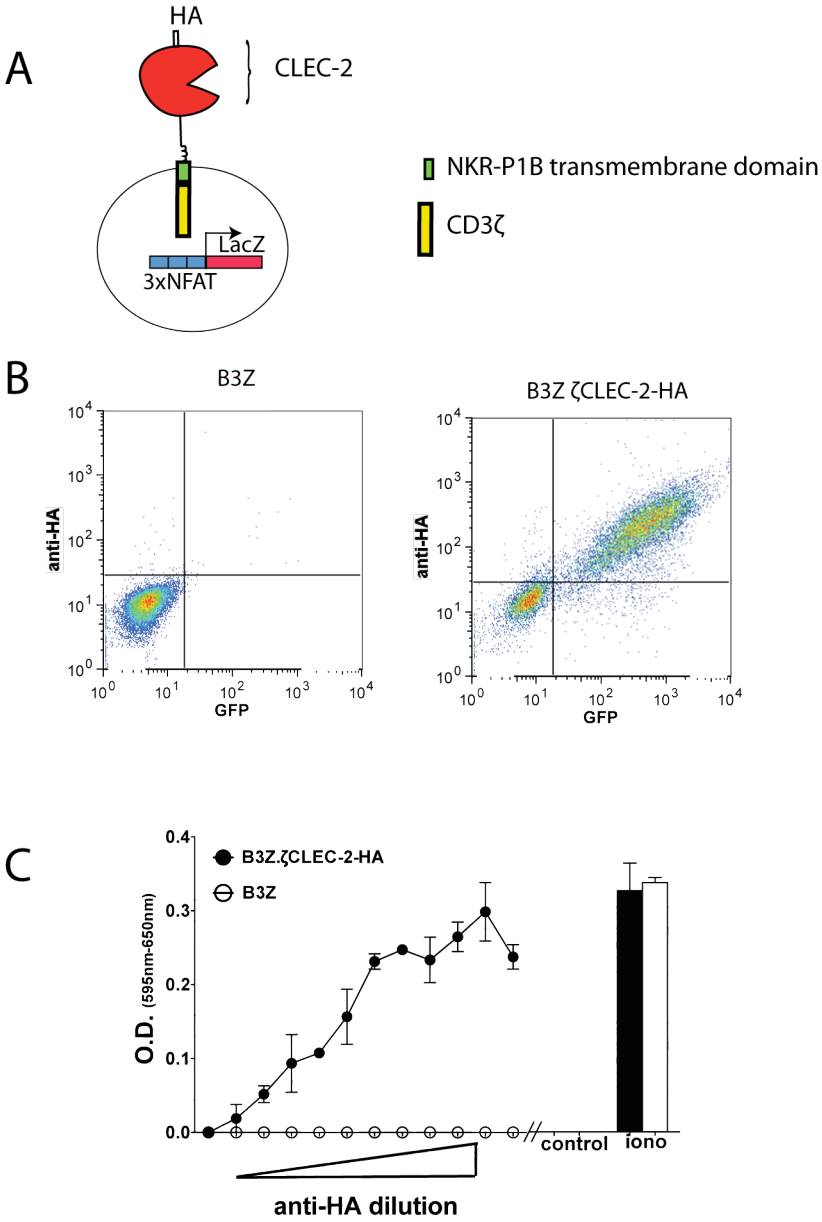

Figure 3.1 CLEC-2 ligand screen
(A) Scheme illustrating the B3Z cells stably expressing an NFAT-lacZ reporter and extracellular CLEC-2 HA-tagged fused to NKRP1B and CD3ζ. (B) B3Z and B3Z. ζ.CLEC-2.HA stained with anti-HA antibody and analysed for HA and GFP expression by FACS. (C) $1x10^5$ B3Z and B3Z. ζ.CLEC-2.HA were cultured for 18 hours on the presence of different dilution of anti-HA monoclonal antibody (higher concentration of 1:50 and two-fold dilution until 1:102.400) or left untreated (control) before quantification of LacZ activity. Data are means ± SD of duplicate wells of at least three independent assays.

3.2.1 Screen for CLEC-2 ligands on pathogens

As dectin-1 recognises β-glucans derived from bacteria and fungi, I hypothesized that CLEC-2, as a dectin-1-like receptor, might also be involved on the recognition of microbes. First of all, I tested different classes of microbes, such as heat-killed gram negative and positive bacteria (*Escherichia coli* and *Staphylococcus aureus*, respectively), *Mycobacterium tuberculosis* and the fungi *Candida albicans*. To control the specificity of the assay, I used B3Z cells expressing ζdectin-1-HA. As shown on figure 3.2, none of the tested pathogens induced NFAT activation in B3Z ζCLEC-2-HA cells whilst anti-HA treatment activated the reporter and *Candida albicans* stimulated NFAT in B3Z ζ.dectin-1-HA cells (fig.3.2A). Zymosan or *Escherichia coli* LPS also did not induce NFAT in B3Z ζCLEC-2-HA cells, but zymosan did in B3Z ζdectin-1-HA cells (fig.3.2B). This assay indicates that CLEC-2, differently to dectin-1, does not interact with any obvious bacteria or fungi-derived molecules. In addition, I decided to look for immunostimulatory complex derived from protozoans or helminthes, such as *Toxoplasma gondii* antigen (STAg) and *Schistosoma* Egg Antigen (SEA). The SEA has been demonstrated to stimulate Th2 responses by acting on DCs (MacDonald et al. 2001) while STAg was shown to induce IL-12 from DCs on a TLR-11-dependent way (Yarovinsky et al. 2005). Nevertheless, neither SEA nor STAg induced NFAT activation in B3Z ζCLEC-2-HA cells, indicating that CLEC-2 does not interact with it (figure 3.2C).

Another class of microbes assayed for binding to CLEC-2 was viruses. The CLR DC-SIGN was already shown to interact with HIV, with the virus exploiting this interaction for infecting DCs (Geijtenbeek et al. 2000). Additionally the CLR CLEC5a was demonstrated to interact with Dengue virus (Chen, Lin et al. 2008). I tested different viruses representing distinct virus families with different genome and capsid types, as: alphavirus single strand positive RNA-strand Semliki Forest Virus (SFV), the paramyxoviridae single strand positive RNA-strand Sendai Virus (SeV), the poxvirus double strand DNA Vaccinia Virus (VV), the double strand DNA Adenovirus (*adenoviridae* family), the

orthomyxoviridae influenza virus (negative single strand RNA) and the picornavirus positive RNA strand encephalomyocarditis virus (EMCV). As represented on figure 3.2D, none of the live viruses tested induced NFAT activation, implying that CLEC-2 does not bind viruses.

Overall, screen for putative CLEC-2 ligands in different classes of microbes was unable to identify any microbe-derived molecule that interacts with CLEC-2. In addition I decided to look for specific pathogens that induce Th17 immunity, a response that is stimulated by the CLR dectin-1 (Leibundgut-Landmann et al. 2007). Consequently, I tried the murine pathogen *Citrobacter rodentium* and *Klebsiella pneumonie*, which induce strong Th17 responses (Mangan et al. 2006) (Happel et al. 2005). Nevertheless, as for the other microbes tested, these two bacteria did not bind to CLEC-2 (fig.3.2E).

In summary, I screened different classes of microbes that contain distinct molecular features, such as bacteria, viruses, protozoans and helminthes. Nonetheless, none of the microbes tried interacted with CLEC-2, suggesting this receptor do not recognise microbe-derived molecules.

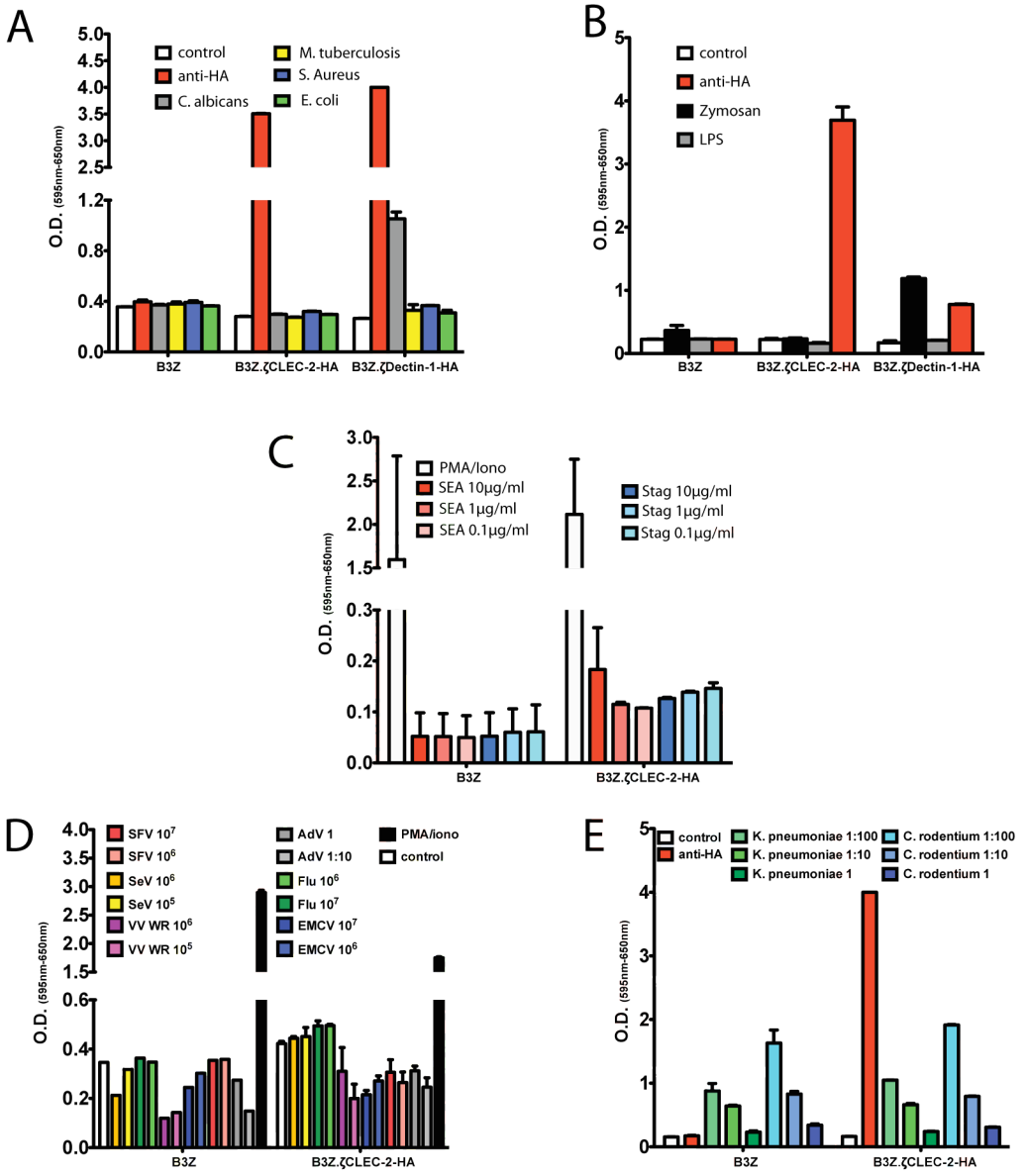

Figure 3.2 CLEC-2 does not interact with microbes

$1x10^5$ B3Z and B3Z. ζ.CLEC-2.HA were cultured for 18 hours on culture medium (control) or on the presence of different stimuli before quantification of LacZ activity. (A) $1x10^6$ fixed *Candida albicans*, 10µg/ml of *Mycobacterium tuberculosis* extract, 100µg/ml of *Staphylococcus aureus* extract, 20µl of fixed overnight *Escherichia coli* overnight culture. (B) 100µg/ml of zymosan, 100ng/ml of LPS. (C) Schistosoma egg antigen (SEA) and soluble tachyzoite antigen (Stag) from *Toxoplasma gondii* were added at indicated concentrations. (D) Semliki forest Virus (SFV), Sendai Virus (Sev), Vaccinia Virus West Nile Reserve (VV WR), Adenovirus (AdV), Influenza virus (Flu) and Encephalomyocarditis virus (EMCV) were added at the indicated concentrations. (E) Overnight cultures of *Klebsiella pneumoniae* and *Citrobacter rodentium* were fixed with paraformaldehyde (PFA) and added to the cells at the indicated dilution. Data are means ± SD of duplicate wells of at least three independent assays.

3.2.2 Endogenous molecules screen for CLEC-2 ligands

As the ligand screen did not show any obvious role for CLEC-2 in the recognition of microbe molecules, I decided to test whether CLEC-2 could interact with endogenous molecules. In fact, many CLRs interact with endogenous molecules, as the case of several NK cell receptors (Raulet and Vance 2006) and the CLR CLEC12a (Pyż, Huysamen et al. 2008). Moreover, dectin-1 was shown to bind to an unidentified ligand on T cells (Ariizumi, Shen et al. 2000).

To screen for endogenous molecules, I cultured the B3Z ζCLEC-2-HA with cell lines from different species, such as monkey, human or mouse (fig.3.3A). Interestingly, among the cell lines tested, the mouse fibroblast cell line 3T3 and the B16 mouse melanoma cell line specifically activated NFAT in B3Z ζCLEC-2-HA cells. 3T3 and B16 cells are transformed cells making it impossible to determine if the ligand(s) represent self or altered self-molecules. To resolve this issue, I obtained cell suspensions from organs isolated from C57BL/6. By doing this I expected to be testing rather self than altered self-molecules. Amongst the cell suspensions tested, the lung cells showed strong NFAT activation in B3Z ζCLEC2-HA, indicative of expression of CLEC-2 ligand(s) (fig.3.3B).

The lung is a mucosal surface that is constantly exposed to airborne microbes and is composed of leucocytes and endothelial cells. Therefore, I decided to test if inflammatory leucocytes could be a source of the endogenous CLEC-2 ligand(s). Thus, I isolated inflammatory peritoneal macrophages by challenging mice with thioglycollate. The co-culture of B3Z ζCLEC-2-HA and thioglycollate-elicited macrophages induced the activation of the reporter, indicating that a CLEC-2 ligand is expressed by these macrophages (fig.3.3C). On this experiment I used the 3T3 cells as positive control for the activation of NFAT in B3Z ζCLEC2-HA cells and tried another endothelial-derived cell, the Chinese Hamster Ovarian (CHO) cells. This experiment demonstrated that CHO cells could, as efficiently as 3T3 cells and thioglycollate-elicited

macrophages, induce NFAT in B3Z ζCLEC2-HA (fig.3.3C). In order to determine if the CLEC-2 ligand(s) expressed by monocytes was generally expressed in myeloid cells, I generated bone marrow derived macrophages (BMM) or bone marrow derived dendritic cells (BMDC). I cultivated Raw 264.7 (macrophage cell line), BMM and BMDCs with B3Z ζCLEC-2-HA. On these co-cultures, unstimulated Raw 264.7 and BMMs, but not BMDC, induced NFAT activation (fig.3.3D), demonstrating that macrophages express a CLEC-2 ligand.

The understanding that CLEC-2 interacts with a self-molecule needed to be expanded to determine if this ligand is a membrane-bound or a soluble molecule. Thus, by culturing B3Z ζCLEC-2-HA with supernatant from 3T3, live or fixed 3T3 cells I tested for the presence of a soluble CLEC-2 ligand (fig.3.3E). This assay showed that live and fixed 3T3 cells, but not 3T3 supernatant, activated NFAT. Furthermore, culture of 3T3 with B3Z ζCLEC-2-HA separated by a permeable membrane showed the requirement of physical interaction between both cells for NFAT activation (fig.3.3F). Overall these two assays indicate that CLEC-2 ligand on 3T3 cells is not a soluble factor, and is in fact a membrane-bound molecule.

To better characterise the specificity of CLEC-2 to its ligand, the human CLEC-2 extracellular portion was fused to the CD3ζ chain on the same way as the mouse CLEC-2 and stable B3Z cells were generated (B3Z.ζ.humanCLEC-2-HA). The human CLEC-2 was compared with its mouse equivalent for specificity for ligands. The figure 3.3G shows that the mouse and the human CLEC-2 have the same specificity and both recognise a ligand on the 3T3, CHO and B16 cells, as well as on human endothelial 293.T and the mouse tumour colo 26 cell lines.

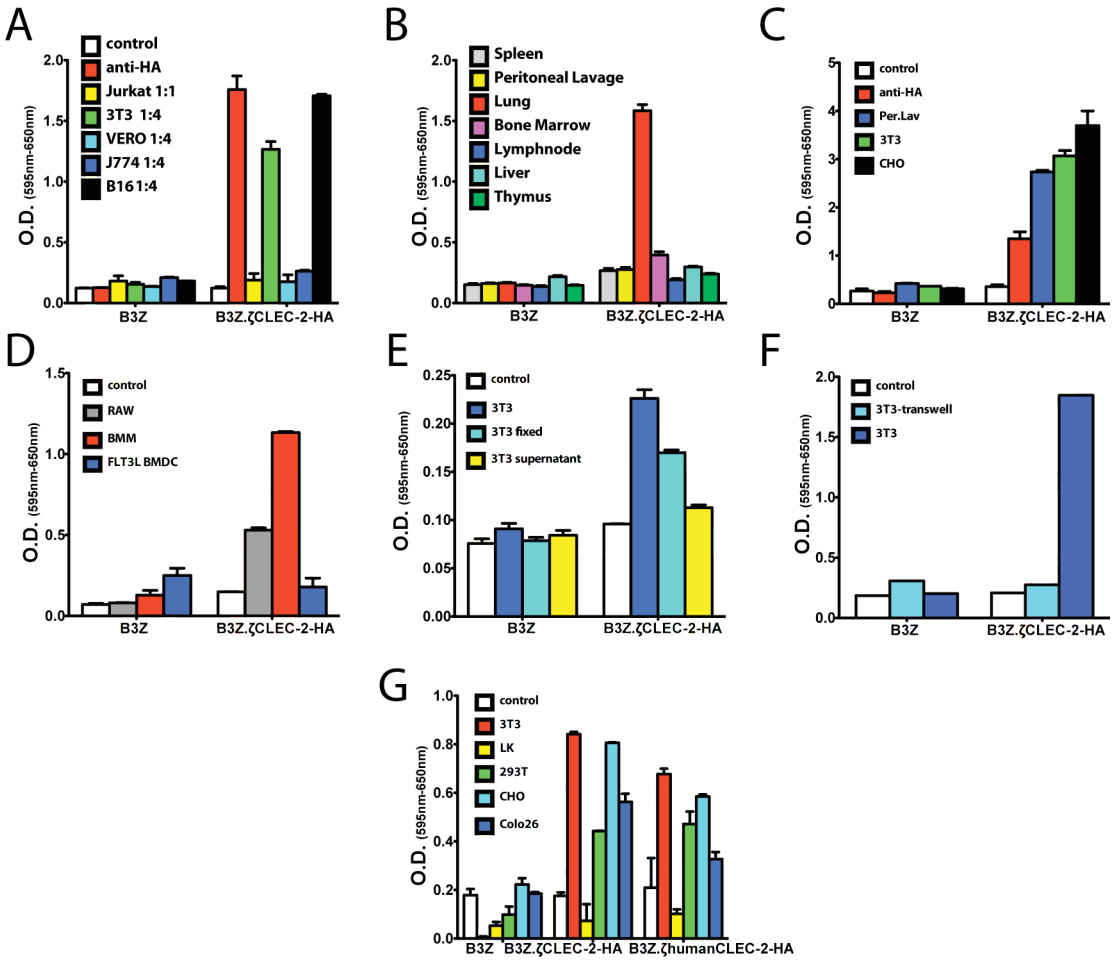

Figure 3.3 Self-molecule(s) bind to CLEC-2

$1x10^5$ B3Z and B3Z.ζ.CLEC-2.HA were cultured for 18 hours on culture medium (control) or on the presence of different stimuli before quantification of LacZ activity. (A) The corresponding cell lines were added at the cell line: B3Z ratio indicated. (B) C57BL/6J tissues were obtained and digested with collagenase and DNase to obtain cell suspensions. From this, $1x10^5$ cells were co-cultured with B3Z clones. (C) $0.25x10^5$ CHO or 3T3 cells or $1x10^5$ thioglycollate-elicited peritoneal lavages cells were added to B3Z clones culture. (D) $1x10^5$ Raw 264.7, bone marrow macrophages (BMM) or FLT3L BMDC were cultured with B3Z clones. (E) $0.25x10^5$ live or PFA fixed 3T3 cells were culture with B3Z clones (F) 3T3 cells were cultured with B3Z clones separated or not by transwell membrane inserts with 8µm pore size and supernatant from 3T3 cell culture was obtained and used to culture B3Z clones. (G) B3Z.ζ.humanCLEC-2.HA cell were tested as the other B3Z clones for induction of lacZ activity by co-culture with $1x10^5$ LK cells or $0.25x10^5$ CHO, 3T3, 293T or colo26 cells. Data are means ± SD of duplicate wells of at least three independent assays.

3.2.3 Biochemical inhibition of CLEC-2 ligand

Mouse and human CLEC-2 recognises a membrane-bound ligand on macrophages, tumour cells and endothelial cell lines. Nevertheless, the biochemical nature of the CLEC-2 ligand is largely unknown.

In order to have an insight into the molecular nature of CLEC-2 ligand, I treated 3T3 cells with protein or mRNA synthesis inhibitors (anisomycin and actinomycin D, respectively). The inhibition of mRNA or protein synthesis impaired the binding of 3T3 to CLEC-2 (fig.3.4A). This suggested that the CLEC-2 ligand expressed by 3T3 cells needs a constant production of protein to be expressed. To exclude that the inhibitor-treated cells had carried over the inhibitor and this way impairing the reporter on B3Z cells, I added the anti-HA antibody to wells with co-cultures with inhibitors. As observed, the response to anti-HA antibody is equal, irrespectively of the presence of inhibitor-treated cells, indicating no carrying over of drugs.

Lectin receptors are largely characterised as carbohydrate-binding proteins (Drickamer 1999). Hence, I decided to treat 3T3 cells with glycosylation inhibitors to check if this could impair NFAT activation, indicative of lack of CLEC-2 ligand(s). Tunicamycin is a drug that inhibits the first enzyme responsible for N-glycosylation of proteins. As a result of N-glycosylation inhibition on 3T3 cells there was an impaired NFAT activation in B3Z ζCLEC2-HA cells (fig.3.4B). This implies that carbohydrate chains on proteins are needed for optimal expression of CLEC-2 ligand. Therefore I decided to evaluate if the specific blockade of certain carbohydrates could block the binding of 3T3 to CLEC-2. Lectins extracted from plants have been commonly used to determine the carbohydrate composition of glycoproteins due to their high specificity for certain sugar chains. Based on this, I incubated 3T3 cells with two different plant lectins: the GlcNAcβ1-4GlcNAcβ1-4GlcNAc, Neu5Ac binding lectin WGA (wheat germ agglutinin) and the Neu5Ac, Galß1-3N-GalNAc (“T antigen”) binding lectin Jacalin. These two sugar are expressed in different cell types, and the “T antigen” is found to be differentially expressed by MUC1

in tumour cells (Taylor-Papadimitriou et al. 1999). The treatment of 3T3 cells with jacalin impaired the activation of the reporter by 3T3 cells, suggesting that carbohydrate chains composed of sialylated galactose and N-acetylgalactosamine are required for optimal binding of CLEC-2 and its ligand on 3T3 cells (fig.3.4C).

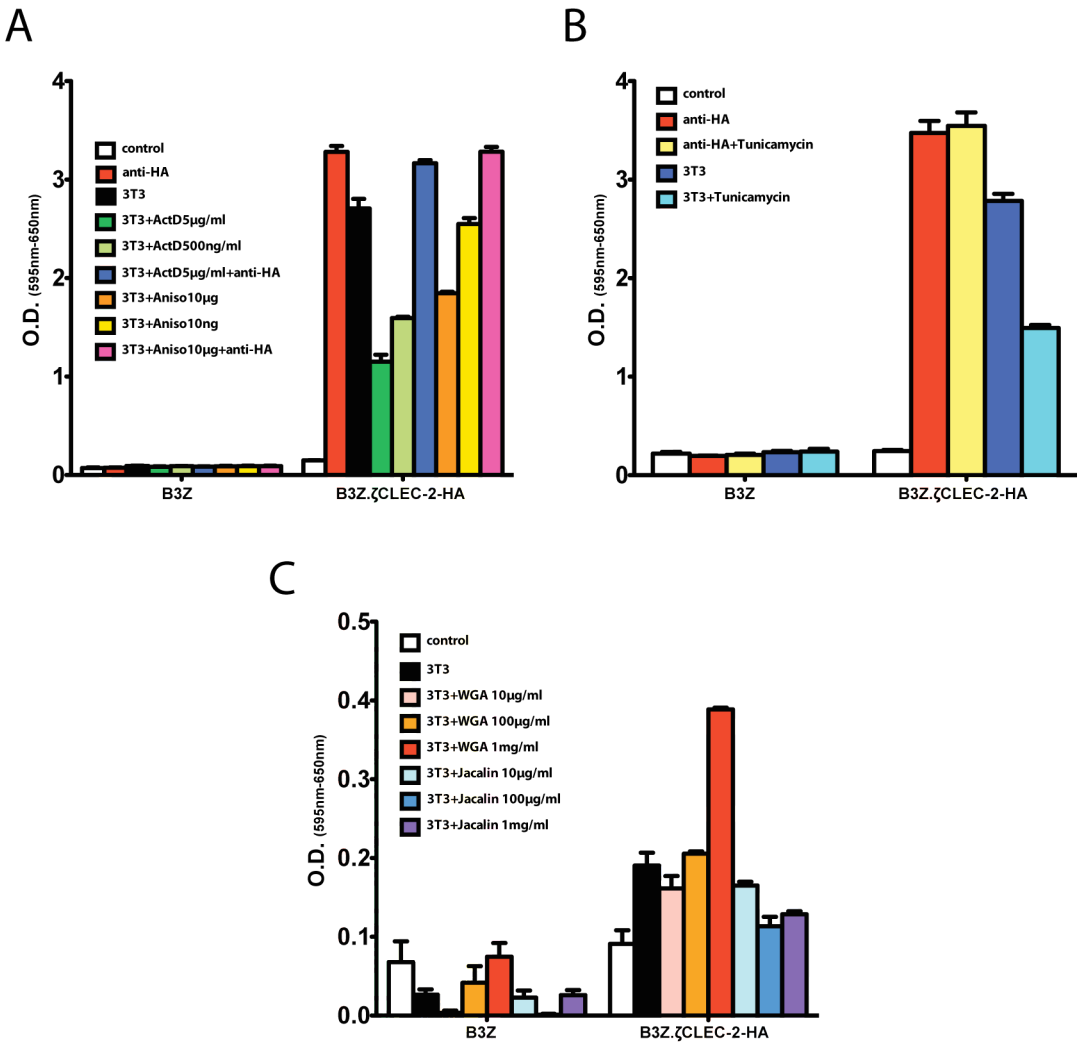

Figure 3.4 Analysis of carbohydrate interaction with CLEC-2

$1x10^5$ B3Z and B3Z. ζ.CLEC-2.HA were cultured for 18 hours on the presence of culture medium (control) or with $0.25x10^5$ 3T3 cells treated with different inhibitors before quantification of LacZ activity. (A) 3T3 were overnight treated with indicated amounts of anisomycin (aniso) or actinomycin D (actD) or (B) 2µg/ml of tunicamycin. (C) 3T3 with the indicated amounts of lectins jacalin or WGA before co-culture with B3Z clones. Data are means ± SD of duplicate wells of at least three independent assays.

To continue assessing the role of specific carbohydrate chains on proteins, I made use of glycosylation deficient CHO cells. These CHO-deficient cells were generated by EMS treatment, a mutagenic compound that induces single nucleotide mutations (Esko 1986). I used four different CHO mutants: CHO pgsD-677 and CHO H661 cells are defective on the generation of heparan sulfate chains (Lidholt et al. 1992; Wei et al. 2000), CHO pgsF-17 are defective on sulfation of heparan sulfate (Bai and Esko 1996) and CHO pgsA-745 are defective on the production of all glycosaminoglycans, including heparan sulfate (Lugemwa and Esko 1991). The co-culture of CHO mutant cells with B3Z ζCLEC-2-HA revealed that the mutants CHO H661 or CHO pgsD-677 did not induce activation of the reporter cells, indicating a lack of CLEC-2 ligand(s) (fig.3.5A). These two CHO lines are defective in Ext1, an enzyme responsible for the generation of heparan sulfate chains (Wei, Bai et al. 2000). Although these two deficient CHO cells suggested that heparan sulfate chains were part of the CLEC-2 ligand, CHO pgsA-745 cells that are also defective in heparan sulfate generation induced the activation of the reporter (fig.3.5A). Staining for heparan sulfate confirmed that the cells correctly identified (fig.3.5B). Reverse-transcription analysis of PDPN expression in CHO cells failed as proper primers for hamster sequences were lacking. Thus it is possible that CHO pgsA-745 still have a CLEC-2 ligand because of PDPN expression. As the presence of the heparan sulfate or other GAGs did not correlate with CLEC-2 ligand presence, I decided to check if the Ext1 enzyme could be responsible to CLEC-2 generation. Hence, I reconstituted expression of Ext1 on CHO H661 cells and tracked its activity by monitoring the surface heparan sulfate (fig.3.5D). Two populations of heparan sulfate-expressing cells are observed, which might be due to a high- and low-expressing Ext1 clones (right and left peaks in figure 3.5D). Given that Ext1 was active and able to produce heparan sulfate I tested these CHO H661-Ext1 on the ligand screen. The Ext1 reconstituted CHO cells were now able to activate the reporter, suggesting that Ext1 is involved in the generation of CLEC-2 ligand(s). As

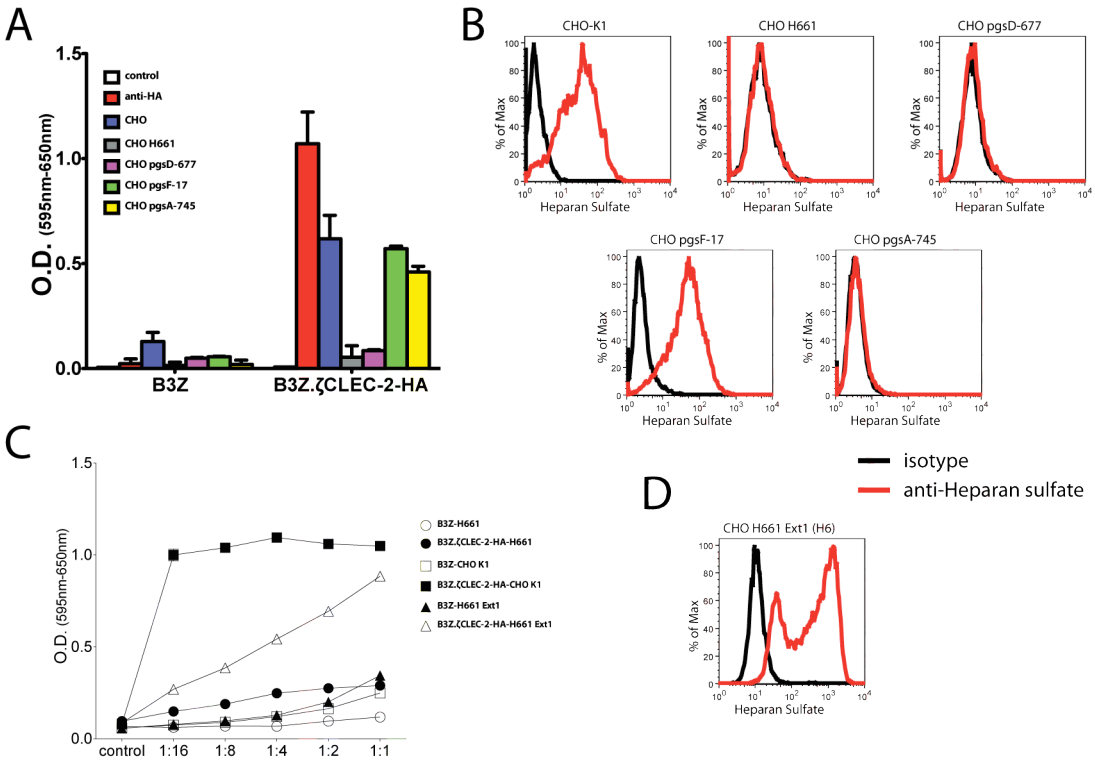

Figure 3.5 The glycosyltransferase Ext1 is involved in CLEC-2 ligand generation

$1x10^5$ B3Z and B3Z. ζ.CLEC-2.HA cells were cultured for 18 hours on the presence of culture medium (control) or with different stimuli before quantification of LacZ activity. (A) $0.25x10^5$ CHO clones (B) CHO clones stained for heparan sulfate. (C) CHO H661 or CHO H661 stably expressing human Ext1 (H661 Ext1) were culture at different numbers with B3Z clones. (D) CHO H661 Ext1 staining for heparan sulfate. Data are means ± SD of duplicate wells of at least three independent assays.

3.2.4 The glycoprotein podoplanin is a CLEC-2 ligand

During the time that I was screening for the involvement of Ext1 and glycoproteins on the binding to CLEC-2, Suzuki-Inoue and colleagues published a paper demonstrating that the endogenous protein podoplanin is a ligand for CLEC-2 (Suzuki-Inoue, Kato et al. 2007). Podoplanin (PDPN) is a mucin-like type I transmembrane glycoprotein largely O-glycosylated (Wicki and Christofori 2007) that had been associated with induction of platelet aggregation via specific extracellular O-glycosylation site named platelet-aggregation (PLAG) domain (Kaneko et al. 2006). The expression of PDPN was described on several human tissues, such as lung (Rishi, Joyce-Brady et al. 1995), kidney (Breiteneder-Geleff, Matsui et al. 1997), skin (Durchdewald et al. 2008) and

lymphatic vessel (Schacht et al. 2003). In addition to these tissues, PDPN expression had been described on the surface of cancer cells promoting metastasis through regulation of spreading and adhesion (Wicki and Christofori 2007). Because of the differential expression of PDPN on lymphatic, but not blood vessels, this protein has been commonly used as a marker for lymphatic endothelia.

In face of this newly reported CLEC-2 ligand, I sought to determine if PDPN was expressed on the cell lines that activated the CLEC-2 reporter on the ligand screen. I stained all the cells tested on the ligand screen for the expression of PDPN. As shown on figure 3.6A, the stimulated peritoneal cells as well as 3T3, colo 26 and B16 cell lines all expressed PDPN. In contrast, the cell lines testing negative in the B3Z assay (B3Z and LK cells) did not show PDPN staining (compare figure 3.3G with 3.6A). The human cell line 293.T and the hamster cell line CHO also did not show PDPN staining, although the anti-podoplanin antibody (anti-PDPN) used for staining was raised against mouse PDPN and probably does not cross-react with human or hamster PDPN. Human 293 cells were later reported to express PDPN and bind to CLEC-2 (Christou, Pearce et al. 2008).

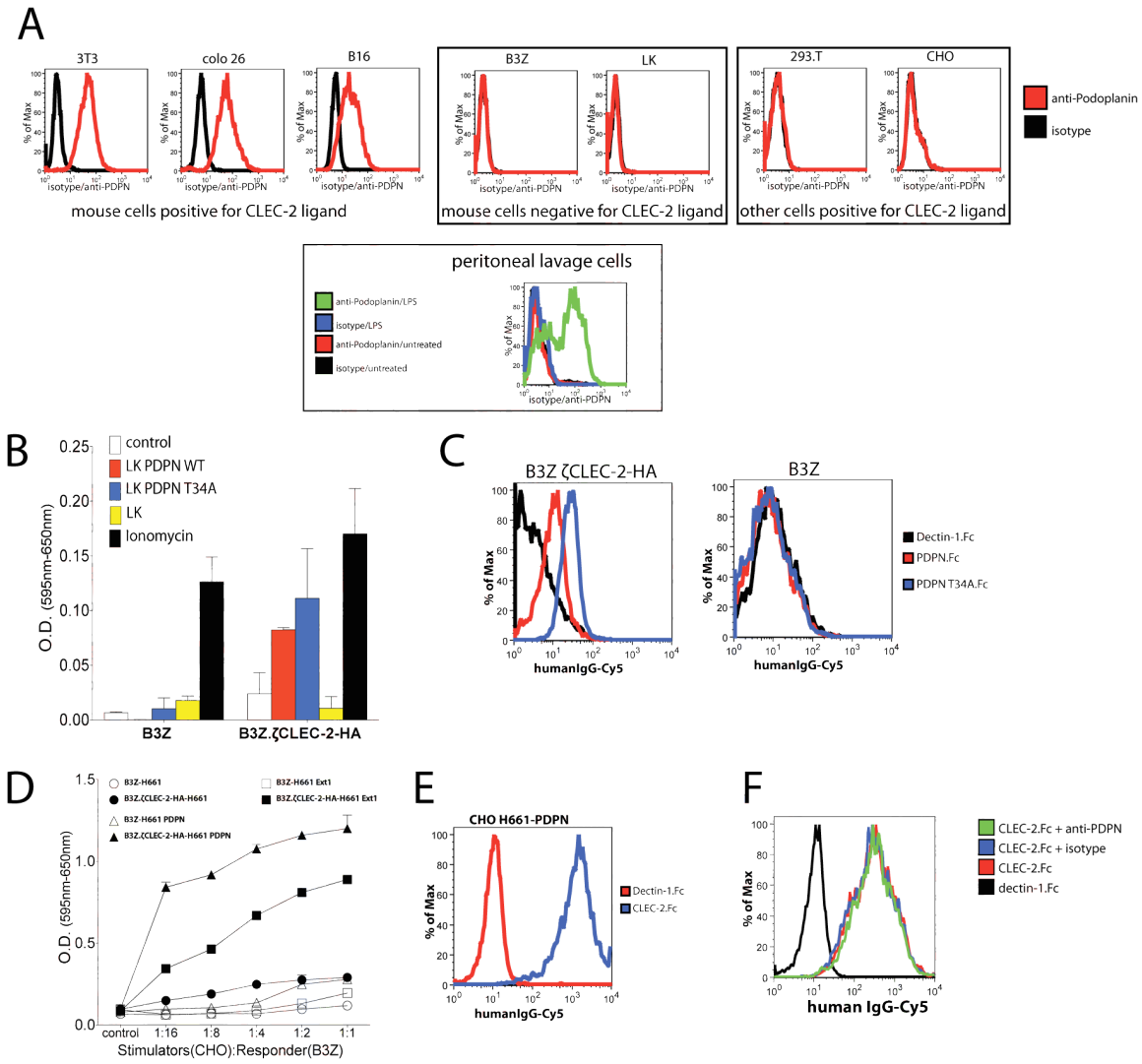

Figure 3.6 The endogenous glycoprotein podoplanin is a CLEC-2 ligand

(A) The indicated cell lines were stained with rat IgG2a-FITC (isotype) or anti-mouse podoplanin-FITC (anti-PDPN). Peritoneal lavages cell were obtained from C57BL/6J mouse and treated overnight with 1µg/ml of LPS before being stained. (B) $1x10^5$ B3Z and B3Z.ζ.CLEC-2.HA were cultured for 18 hours in the presence of culture medium (control), 1µg/ml of ionomycin or $1x10^5$ LK clones before quantification of LacZ activity. (C) B3Z or B3Z. ζ.CLEC-2.HA were incubated for 20 minutes with dectin-1.Fc, PDPN.Fc or PDPN T34A.Fc supernatants before wash and addition of anti-human IgG-Cy5 antibody for another 20 minutes. (D) $1x10^5$ B3Z or B3Z.ζ.CLEC-2.HA cells were incubated with CHO H661, CHO H661 expressing mouse podoplanin (H661 PDPN) or H661 Ext1 cells at different ratios and LacZ activity was quantified after 18 hours. (E) H661 PDPN cells were stained with dectin-1.Fc or CLEC-2.Fc supernatant as described above. (F) H661 PDPN cells were incubated with isotype or anti-PDPN for 20 minutes before staining with dectin-1.Fc or CLEC-2.Fc as described above. Data are means ± SD of duplicate wells of at least three independent assays.

Although I could not formally demonstrate that PDPN was the CLEC-2 ligand on the cell lines tested, I decided to do a gain of function experiment to demonstrate the sufficiency of PDPN to induce CLEC-2 binding. The figure 3.6B indicates that ectopic expression of PDPN on LK cell (negative on ligand screen and for PDPN expression) is sufficient to induce activation of the reporter in B3Z ζCLEC-2-HA, suggestive of binding to CLEC-2 (fig.3.6B).

Ectopic expression of PDPN on cells was sufficient to stimulate binding to CLEC-2. As my previous results pointed to a role of glycoproteins on CLEC-2 interaction, I decided to investigate the requirement of PDPN glycosylation for CLEC-2 binding. Mouse PDPN threonine 34 was shown to be an O-glycosylation site required to induction of platelet aggregation (Kaneko, Kato et al. 2006). Therefore, I mutated this aminoacid and asked whether PDPN was still able to bind to CLEC-2. The ectopic expression of PDPN T34A on LK cell and culture with B3Z ζCLEC-2-HA cells lead to reporter activation, suggesting that PDPN T34A still binds to CLEC-2 (fig.3.6B). Additionally, I cloned and produced PDPN or PDPN T34A as a soluble protein tagged with human IgG1 Fc portion. Staining of CLEC-2 expressing cells with these proteins allowed me to assess the binding of this mutated PDPN to CLEC-2. Both PDPN.Fc and PDPN T34A.Fc stained B3Z ζCLEC-2-HA cells, but not to B3Z cells (fig.3.6C), indicating that PDPN binds to CLEC-2 independent of this platelet aggregation-inducer O-glycosylation site.

As a result of failing to determine the site of PDPN responsible for CLEC-2 interaction, I asked about the involvement of Ext1 on the generation of glycosylated PDPN able to interact with CLEC-2. For this, I expressed mouse PDPN on CHO H661 cells and assayed these cells for activation of NFAT in B3Z ζCLEC-2-HA cells. As shown on figure 3.6D, the ectopic expression of PDPN on Ext1-deficient CHO H661 is sufficient to induce NFAT activation. Due to PDPN and Ext1 being expressed by different vector and, presumably, at different levels it is difficult to state if there are significant differences between

ectopic expression of PDPN and Ext1 in CHO H661 cells. This demonstrated that there is no obvious involvement of Ext1 on glycosylation of PDPN and interaction with CLEC-2. To further verify this fact I produced soluble CLEC-2.Fc chimeric protein and stained CHO H661-PDPN cells. CLEC-2.Fc, but not dectin-1.Fc, stained CHO H661-PDPN cells (fig.3.6E), confirming that Ext1 deficiency does not impair PDPN-CLEC-2 binding.

After determining that the binding of the cell lines to CLEC-2 could be explained by PDPN expression by the cell lines, I tried to determine if PDPN was the only ligand on these cells by specifically blocking PDPN-CLEC-2 interaction. To do this, I needed cells that knowingly express PDPN as the only CLEC-2 ligand and a method to assess this interaction. I decided to treat CHO H661-PDPN with an anti-PDPN antibody or an isotype-matched antibody and then add CLEC-2.Fc to these cells. On figure 3.6F it is shown that anti-PDPN antibody did not block the interaction of PDPN with CLEC-2, thus not being suitable as a tool to determine if PDPN is the only CLEC-2 ligand. Other ways of interfering with CLEC-2-PDPN interaction are yet to be tested such as incubating B3Z ζCLEC-2-HA with PDPN.Fc and incubating PDPN-expressing cells with CLEC-2.Fc before performing the B3Z assay.

3.2.5 Summary and discussion

Previous studies had identified rhodocytin as a CLEC-2 agonist (Suzuki-Inoue, Fuller et al. 2006). Rhodocytin is a known snake venom protein that induces platelet aggregation, and for many other snake venoms this is regarded as the mechanism of toxicity (Andrews et al. 2001). It is reasonable to speculate that CLEC-2 has ligands others than rhodocytin. I decided to look for CLEC-2 ligands by using an established cell-based screen (Carlyle, Jamieson et al. 2004). As CLEC-2 has a structural similarity with the β-glucan receptor dectin-1, I hypothesized that CLEC-2 might recognise pathogens-derived molecules and thus, I screened several microbes for CLEC-2 binding. Remarkably, I could not find any microbe that bound to CLEC-2, which diverted my screen to examine possible CLEC-2 interactions with self-derived molecules. As is the case for other CLRs from the same sub-group, I found that CLEC-2 interacted with a

self-derived molecule present on the surface of endothelial cells and macrophages (Zelensky and Gready 2005).

Several approaches were used to characterise the self-molecule that could bind to CLEC-2. An important finding came from the fact that a ligand was present on CHO cells, but not a CHO mutant defective on the heparan sulfate polymerase enzyme Ext1 (Wei, Bai et al. 2000). Heparan sulfate (HS) is a member of the glycosaminoglycans (GAGs) family that are complex sugars found covalently attached to proteins, forming proteoglycans (Bernfield et al. 1999; Gandhi and Mancera 2008). Notably while this work was ongoing, the heavily glycosylated mucin-like protein podoplanin (PDPN) was described as a CLEC-2 ligand and agonist (Suzuki-Inoue, Kato et al. 2007). However, PDPN binding to CLEC-2 was not dependent of Ext1 or HS, suggesting that there might exist two distinct CLEC-2 ligands: PDPN and an Ext1-dependent ligand. Alternatively, it is possible that Ext1 is involved in promoting PDPN expression, although this involvement was not observed upon ectopic over-expression of PDPN. Hence, it would be attractive to evaluate normal PDPN expression on mouse and human cells deficient of Ext1. Unfortunately, I did not have access to neither human Ext1-deficient cells nor Ext1 knockout mouse cells. Furthermore my approaches to knockdown Ext1 by siRNA failed, as I could not make sure about siRNA transfection efficiency.

The O-glycosylation site (threonine 34) of PDPN is involved on its platelet-aggregation activity, as mutation on this site does not abolish PDPN expression but impair platelet aggregation induced by PDPN (Kato et al. 2003). However, mutations in this O-glycosylation site (T34A) did not impair CLEC-2 binding (Figure 3.6). As a matter of fact, PDPN T34A remained a CLEC-2 ligand, suggesting that PDPN O-glycosylation is likely to play a role in triggering other receptors to induce platelet aggregation.

Intriguingly, my assays showed that sialylated galactose (ß 1-3) N-acetylgalactosamine, (or “T antigen”) could be involved in CLEC-2 binding, as the lectin jacalin bound to this sugar inhibit CLEC-2 binding (Figure 3.4C). Accordingly, “T antigen” is expressed by PDPN and is necessary for platelet-aggregation activity (Kaneko et al. 2004). It is thus suggestive that “T antigen”

expression on PDPN is involved on CLEC-2 interaction and induction of platelet-aggregation. Nevertheless, jacalin binding to “T antigen” could allosterically inhibit CLEC-2 interaction with the ligand. Thus, “T antigen” does not necessarily need to be part of the ligand.

N-glycosylation inhibition blocked 3T3 binding to CLEC-2 (see figure 3.4B), indicating a role for N-linked carbohydrate chains in CLEC-2 binding. Analysis of PDPN glycosylation sites predicted that mouse PDPN have an N-glycosylation site but it is not present on human PDPN indicating that it might not be involved on CLEC-2 interaction. Even so, it is conceivable that PDPN N-glycosylation site might affect its correct folding and expression on the cell surface. Thus it would be interesting to evaluate PDPN expression upon N-glycosylation inhibition.

Many cancer cells have abnormal expression and activities of glycosyltransferases, which changes the expression of different glycoproteins. This is the case for MUC1 (Taylor-Papadimitriou, Burchell et al. 1999), a heavily O-glycosylated protein like PDPN. It is thus conceivable that PDPN glycosylation is differentially regulated between steady-state conditions and upon inflammation or cancer, and Ext1 could be involved in some of these modifications. Nevertheless, Kaneko and colleagues showed that both lymphatic endothelial cells (LECs) and cancer cells induced platelet-aggregation. Moreover, western blot analysis of PDPN showed the same profile when compared LECs and cancer cells (Kaneko, Kato et al. 2004), indicating no differences on PDPN on normal and cancer cells.

Overall, I tried several approaches to biochemically characterise the CLEC-2 ligand. Many of these approaches were based on the fact that CLEC-2, as a CLR, could recognise carbohydrates. As described above, there is no clear answer whether CLEC-2 recognise N-linked, O-linked or an Ext1-dependent carbohydrate. Interestingly, the CLEC-2 ligand rhodocytin does not have any predicted glycosylation site and no glycosylation confirmed by mass spectrometry analysis (Watson, Brown et al. 2007). Accordingly, the CTLD of CLEC-2 lacks a consensus carbohydrate recognition domain (CRD) and, as many other members of the same sub-group of CLR, may interact with proteins

rather than sugars (Zelensky and Gready 2005). Therefore it is possible that glycosylation modifications might play a role on the exposure of amino acid sequences that interact with CLEC-2.

3.3 CLEC-2 distribution on the immune system

3.3.1 Anti-CLEC-2 antibody specificity

CLEC-2 expression has been largely investigated on human and mouse platelets and by mRNA expression. Therefore, I decided to generate monoclonal antibodies to better characterise CLEC-2 expression in the immune system (see chapter 2, section 2.4).

Monoclonal antibodies have been used for many years on a range of applications, including flow cytometry staining and western blots. I decided to validate the newly generated anti-mouse CLEC-2 antibodies for these two applications. The screen for hybridomas producing anti-mouse CLEC-2 allowed isolating two clones producing rat IgM and rat IgG2b anti-mouse CLEC-2 (described and shown chapter 2). For reasons of stability of antibody and purification I chose to primarily use the rat IgG2b anti-mouse CLEC-2, which will be referred throughout this document as anti-CLEC-2.

In order to clearly demonstrate the specificity of anti-CLEC-2 I used B3Z ζCLEC-2-HA cells mixed to the parental B3Z cells. As B3Z ζCLEC-2-HA cells co-express GFP, CLEC-2-HA cells appear as GFP positive, while the parental B3Z cells do not express either GFP or HA and appear as GFP negative. On figure 3.7A, it is shown that anti-CLEC-2 antibody biotinylated can selectively label GFP positive cells, like the anti-HA antibody. This demonstrates that anti-CLEC-2 can be used as a tool to determine CLEC-2 expression on mouse cells.

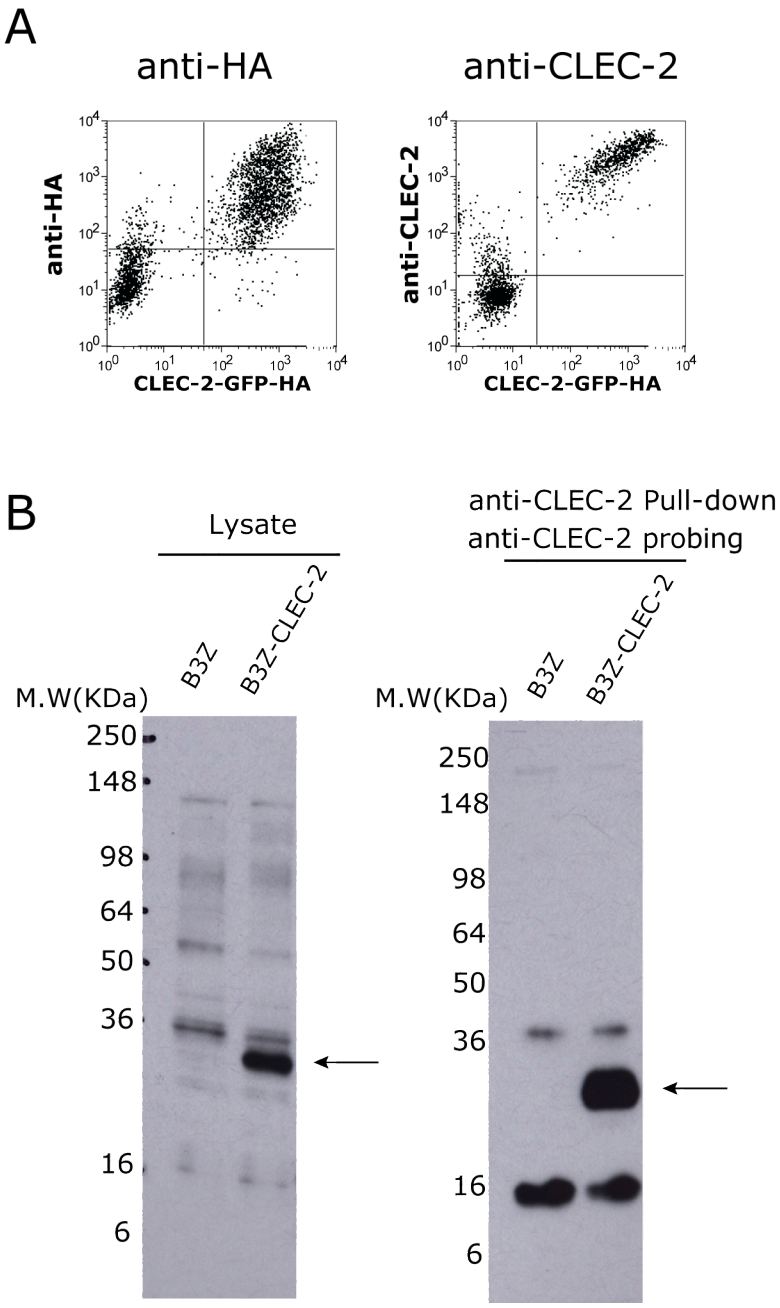

Figure 3.7 Anti-CLEC-2 monoclonal antibody specificity

(A) Biotinylated anti-mouse CLEC-2 monoclonal antibody (anti-CLEC-2) or rat IgG2b (isotype) were incubated with at a concentration of 5µg/ml in a mixture of B3Z or B3Z.ζ.CLEC-2.HA cells. After wash streptavidin-PE was added to the cells for another 20 minutes before further washing and analysis. (B) lysates (left side) or CLEC-2 immunoprecipitations (right) from B3Z or B3Z.CLEC-2 cells were separated in denaturing non-reducing gels and membrane blots were probed with 2 µg/ml of anti-CLEC-2. In the left side membrane was probed with anti-rat IgG-HRP and in the right side membrane was probed with streptavidin-HRP. Arrows indicate specific band and number represent molecular weight in KDa.

Subsequently, I analysed the ability of this antibody to detect CLEC-2 by western blot. The western blot analysis of CLEC-2 protein can allow me to verify the molecular weight of CLEC-2, thus determining whether CLEC-2 is glycosylated or is expressed as a monomer or dimer. To achieve this, I lysed B3Z cells ectopically expressing full length CLEC-2 (B3Z-CLEC-2) or not (B3Z) and separated the proteins by SDS-PAGE, transferred to a membrane and probed with anti-CLEC-2. As shown on figure 3.7B, there is a specific band on B3Z-CLEC-2 lysate with a molecular weight of approximately 30KDa. Further bands are observed, which might be due to the cross-reactivity of the secondary antibody (anti-rat HRP conjugated antibody) to other proteins. Immunoprecipitation of CLEC-2 also showed that anti-CLEC-2 antibody has a high specificity as it pull-down a CLEC-2 specific band (fig.3.7B, right). Accordingly, the western blot approach revealed that mouse CLEC-2 is expressed as a protein of approximately 30KDa, which is similar as the reported human CLEC-2 found on platelets (Suzuki-Inoue, Fuller et al. 2006).

3.3.2 CLEC-2 expression on myeloid cells

Around one decade ago, Colonna and colleagues described human CLEC-2 as a transcript expressed on all myeloid cells in addition to a NK cell clone (Colonna, Samaridis et al. 2000). Some years after this, human CLEC-2 protein was found on the surface of platelets (Suzuki-Inoue, Fuller et al. 2006). Remarkably, CLEC-2 expression was more recently reported on the surface of myeloid cells (Kerrigan, Dennehy et al. 2009; Tang, Li et al. 2010). Using the newly developed anti-CLEC-2 mAb I assessed CLEC-2 expression throughout the immune system.

I decided to firstly determine CLEC-2 expression on myeloid cells. I stained peripheral blood with Gr1 and CD11b, and identified neutrophils as a cell population that is Gr1hi and CD11bhi. The anti-CLEC-2 staining confirmed the previous report (Kerrigan, Dennehy et al. 2009) that neutrophils express CLEC-2 (fig.3.8B). To analyse CLEC-2 expression on macrophages, I induced

macrophage recruitment to the peritoneal cavity by treating mice with thioglycollate. With this method I identified macrophages by size and by expression of F4/80 marker. These thioglycollate-elicited macrophages showed CLEC-2 expression (fig.3.8C). Finally, I checked for CLEC-2 expression on dendritic cells (DCs) isolated from spleen, by isolating CD11c cells by magnetic enrichment. The analysis of FSChi and CD11c+ cells indicate that CLEC-2 is expressed on the dendritic cell population (fig. 3.8A). Splenic DCs are not a homogenous population, and they can be sub-divided into at least four subsets by expression of CD4, CD8α, and B220. Conventionally splenic DC subsets are named according to the expression or not of the CD4 and CD8α marker, thus generating the double negative DCs, the CD4+ DCs and the CD8+ DCs. The other subset is the plasmacytoid DC, which is identified by the lower expression of CD11c and by the presence of the B220. By analysing CLEC-2 expression on the splenic DC subsets, I determined that CLEC-2 is homogeneously expressed on all DC subsets, as indicated by the general analysis of splenic CD11c+ cells (fig.3.8A).

To finalise, I verified the expression of CLEC-2 on some myeloid cell models, which are generally used to study macrophages and DCs function on in vitro culture experiments. The RAW 264.7 (mouse macrophage cell line), GMCSF and FLT3L bone marrow-derived DCs (BMDCs) showed CLEC-2 expression (fig.3.8D). This confirms previous report that showed CLEC-2 mRNA expression on human samples of myeloid cells (Colonna, Samaridis et al. 2000), indicating that mouse CLEC-2 might show a similar expression profile. Additionally, it characterises myeloid cell models that will be used further on to study CLEC-2 signalling and function.

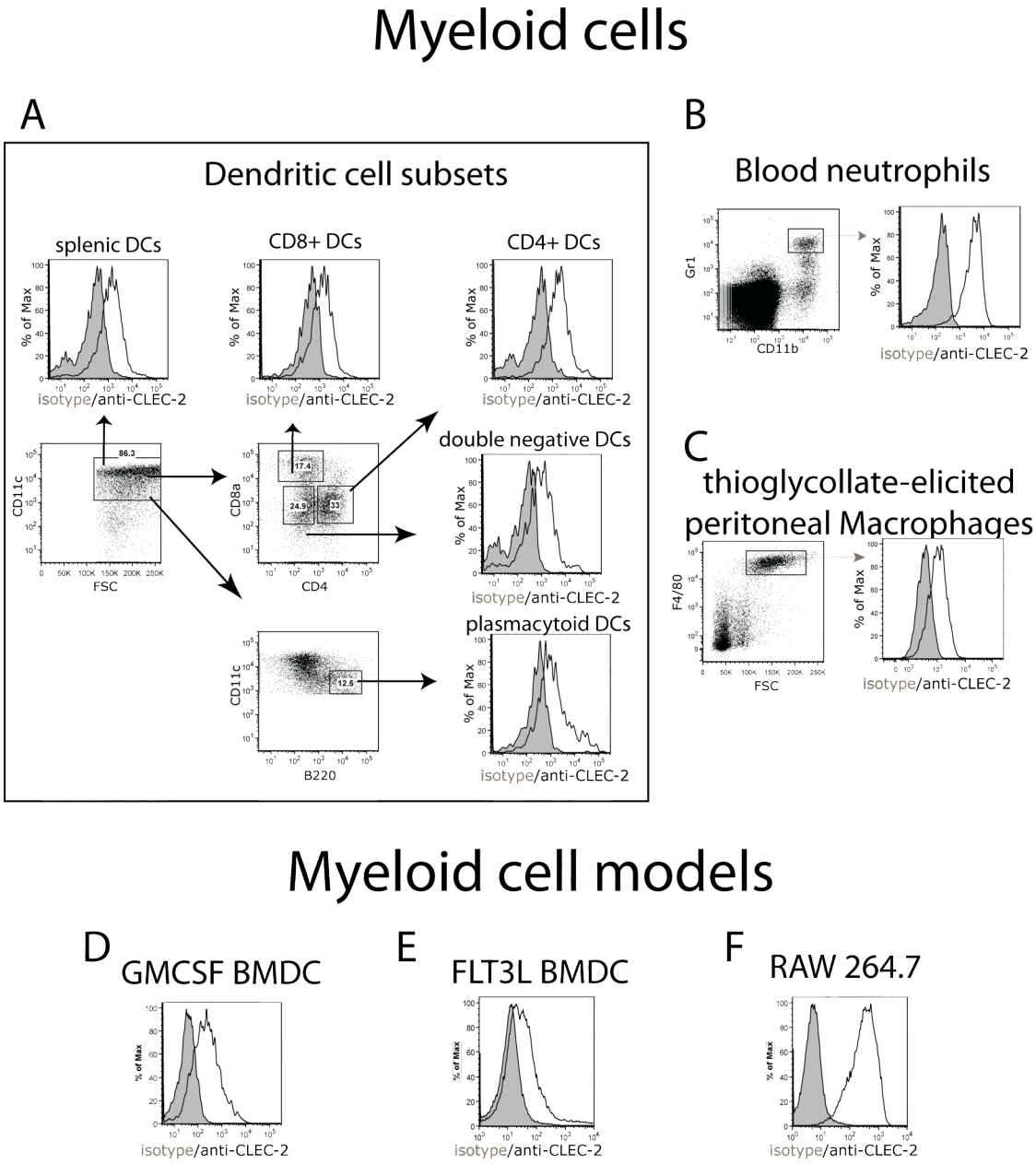

Figure 3.8 CLEC-2 expression on myeloid cells

(A) DCs were isolated from spleen of C57Bl/6J mouse by MACS and staining with anti-CD11c, anti-CD4, anti-CD8, anti-B220 and anti-CLEC-2 or isotype biotinylated. Numbers in graphs indicate the percentage of cells in the gated region. (B) Blood from C57Bl/6J mouse was obtained and stained with anti-Gr1, anti-CD11b and anti-CLEC-2 or isotype biotinylated. (C) Peritoneal lavage from thioglycollate-elicited C57Bl/6J mouse was obtained and stained with anti-F4/80 and anti-CLEC-2 or isotype biotinylated. Regions and arrows indicate the population depicted on histograms (D) GMCSF BMDC, FLT3L BMDC or Raw 264.7 were stained with anti-CLEC-2 or isotype biotinylated antibodies.

3.3.3 CLEC-2 cellular distribution on normal or inflammatory conditions

Several proteins change expression during inflammation, I sought to determine if CLEC-2 expression could be modulated during inflammatory conditions. To do so, I challenged C57Bl/6 mice with PBS or 5µg of LPS intravenously and analysed CLEC-2 expression on spleen, bone marrow or lymph node cell suspensions 16 hours after.

The spleen is a secondary lymphoid organ that can be used as a source of several mature hematopoietic cells, like myeloid and lymphoid cells. I used the staining of CLEC-2 on platelets as a positive control, and I identified platelets by analysing FSC low (small cells) and staining with CD41 marker (an integrin highly expressed on platelets). Hence, platelets showed a high CLEC-2 expression, which did not change upon LPS challenge *in vivo* (fig.3.9A).

I also analysed CLEC-2 expression on lymphoid cells by staining with B220, NK1.1 and CD3. B220 identifies B cells, while the latter markers identify NK cells (NK1.1+ and CD3-), NKT cells (NK1.1+ and CD3+) and T cells (NK1.1- and CD3+). As seen on figure 3.9A, CLEC-2 is found on steady state condition on a minor population of NK cells. When CLEC-2 expression is analysed after challenge with LPS, we observe that the B cells up-regulated CLEC-2 expression, but not T, NK or NKT cells. Intriguingly, the frequency of NK cells expressing CLEC-2 increased after LPS challenge, although CLEC-2 expression was not up regulated (fig.3.9A).

Myeloid cells were also analysed in the spleen with CD11c+ cells being identified as DCs, and CD11b and Gr1 identifying neutrophils and monocytes. DCs showed expression of CLEC-2, which was greatly increased upon LPS challenge (fig.3.9A). Neutrophils (Gr1 hi CD11b hi) showed a similar phenotype, with increased CLEC-2 expression upon LPS treatment of mice (fig.3.9A). Monocytes on the spleen can be identified by the lower expression of CD11b, compared to neutrophils, and/or presence of the Gr1 marker. The analysis of CLEC-2 expression on these monocytes showed no CLEC-2 expression.

Nevertheless, upon LPS challenge CLEC-2 expression was observed on these populations (fig.3.9A).

The lymph nodes are secondary lymphoid organs where the activated antigen-presenting cells (APCs) encounter and activate naïve T cells. For the analysis of CLEC-2 expressing cells on these sites, I extracted the inguinal and brachial lymph nodes of resting or LPS-challenged mice. Analysis of CLEC-2 expression on T cells showed no CLEC-2 expression in resting or LPS-treated mice (fig.3.9B). B cells in lymph nodes do not express CLEC-2 in resting mice and, differently from the splenic B cells, in LPS-treated mice (fig.3.9B). Also distinct from the DCs found in the spleen, a very small DC population express CLEC-2 at the steady state (fig.3.9B, compare shaded grey with thin black line histogram). But, upon LPS challenge all DC express CLEC-2 at increased levels.

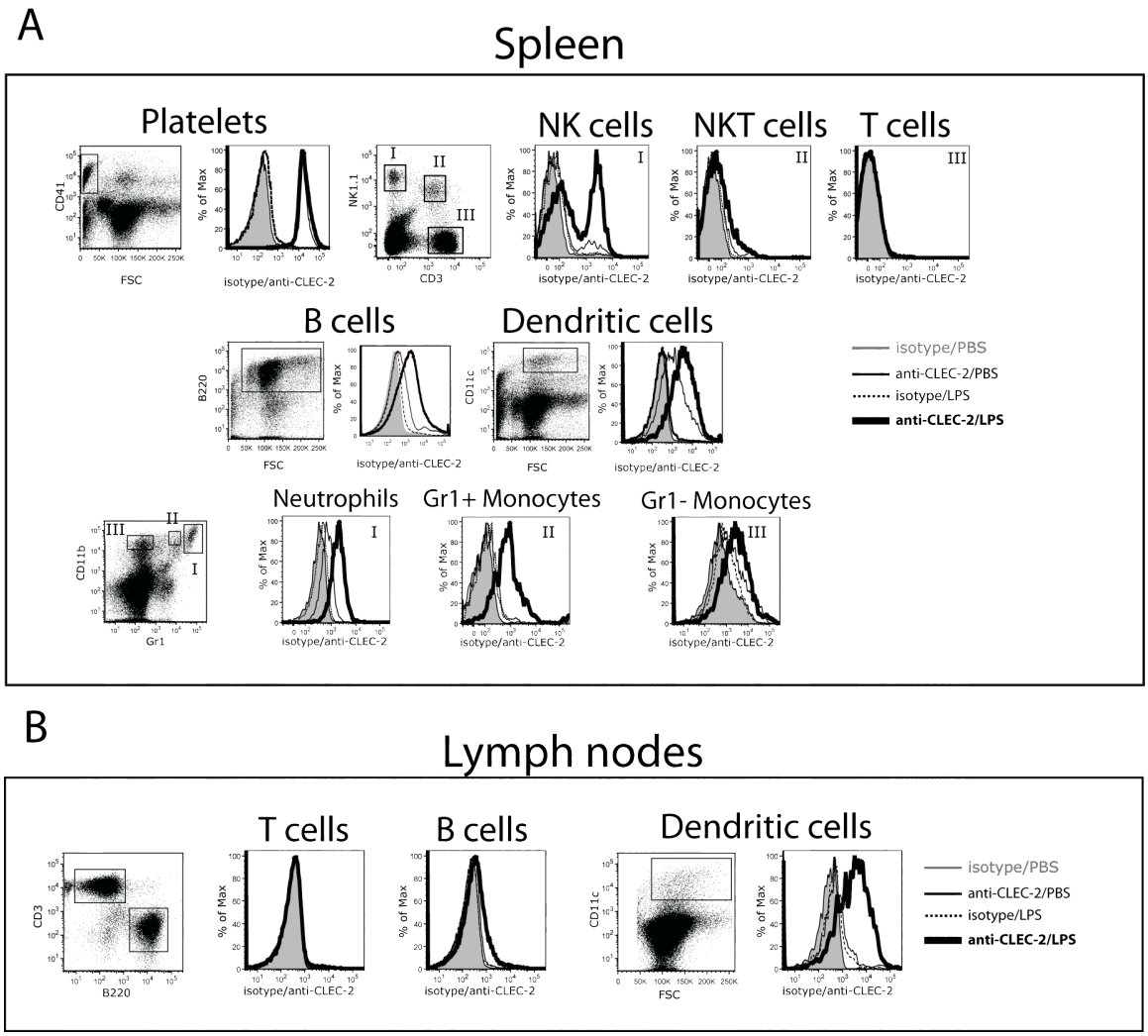

Figure 3.9 Inflammation modulates CLEC-2 expression

C57Bl/6 mice were injected intravenously with 5µg of LPS or with PBS. After 16 hours (A) splenocytes were stained with anti-CLEC-2 biotinylated or with isotype matched control antibody and anti-CD41, anti-CD11c, anti-B220, anti-NK1.1and anti-CD3. (B) Lymph node cells were stained with anti-CD3, anti-B220 and anti-CD11c. Regions on scatter plots indicate the population analysed for CLEC-2 expression on histograms.

3.3.4 Summary and discussion

The generation of an anti-mouse CLEC-2 mAb allowed me to analyse mouse CLEC-2 biochemically and its expression. Analysis of mouse CLEC-2 by western blot identified it as a monomer of approximately 30 KDa. However, the aminoacid sequence of mouse CLEC-2 predicts a polypeptide chain of 26 KDa, which suggests that mouse CLEC-2 might by expressed as a glycosylated protein. Interestingly, human CLEC-2 was identified as a N-glycosylated protein of approximately 32 KDa (Suzuki-Inoue, Fuller et al. 2006).

Using anti-CLEC-2 mAb I could expand previous finding of CLEC-2 expression on platelets. I showed that CLEC-2 was expressed on all myeloid cells (DCs, macrophages and neutrophils) and NK cells. This confirmed previous reports that showed CLEC-2 in human myeloid and NK cells (Colonna, Samaridis et al. 2000) and in mouse neutrophils and monocytes (Kerrigan, Dennehy et al. 2009; Tang, Li et al. 2010). Interestingly, I found that inflammation induced by LPS in mice could increase CLEC-2 expression in myeloid and B cells. Additionally, LPS did not increase CLEC-2 expression on NK cells, but increased the frequency of CLEC-2 positive NK cells. This might be because LPS induce the expansion of this population of NK cells. Accordingly, Colonna and colleagues demonstrated that CLEC-2 transcripts were only found on particular human NK cell clones (Colonna, Samaridis et al. 2000). The increased frequency of NK cells after LPS challenge might be due to induction of a mediator, such as IL-15, which induces NK cell development and proliferation (Colonna 2009). This increased frequency of NK cells might be important to increase the chances of a NK cell to get in contact with a PDPN-expressing tumour cell during developing tumour.

Analysis of monocytes in the spleen showed CLEC-2 expression upon LPS-treatment, but not in resting conditions. Although I could demonstrate that CLEC-2 can be expressed on monocytes in the spleen, these cells are knowingly auto-fluorescent, which might have impaired the analysis on steady state conditions.

Additionally I found that, in lymph nodes, CLEC-2 is expressed only on a minor subset of DCs. Nevertheless, upon LPS-induced inflammation B cell did not up-regulate CLEC-2, while DCs did. It is interesting that CLEC-2 is not found similarly regulated on the spleen and lymph nodes. It is tempting to speculate that on the steady state, CLEC-2 expressed on a minor DCs subset represents migratory DCs. PDPN expression on afferent lymphatic vessel (Pflicke and Sixt 2009) and lymph node stroma (Lee et al. 2007) might play a role in the migration of DCs from tissues to lymph nodes.

Chapter 4. Study of CLEC-2 signalling and function on myeloid cells

4.1 Introduction

Most studies on CLEC-2 function and signalling have been performed using platelets and focusing on the role of CLEC-2 on the induction of platelet aggregation. Nevertheless, the finding that CLEC-2 is expressed on a variety of myeloid cells suggests that it might have other functions beside platelet aggregation. CLEC-2, as dectin-1, is a hemITAM Syk-coupled receptor and might therefore behave similarly to dectin-1 in terms of its ability to activate myeloid cells. Syk signalling downstream of dectin-1 can lead to cytokine (Rogers, Slack et al. 2005) and ROS production (Underhill, Rossnagle et al. 2005). Indeed, a chimeric dectin-1 receptor bearing the intracellular tail of CLEC-2 was recently described as able to induce TNF, but not ROS production (Kerrigan, Dennehy et al. 2009).

However, studies of signalling and function of the intact receptor in myeloid cells are limited. The only report available focused on the role of CLEC-2 on neutrophils and used rhodocytin as a CLEC-2 agonist (Kerrigan, Dennehy et al. 2009).

In this chapter I will focus on the function of CLEC-2 on myeloid cells, using DCs as a model. I will primarily concentrate on the fact that CLEC-2 is a dectin-1-like receptor, thus allowing Syk signalling and modulation of gene induction. I will use a system to study the agonistic activity of different CLEC-2 agonists and study its consequences in terms of signalling and function.

4.2 Rhodocytin as a CLEC-2 agonist

The snake venom toxin rhodocytin was the first reported agonist for CLEC-2 function, inducing a strong Syk-dependent signalling and platelet aggregation (Suzuki-Inoue, Fuller et al. 2006); therefore I decided to test it as a selective CLEC-2 agonist able to induce cytokine production by DCs.

The analysis of cytokine production via Myd88 or Syk pathways can indicate the ability of an agonist to activate the TLR or the CLR family. As shown on figure 4.1A, the stimulation of BMDCs with the dectin-1 agonist curdlan leads to TNF production on WT DCs and Myd88-deficient (Myd88-/-) DCs, but not on Syk-deficient (Syk-/-) DCs. Additionally, the TLR4 agonist LPS induce TNF production on WT and Syk-/- DCs but not on Myd88-/- DCs. Despite no dose response was observed, LPS at 10 ng/ml is already at saturating levels (Fig.4.1A). To test rhodocytin-dependent DC activation, I incubated increasing amounts of rhodocytin with WT, Syk-/- or Myd88-/- DCs and measured IL-10, TNF and IL12/IL23p40. Dectin-1-Syk signalling in DCs induces such cytokines (Rogers, Slack et al. 2005; LeibundGut-Landmann, Gross et al. 2007). As observed on figure 4.1B, rhodocytin induced increased production of these cytokines. Nevertheless, such production was severely impaired on Myd88-/- DCs, suggesting that rhodocytin is inducing cytokine production via TLRs. Moreover, Syk-/- DCs were not impaired on the production of these cytokines, supporting the notion that rhodocytin is not inducing cytokine production via Syk signalling.

Rhodocytin was demonstrated to bind CLEC-2 and induce Syk-dependent signalling on platelets and B cell line (Suzuki-Inoue, Fuller et al. 2006; Fuller, Williams et al. 2007), thus I decided to further characterise rhodocytin as a CLEC-2 agonist of gene induction. Rhodocytin selectivity was tested with the generation of a gain of function assay, exploiting the ability of CLEC-2 to signal to downstream function by its intracellular hemITAM motif (tyrosine 7 of YxxL motif) (Fuller, Williams et al. 2007). This assay consist of expressing CLEC-2 (WT) or tyrosine-mutant CLEC-2 (Y7F) on the mouse B cell line LK (expression levels shown on figure 4.1C right). I chose LK cells because of their ability to produce cytokines, express an intact Syk signalling pathway and their previous use to study dectin-1 signalling (Rogers, Slack et al. 2005). The incubation of LK CLEC-2 WT cells with rhodocytin induced increased production of IL-2 (fig.4.1C). Furthermore, the co-treatment of LK cell with the TLR4 agonist LPS increased the production of this cytokine, reminiscent of reports showing collaboration of dectin-1 and TLR signalling (Gantner,

Simmons et al. 2003; Dennehy et al. 2008). However, the treatment with high amounts of rhodocytin induces IL-2 production on LK CLEC-2 Y7F cells at similar levels of LPS, indicating that my rhodocytin preparation is likely to be contaminated with a TLR agonist. The ability of low amounts, but not high amounts, of rhodocytin to collaborate with TLR agonist also suggests that rhodocytin is triggering multiple receptors.

In conclusion, rhodocytin is able to act as a CLEC-2 agonist and suggests that CLEC-2 is likely to collaborate with TLRs to induce increased cytokine production. However, rhodocytin preparations also activate TLRs and therefore are not a suitable tool to selective trigger CLEC-2 and study its function in myeloid cells.

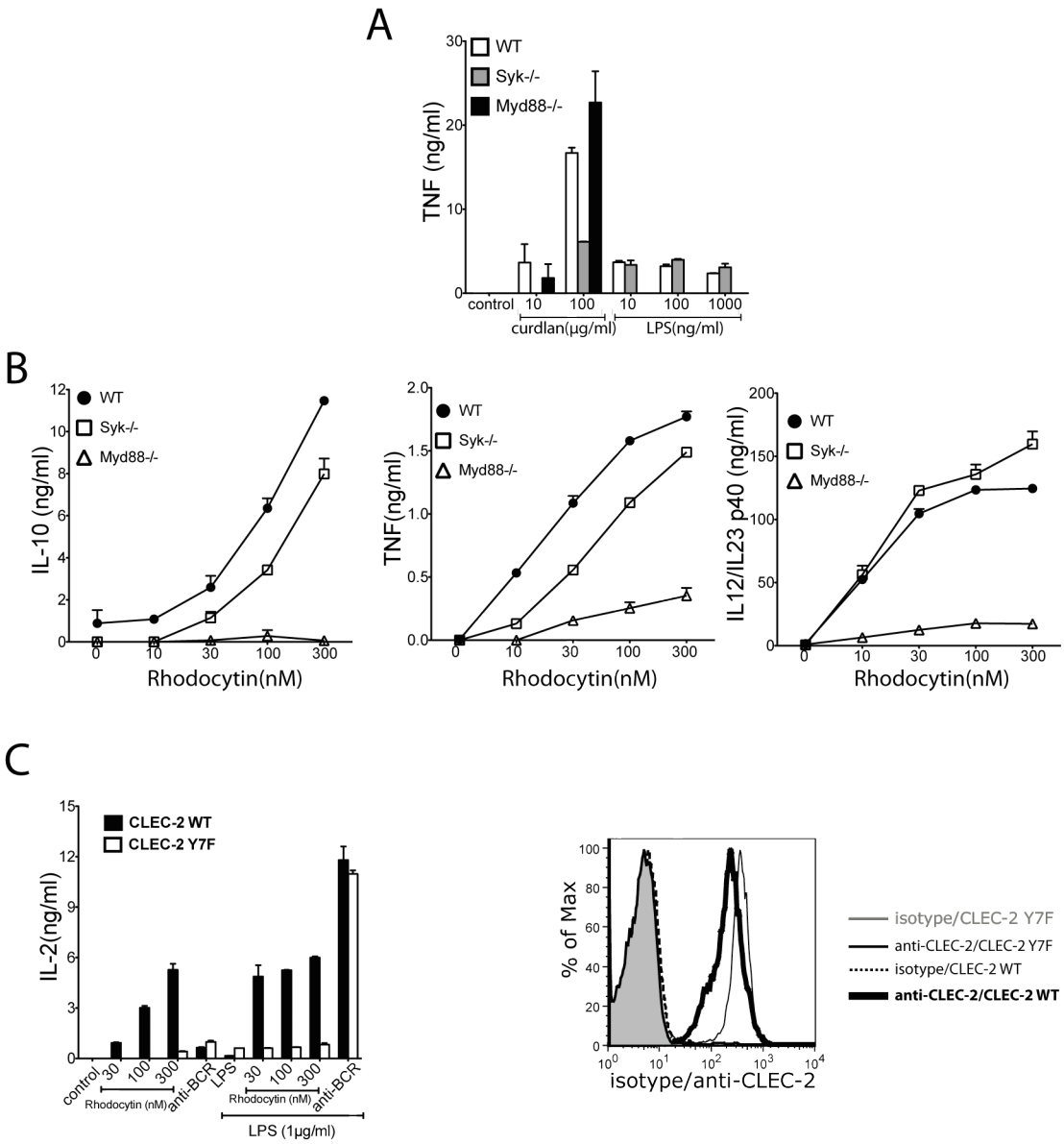

Figure 4.1 Rhodocytin triggers cytokine production via multiple receptors
(A) C57Bl/6J (WT), Myd88-/- or Syk-/- GMCSF BMDC were incubated for 18 hours in culture medium (control) or the indicated concentrations of curdlan and LPS. TNF was measured in the supernatants by ELISA. (B) WT, Myd88-/- or Syk-/- GMCSF BMDC were incubated for 18 hours with indicated amounts of rhodocytin. IL-10, TNF and IL12/IL23p40 were measured in the supernatants by ELISA. (C) $1x10^5$ LK CLEC-2 WT or Y7F were cultured for 18 hours in culture medium (control) or with the indicated concentrations of rhodocytin, LPS or 1µg/ml of soluble anti-mouse IgG (anti-BCR). Right panel shows an anti-CLEC-2 staining of LK CLEC-2 WT and Y7F. IL-2 was quantified in the supernatants by ELISA. Data are means ± SEM of duplicate wells of at least three independent assays.

4.3 Study of Podoplanin as stimulator of gene induction by DCs

CLEC-2 was reported to have two different agonists, the snake venom toxin rhodocytin and the endogenous glycoprotein PDPN (Suzuki-Inoue, Kato et al. 2007). As rhodocytin did not show to be a selective CLEC-2 agonist in myeloid cells, I decided to test the role of PDPN.

To evaluate the potential of cells expressing PDPN to act as a CLEC-2 agonist, I co-cultured PDPN-expressing cells with LK cells expressing CLEC-2 WT or Y7F with or without LPS. I chose to use LK PDPN cells because it only binds to CLEC-2 when ectopically expressing PDPN (figure 3.6B). Increased IL-2 amounts were observed on co-cultures with CLEC-2 WT cells, suggesting that PDPN-expressing cells can act as agonist of CLEC-2 (fig.4.2A).

Based on that, I cultured one LK or LK PDPN cell for each BMDC on culture. The quantification of cytokine production after overnight co-culture showed no differences to co-cultures of LK or LK PDPN with BMDCs. Furthermore, no differences on the production of IL-10, TNF or IL-12/IL-23p40 were observed when the co-cultures were treated with different concentration of LPS (fig.4.2B). This result suggests that PDPN-expressing cells are not major stimulators or modulators of IL-10, TNF or IL12/IL23p40 production.

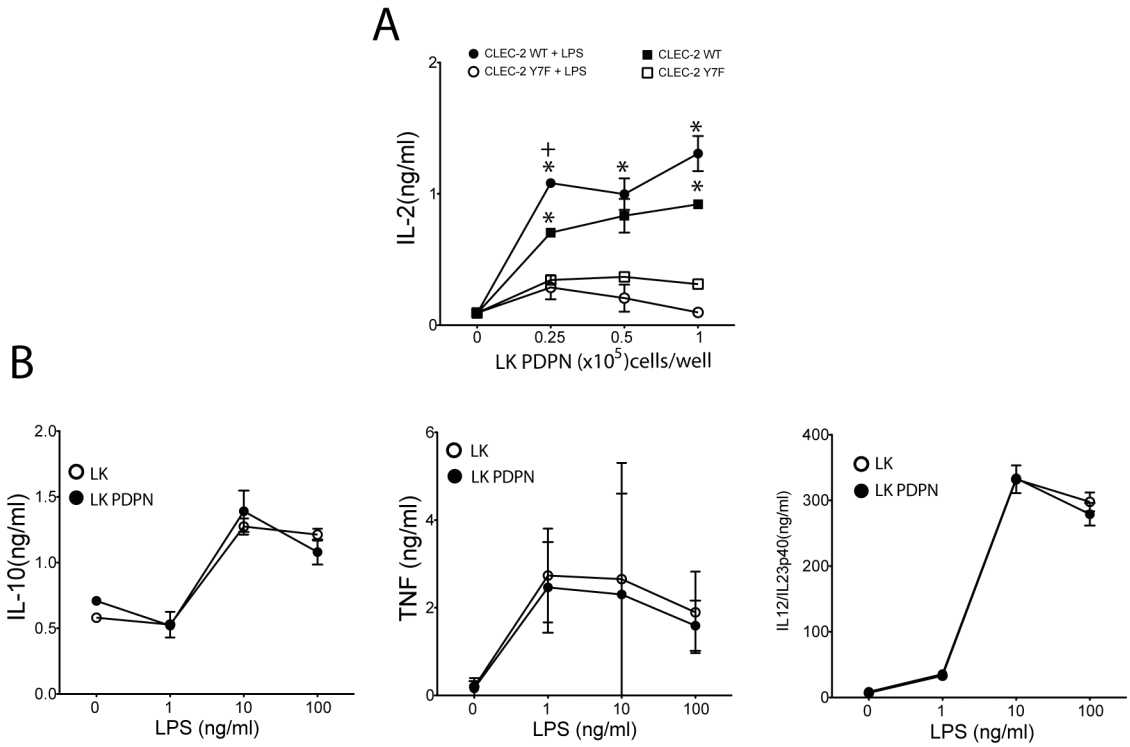

Figure 4.2 Cells expressing PDPN are CLEC-2 agonists but do not stimulate cytokine production by DCs
(A) $1x10^5$ LK CLEC-2 WT or Y7F were cultured for 18 hours on culture medium (0) or with indicated numbers of LK PDPN with or without 1µg/ml of LPS. (B) $1x10^5$ GMCSF BMDCs were cultured with $1x10^5$ LK PDPN cells and indicated amounts of LPS for 18 hours. Cytokines were measured on the supernatants by ELISA. Data are means ± SEM of duplicate wells of at least three independent assays. Unpaired t test was performed on all the samples and statistical differences ($p<0.05$) between CLEC-2 WT+LPS and CLEC-2 Y7F+LPS are indicated by * while statistical differences between CLEC-2 WT and CLEC-2 WT+LPS is indicated with +.

To avoid the use of cells expressing PDPN and exclude multiple receptor triggering, the ideal scenario would be to incubate DCs with PDPN protein and then measure cytokine production. For this, I made use of concentrated supernatant from PDPN.Fc-secreting mammalian cells on wells coated with Fab2 anti-human IgG. I cultured DCs on these wells with increasing amounts of LPS. AS the purification of Fc-tagged proteins involves flow through columns of microbe-derived protein A and elution with acid buffer I decided to capture PDPN.Fc on the plate and avoid a possible contamination or degradation of PDPN.Fc during purification. As shown on figure 4.3A, the incubation of LK

CLEC-2 WT cell on PDPN.Fc-coated wells leads to increased IL-2 amounts that are not observed with CLEC-2 Y7F LK cells or control stimulations (dectin-1.Fc-coated wells). Furthermore, it shows that LPS increased IL-2 production induced by PDPN.Fc by PDPN.Fc. This indicates that PDPN.Fc can induce CLEC-2 signalling in LK cells over expressing the receptor and that PDPN.Fc-coated wells are suitable for inducing this signalling. Thus I sought to use the same system to stimulate DCs. The incubation of BMDCs in PDPN.Fc-coated wells did not lead to production of IL-10, TNF or IL-12/IL-23p40 or modulated LPS-induced cytokine production (fig.4.3B). In conclusion, PDPN can act as a CLEC-2 agonist in LK cells over expressing CLEC-2, but not in DCs.

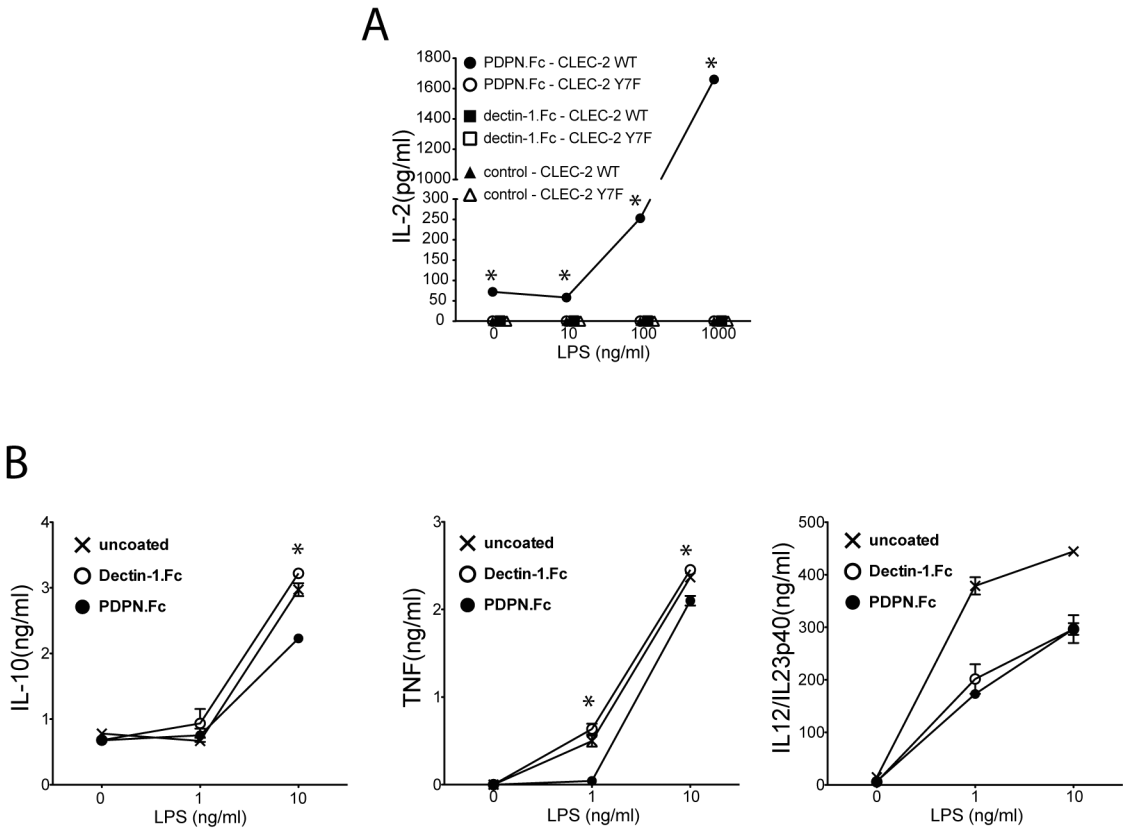

Figure 4.3 No effects of PDPN on gene induction by DCs
(A) 1x10^5 LK CLEC-2 WT or Y7F were cultured for 18 hours with or without increasing amounts of LPS in wells coated with Fab2' anti-human IgG and culture medium (control) or supernatants containing PDPN.Fc or dectin-1.Fc. ELISA. (B) GMCSF BMDCs were culture as in (A). All cytokines were measured by ELISA. Data are means ± SEM of duplicate wells of at least two independent assays. Statistical Unpaired t test was performed in all samples and * indicates statistical differences ($p<0.05$) between dectin-1.Fc and PDPN.Fc.

4.4 Anti-CLEC-2 monoclonal antibody as agonist of CLEC-2

Several monoclonal antibodies against CLRs have been used as selective agonists to study responses induced by these receptors (Caparrós et al. 2006; Chen 2006; Suzuki-Inoue, Fuller et al. 2006; Röck, Schneider et al. 2007; Chen, Lin et al. 2008; Robinson, Osorio et al. 2009). Moreover, polyclonal anti-human CLEC-2 antibodies have been used as agonists to trigger CLEC-2 signalling (Suzuki-Inoue, Fuller et al. 2006). Based on these findings, I sought to determine if anti-CLEC-2 mAb was able to induce gene changes on DCs. To do so, I cultured FLT3L BMDCs that are considered to be representative of all DCs subsets found in mouse spleen (Xu et al. 2007). The culture of FLT3L BMDC on plates coated with anti-CLEC-2 mAb or an isotype-matched antibody and measured IL-10 and IL12/IL23p40 after overnight incubation. No cytokine was observed on the supernatants upon this treatment. However, when DCs were cultured on anti-CLEC-2-coated wells, but not on isotype-matched-coated wells, in the presence of LPS or Pam3Cys (TLR2 agonist), an increased production of IL-10 was observed (fig.4.4A). Noteworthy, anti-CLEC-2 treatment of BMDCs increased the production of IL-10 at all concentrations of TLR agonists tested. Remarkably, no differences on the production of IL-12/IL-23p40 were observed, indicating that anti-CLEC-2 treatment was inducing the modulation of certain genes only (fig.4.4A). This indicates that anti-CLEC-2 induced a qualitative change rather than a quantitative change of TLR responses by DCs.

Antibodies can induce increased IL-10 production together with LPS treatment by engaging Fc receptors (Gerber and Mosser 2001). Even though the isotype-matched control mAb treatment did not induce increased IL-10 production, I decided to design an experiment to rule out the possibility that the anti-CLEC-2 mAb treatment was engaging Fc receptors. I generated anti-CLEC-2 and control Fab (chapter 2, section 2.4.4) to make sure that the stimulus for increased IL-10 production was coming by signalling mediated by CLEC-2 and not from Fc triggering. The incubation of BMDCs on wells coated

with anti-CLEC-2 Fab and LPS led to an increased production of IL-10, but not of IL-12/IL-23p40 (fig.4.4B). The DCs cultured on uncoated or wells coated with control Fab did not show any difference on the production of IL-10 or IL-12/IL-23p40 (fig.4.4B). Overall, this experiment indicates that CLEC-2 cross-linking can modulate TLR responses on DCs. Furthermore, no changes on IL-6 and TNF were observed on samples treated with LPS, LPS and anti-CLEC-2 Fab and LPS and control Fab (data not shown).

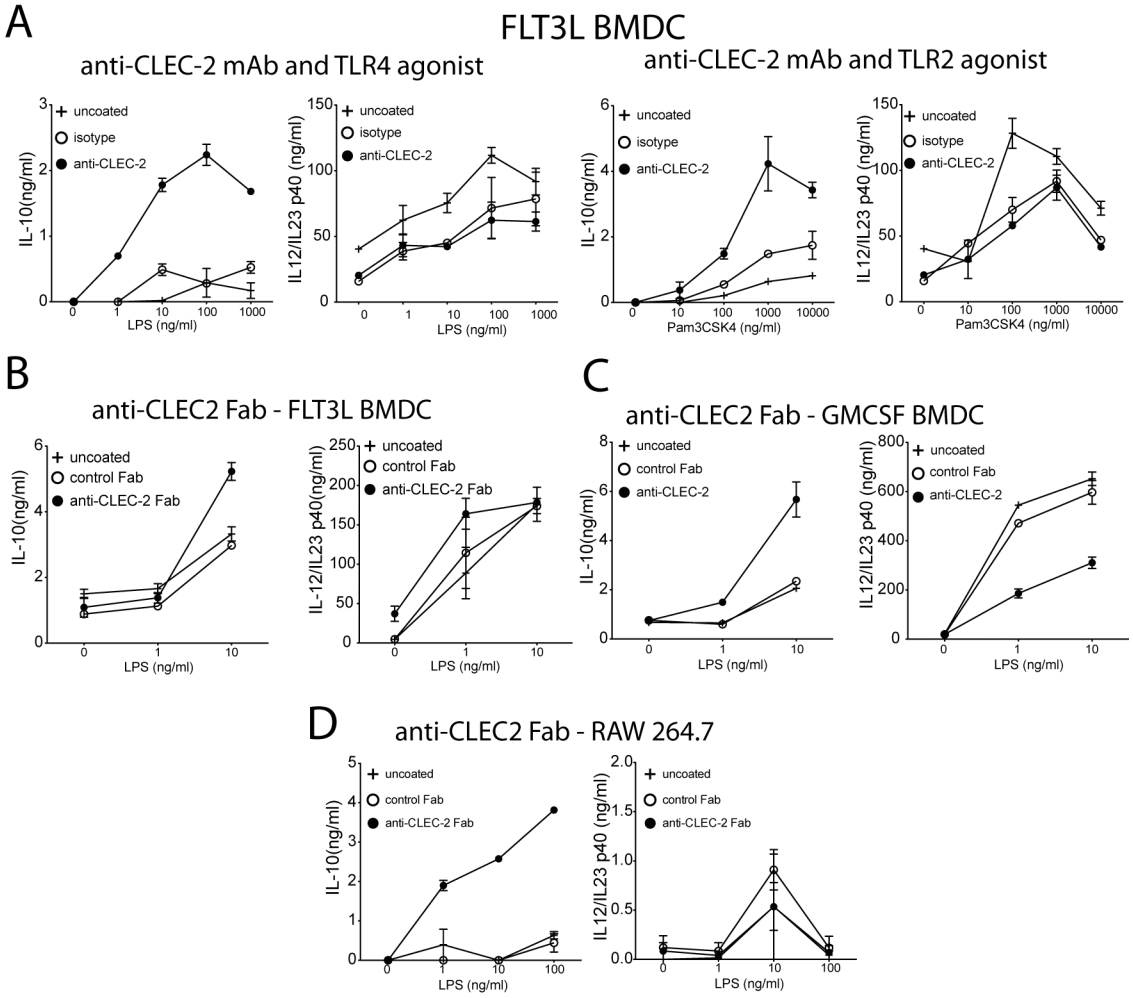

Figure 4.4 Anti-CLEC-2 modulates TLR-induced cytokine production
(A) 1x10^5 FLT3L BMDCs were culture for 18 hours on wells coated with isotype or anti-CLEC-2 mAb with increasing amounts of LPS (left) or Pam3CSK4 (right). (B) 1x10^5 FLT3L BMDCs were cultured on wells coated with control or anti-CLEC-2 Fab for 18 hours in the presence of increasing amounts of LPS. (C) GMCSF BMDCs were cultured as in B. (D) Raw 264.7 cells were cultured as in B. All cytokines were quantified on supernatants by ELISA. Data are means ± SEM of duplicate wells of at least three independent assays.

To exclude the possibility that CLEC-2 modulation of TLR response was observed only with a specific DC model, I repeated the experiments using GMCSF BMDC instead of FLT3L BMDC. Interestingly, on GMCSF BMDC the ligation of CLEC-2 on the presence of LPS induced increased IL-10 and reduced IL-12/IL-23p40 production (fig.4.4C). Moreover, the same anti-CLEC-2 Fab and LPS treatment induced increased IL-10 production by the macrophage cell line RAW 264.7 (fig.4.4D). Collectively, these data suggests that CLEC-2 ligation by mAbs can modulate TLR responses in different myeloid cell models (FLT3L BMDC, GMCSF BMDC and RAW 264.7) with a reproducible phenomenon of increased IL-10 production.

4.5 Molecular requirements for CLEC-2 modulation of LPS responses

As anti-CLEC-2 ligation on different myeloid cells was able to modulate TLR responses, I sought to determine which molecular requirements on CLEC-2 and downstream of CLEC-2 were necessary for this modulation.

Here, I made use of LK cells expressing CLEC-2 (WT) or the hemITAM mutant CLEC-2 (Y7F), to determine the need for CLEC-2 intracellular tyrosine on the modulation of LPS responses. The culture of these LK cells on wells coated with anti-CLEC-2 or control Fab on the presence of LPS led to increased IL-10 and IL-2 production only when the cells were CLEC-2 WT and treated with anti-CLEC-2 Fab (figures 4.5A and 4.5B). Importantly, LK CLEC-2 Y7F and WT cells were equally able to produce IL-2 and IL-10 when stimulated in a CLEC-2-independent manner (anti-BCR antibody). Additionally, no difference on the expression of CLEC-2 WT or Y7F was observed on the surface of LK cells (fig.4.5D), indicating that CLEC-2 hemITAM is necessary for modulation of LPS response and increased production of IL-10. These data again confirm that the phenomenon is not an artefact from Fc receptor cross-linking.

Noteworthy, LK cells are able to produce IL-10 once treated with LPS, but CLEC-2 ligation was able to signal to an increased production of IL-10

(fig.4.5A). Moreover, CLEC-2 ligation and LPS treatment induced increased IL-2 production by CLEC-2 WT cell, but not CLEC-2 Y7F cells (fig.4.5B), on a phenomenon similar to the treatment of these cells with rhodocytin and PDPN (fig.4.2 and 4.3). Seeing that expression of CLEC-2 on LK cells confer them the ability to respond to CLEC-2 ligation with the production IL-2, I decided to use these cells to compare the agonistic activity of different CLEC-2 ligands. As shown on figure 4.5C, LK CLEC-2 WT cells were able to produce low levels of IL-2 when stimulated with anti-CLEC-2 antibodies (the rat IgG2b or the rat IgM) or PDPN.Fc or PDPN T34A.Fc. Noteworthy, the anti-CLEC-2 (IgG2b) was the best for inducing IL-2 production without LPS, suggesting that it might be the best to cross-link CLEC-2, inducing downstream signalling and modulation of LPS response.

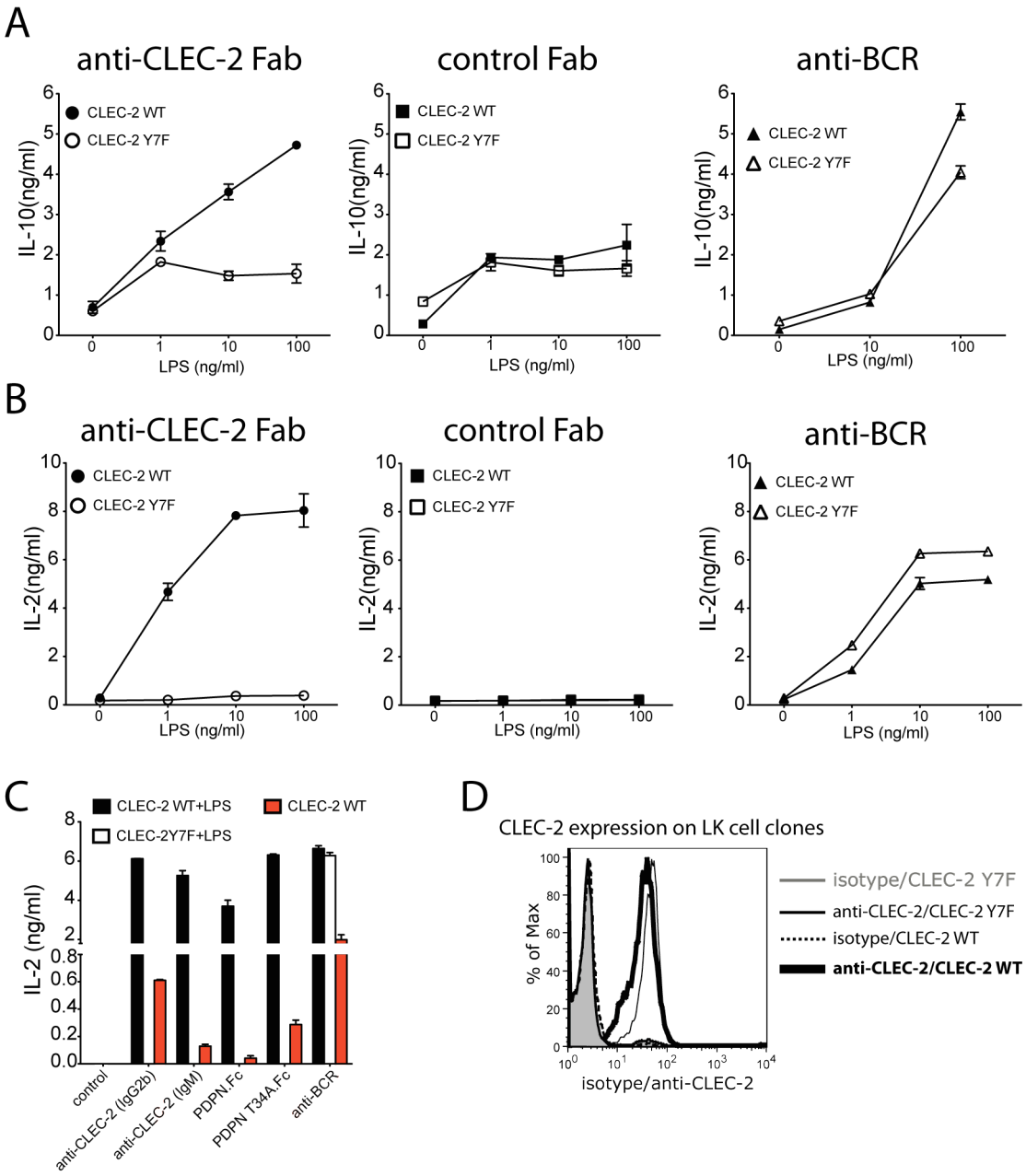

Figure 4.5 CLEC-2 hemITAM is required for modulation of IL-10 production
(A) and (B) 1x10^5 LK CLEC-2 WT or Y7F were cultured for 18 hours on wells coated with control or anti-CLEC-2 Fab or 1µg/ml of soluble anti-mouse IgG (anti-BCR) in the presence of increasing amounts of LPS. (C) LK cell clones were cultured as in A on wells coated with anti-CLEC-2 IgG2b or IgM, PDPN.Fc or PDPN T34A.Fc, anti-BCR was added soluble at 1µg/ml. LPS was added at 1µg/ml to the indicated groups. Data are means ± SEM of duplicate wells of at least three independent assays. (D) CLEC-2 staining of LK CLEC-2 WT or Y7F cells.

In order to understand how anti-CLEC-2 mAb ligation modulates the TLR response, I decided to evaluate the induction of downstream signalling emanating from CLEC-2. To do so, I cultured FLT3L BMDC on wells coated with anti-CLEC-2 or isotype mAb and analysed the activation of downstream signalling molecules. I analysed the protein lysate on a western blot, probing the membrane with an antibody that detects phosphorylated tyrosine. As observed on figure 4.6A, CLEC-2 ligation on DCs by anti-CLEC-2 mAb, but not an isotype-matched mAb, induces the phosphorylation of, at least, two different proteins: one of approximately 70Kda and another of around 20Kda. Given that CLEC-2 was demonstrated to be a Syk-coupled receptor and Syk is a 72Kda protein that is tyrosine-phosphorylated when active (Mócsai, Ruland et al. 2010), I decided to evaluate if anti-CLEC-2 was inducing Syk activation on DCs. When I probed the western blot with a specific anti-phosphorylated Syk antibody, I observed that phosphorylated (active) Syk is present only on cells treated with anti-CLEC-2 and not with isotype mAb (fig.4.6B). Total Syk (inactive or active) is present at very similar levels on all samples tested, indicating that the difference on the activation of Syk is due to signalling and not to differential protein content (fig.4.6C). Several proteins of 20KDa can be tyrosine phosphorylated and I did not pursue the identity of this proteins, nevertheless an attractive hypothesis is that this might correspond to caveolin-1 and CLEC-2 is localised to caveolae.

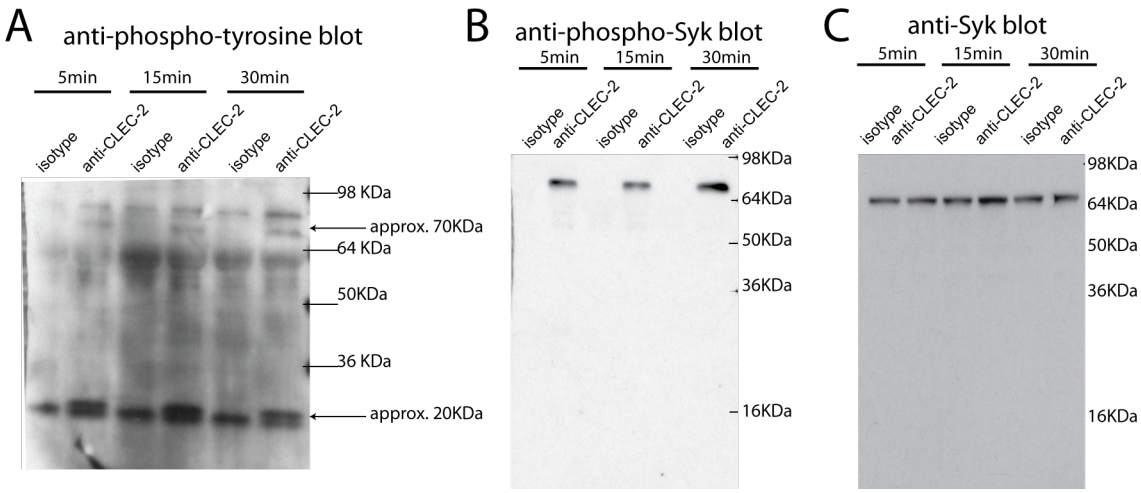

Figure 4.6 Anti-CLEC-2 induces tyrosine phosphorylation and Syk activation in DCs
FLT3L BMDCs were centrifuged on wells coated with isotype or anti-CLEC-2 mAb and incubated at 37oC for the indicated time before lysis. Proteins were separated by non-reducing denaturing gels and analysed by western blot with (A) mouse anti-phospho tyrosine (4G10 antibody) (B) rabbit anti-phospho Syk (Tyr352) or (C) rabbit anti-Syk.

To test if CLEC-2 signalling can induce Ca^{2+} flux by anti-CLEC-2 mAb cross-linking I expressed CLEC-2 WT or Y7F on B3Z cells expressing Syk (B3Z.Syk cells), and I loaded these cells with indo-1, a dye that changes fluorescence in a Ca^{2+}-binding dependent manner. Thus, FACS acquisition of indo-1-loaded cells can immediately indicate the changes on the ratio between indo-1 free Ca^{2+} and bound Ca^{2+}. The method that I chose to test anti-CLEC-2 cross-linking on cell suspension was to stain the indo-1-loaded cells with the biotinylated anti-CLEC-2 antibody, and induce the mAb cross-linking by adding streptavidin (protein with four biotin binding sites). Hence, the addition of streptavidin can cross-link anti-CLEC-2 biotinylated mAb to induce signalling. As shown on figure 4.7A, the addition of streptavidin to anti-CLEC-2-stained CLEC-2 WT cells induced a strong calcium flux on these cells. Remarkably,

CLEC-2 WT cells stained with an isotype antibody, or CLEC-2 Y7F cells stained with anti-CLEC-2 did not increase cytoplasmic calcium upon antibody cross-linking by streptavidin addition. Moreover, both CLEC-2 WT and Y7F cells expressed similar levels of CLEC-2 on their surface and fluxed calcium upon ionomycin treatment (fig.4.7A bottom and 4.7C). As shown, ionomycin induced normal calcium flux on CLEC-2 WT and Y7F cells, indicating that CLEC-2 Y7F cells calcium flux deficiency is only for CLEC-2 specific stimulation.

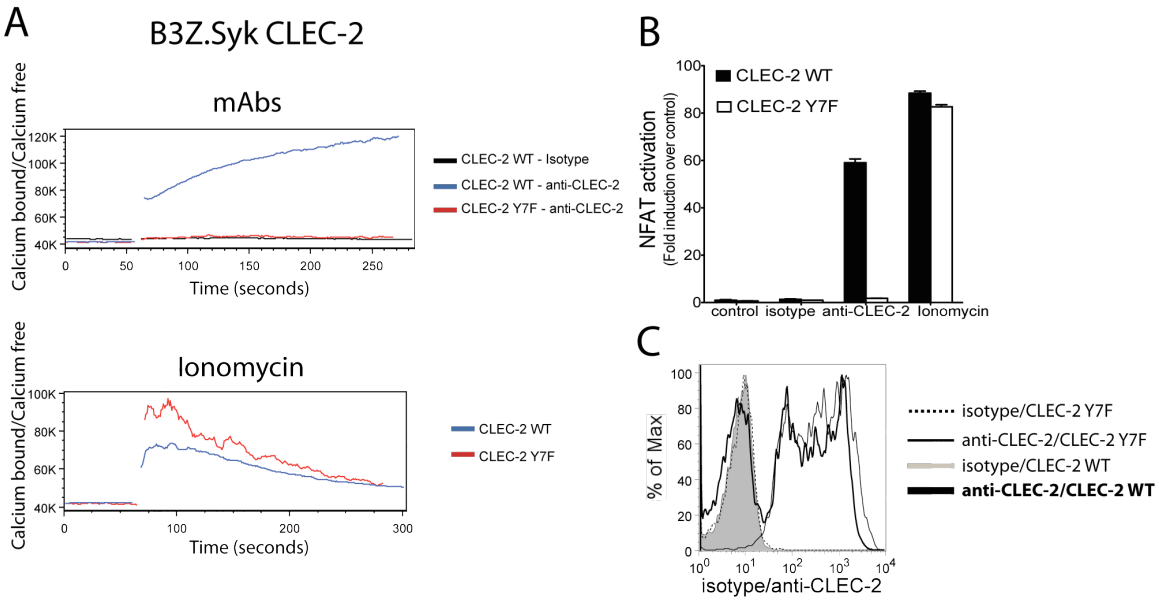

Figure 4.7 CLEC-2 triggering leads to calcium flux and NFAT activation on a hemITAM dependent manner
(A) 2x10^6 B3Z.Syk CLEC-2 WT or Y7F cells were loaded with indo-1 and the calcium flux was analysed by FACS. (B) 1x10^5 B3Z.Syk CLEC-2 WT or Y7F cells were cultured for 18 hours with 1µg/ml of ionomycin or on wells coated with of isotype or anti-CLEC-2 mAb. NFAT induction was quantified by measuring LacZ activity as for the ligand screen. (C) B3Z.Syk CLEC-2 WT and B3Z.Syk CLEC-2 Y7F were stained with anti-CLEC-2-Alexa647 or isotype-matched-Alexa647.

Intracellular calcium fluxing is able to mediate activation of the calcineurin-NFAT pathway. To quantify NFAT activation by CLEC-2 I quantified LacZ activity induced by the NFAT-LacZ reporter stably expressed on B3Z cells (described in chapter 3). As shown on figure 4.7B, plate-coated anti-CLEC-2 antibody strongly induced the NFAT reporter on B3Z.Syk expressing CLEC-2 WT, but not Y7F. Furthermore, the treatment with an isotype-matched control antibody did not activate the reporter. Treatment with ionomycin induced normal NFAT activation on cells expressing CLEC-2 WT or Y7F. Collectively, this data demonstrate the need for the hemITAM of CLEC-2 for calcium flux and NFAT activation in response to receptor cross-linking.

Using NFAT activation as a parameter, I compared different CLEC-2 agonists for their ability to activate CLEC-2. I used B3Z.Syk CLEC-2 cells to test the ability of anti-CLEC-2, rhodocytin or PDPN-expressing cells to activate NFAT (figure 4.8). The anti-CLEC-2 mAb proved to be the best stimulator of NFAT activation. Intriguingly, B3Z.Syk CLEC-2 WT cells showed an increased basal activation of NFAT (compare control on different cells on figure 4.8), suggesting a ligand independent activation of NFAT on a CLEC-2 hemITAM dependent way.

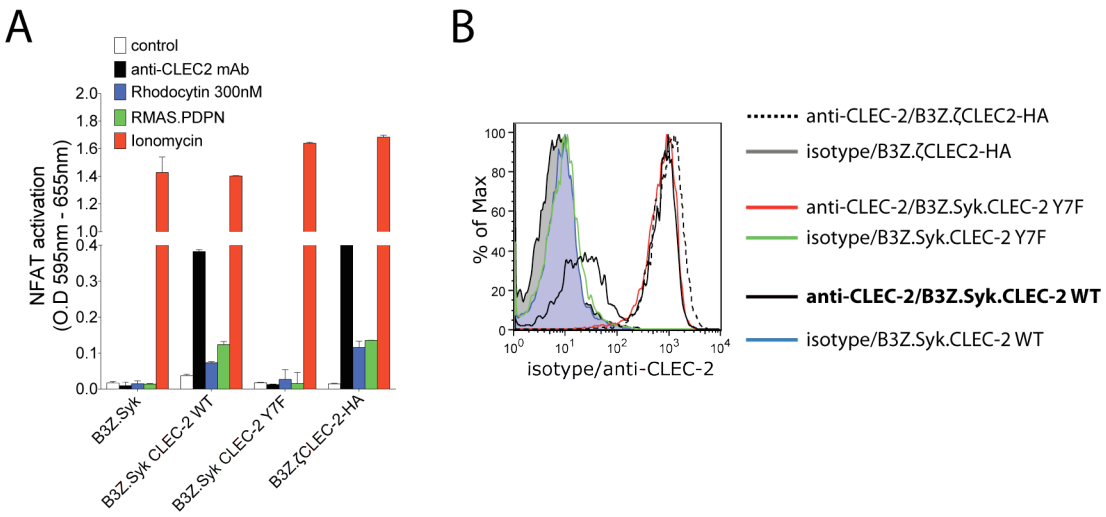

Figure 4.8 Comparison of NFAT activation by CLEC-2 agonists
(A) 1x10^5 B3Z.Syk CLEC-2 WT or Y7F cells were cultured for 18 hours with 1µg/ml of ionomycin, on wells coated with anti-CLEC-2 mAb, co-culture with 1x10^5 RMAS cells expressing PDPN (RMAS.PDPN) or rhodocytin. NFAT induction was quantified by measuring LacZ activity. (B) CLEC-2 expression on B3Z clones was determined by FACS with isotype or anti-CLEC-2 biotinylated staining.

Altogether, the data show that CLEC-2 triggering can induce Ca^{2+} flux and NFAT activation in hemITAM-dependent fashion indicating that a CLEC-2-Syk-NFAT axis pathway might be responsible for CLEC-2 modulation of TLR responses.

As mentioned previously, dectin-1 signalling via Syk was demonstrated to collaborate with TLR signalling for induction of cytokines (Dennehy, Ferwerda et al. 2008) (Gantner, Simmons et al. 2003). Nevertheless, a Syk-independent Raf1 signalling has also been implicated on dectin-1 modulation of TLR responses (Gringhuis, den Dunnen et al. 2009). Curiously, the CLR DC-SIGN was reported to modulate TLR-induced IL-10 production via Raf1-dependent signalling (Gringhuis, den Dunnen et al. 2007). Therefore, I analysed the requirement for Raf1 and Syk in how CLEC-2 signalling modulates IL-10 production induced by TLR agonists. Firstly, I decided to examine if Raf1 is involved on the increased IL-10 production induced by LPS and CLEC-2 triggering. As shown on figure 4.9A, Raf1 inhibitor did not impair the production of IL-10 induced by anti-CLEC-2 and LPS, suggesting that this pathway is not involved on CLEC-2 modulation of TLR responses. Consequently, I decided to evaluate the involvement of Syk on this modulation. As shown on figure 4.9B the incubation of GMCSF BMDC with LPS and anti-CLEC-2 Fab induced increased IL-10 and reduced IL-12/IL-23p40 production. The pharmacological inhibition of Syk significantly impaired the CLEC-2-induced increase of IL-10 in the presence of LPS, whilst it had no effect on IL-12/IL-23p40 production (figure 4.9B). Accordingly, anti-CLEC-2 could not induce increased IL-10 production in Syk-/- DCs, whilst it greatly increased the IL-10 production by WT DCs. The Syk-/- DCs were not impaired on the production of IL-12/IL-23p40, as they produced increased amounts of this cytokine when compared to their WT counter-part (figure 4.9C). In fact, Syk-/- DCs have been reported to hyper-produce IL-12/IL-23p40 upon TLR stimulation (Hamerman et al. 2005).

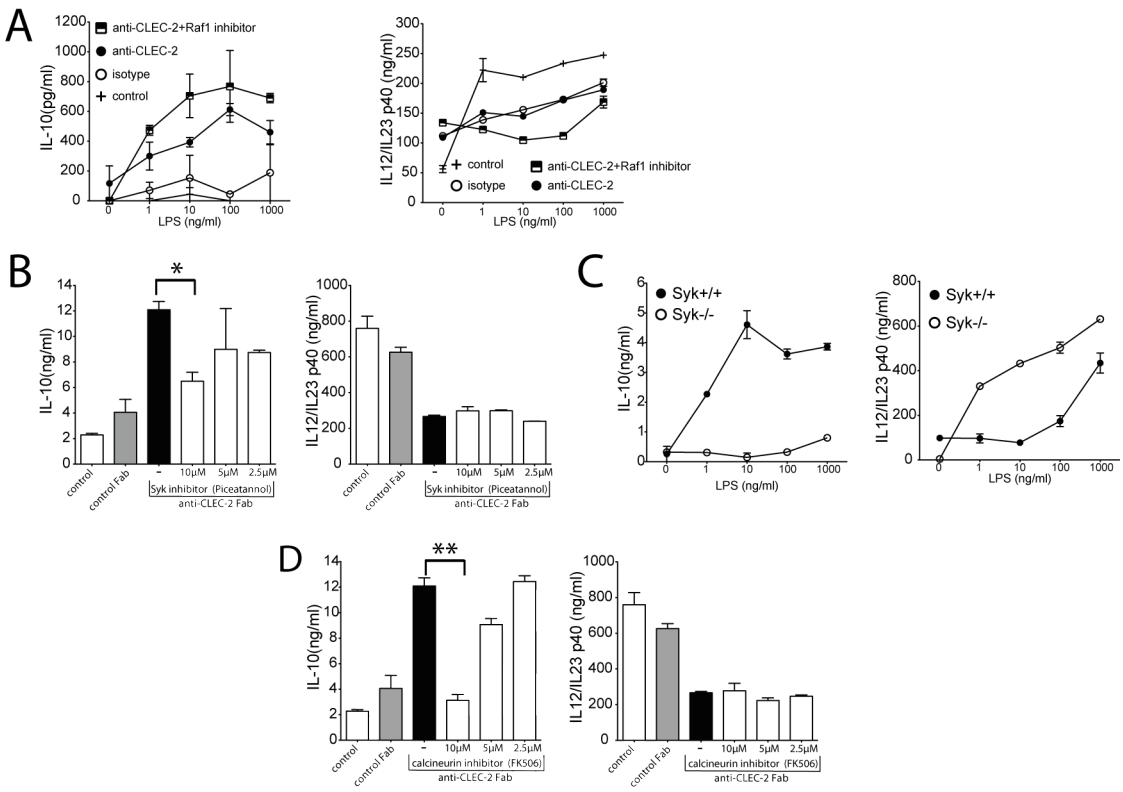

Figure 4.9 IL-10 modulation by CLEC-2 requires Syk and calcineurin, but not Raf1

(A) FLT3L BMDCs were cultured for 18 hour with or without increasing amounts of LPS on wells coated with isotype or anti-CLEC-2 mAb. Raf1 inhibitor (GW5074) was used at 1µM. (B) GMCSF BMDCs were cultured for 18 hours with 10ng/ml of LPS on wells uncoated (control) or coated with control or anti-CLEC-2 Fab and Syk inhibitor (piceatannol) used at the indicated concentrations. (C) FLT3L BMDC from C57BL/6J mouse (WT) or Syk-/- were cultured as in A. (D) GMCSF BMDCs were culture as in (B) using calcineurin inhibitor (FK506) at the indicated concentrations. Cytokines were quantified on the supernatants by ELISA. Unpaired t-test was performed for statistical differences. *p<0.05, **p<0.01

As CLEC-2 triggering was able to induce Ca^{2+} flux and NFAT activation in a hemITAM-dependent way, I decided to examine if this pathway could be involved in CLEC-2 modulation of LPS. I made use of FK506, a strong immunossupressor that blocks NFAT activation (dephosphorylation) by inhibiting calcineurin (Liu et al. 1991). Strikingly, calcineurin inhibition by FK506 strongly reverted the anti-CLEC-2 Fab-induced IL-10 production in the presence of LPS (figure 4.9D). In line with the inhibition of Syk, calcineurin inhibition did not change the production of IL-12/IL-23p40 (figure 4.9D), indicating that the drug is not toxic, at the tested concentrations. Collectively, these experiments indicate that Syk and calcineurin, but not Raf1, are required for CLEC-2 signalling to modulate LPS responses.

4.6 Genome-wide analysis of CLEC-2 modulation of LPS response

To investigate different genes that CLEC-2 signalling can modulate I decided to do a microarray analysis. This approach allows me to do a genome-wide screen of genes by monitoring mRNA levels. Therefore I can extend my findings to genes different from IL-10 and IL12/IL23p40.

While the genome-wide screen assay can be easily performed, the design of the experiment is crucial for the subsequent analysis. As mRNA levels change during time, it is very important to analyse them at different time points. For this, I treated GMCSF BMDC with control or anti-CLEC-2 Fab and 10ng/ml of LPS for 3 or 6 hours prior to RNA extraction. As observed, CLEC-2 triggering greatly increased the induction of *il2*, whilst mildly changed the induction of *il10* observed at 3 hours. In contrast, *il2* mRNA was barely induced at 6 hours, while the production of *il10* mRNA even increased (figure 4.10A). Therefore, this indicate that CLEC-2 can modulate LPS response at the mRNA level opening the perspective to a broadly analysis of genes modulated by CLEC-2.

The microarray assay was performed using RNA extracted from 3 and 6 hours GMCSF BMDC culture with LPS on wells coated with control or anti-

CLEC-2 Fab. The experiment was done twice and the samples were pooled to reduce variability. Therefore, I analysed mRNA samples from 2 independent experiments stimulated for different time points with LPS and anti-CLEC-2 Fab or control Fab (fig.4.10B).

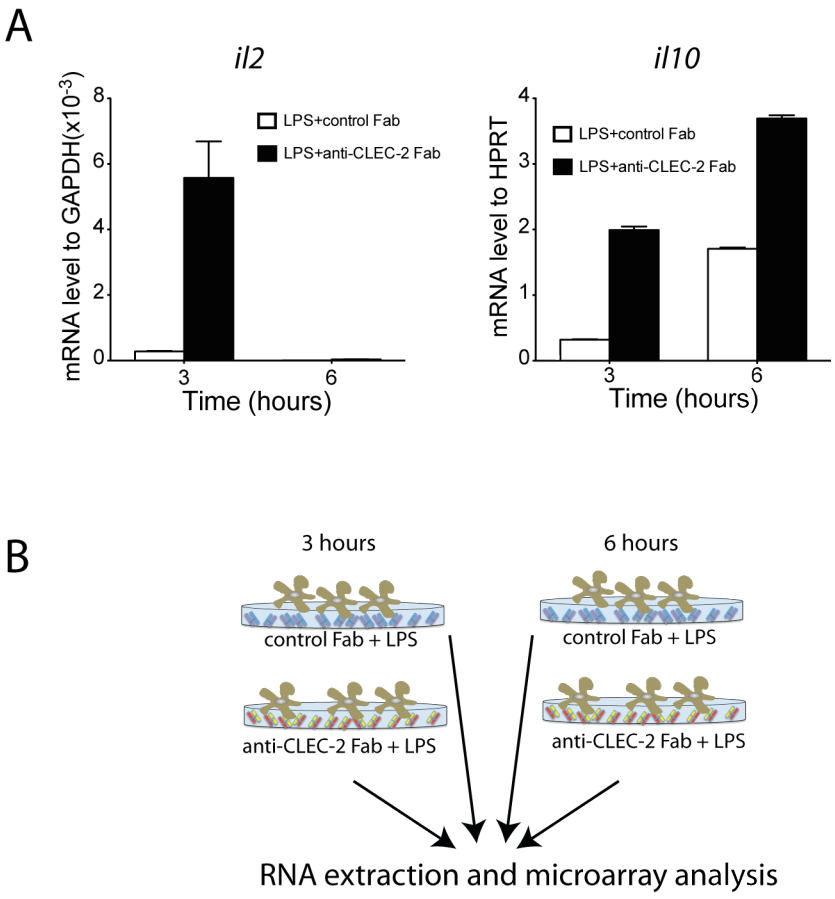

Figure 4.10 Genome-wide analysis of anti-CLEC-2 modulation of LPS responses
(A) GMCSF BMDC were culture for 3 or 6 hours with 10ng/ml of LPS on wells coated with control or anti-CLEC-2 Fab before RNA extraction. The mRNA induction of *il2* and *il10* were quantified by qPCR. (B) Scheme representing the samples used to perform the microarray. Cells were treated as in (A) and RNA was probed with Affymetrix microarray GeneChip mouse genome 430 2.0.

For the analysis of pathways and responses triggered by anti-CLEC-2 and LPS treatment of DCs I considered genes that were changed 2-fold or more, discarding all the other genes. These genes, in collaboration with the Bioinformatics & Biostatistics Service, were analysed clustering according to a database of genes. This gene database characterise genes according to its published functions and ontology (Ekins et al. 2007).

Analysis performed using 274 genes differentially modulated by CLEC-2 at either 3 or 6 hours indicate a gene program that signalling downstream of CLEC-2 can elicit. The genes found differentially induced by CLEC-2 were genes involved on pathways of immune responses, resembling NFAT and PGE_2 signalling (fig.4.11A). Appropriately, my results indicate that CLEC-2 signalling via its hemITAM is able to induce calcium signalling that activates NFAT. Furthermore, using calcineurin inhibitors NFAT signalling was suggested to be implicated on the increased production of IL-10 induced by CLEC-2 and LPS. Interestingly, dectin-1 signalling also induces NFAT and PGE_2 signalling (Goodridge, Simmons et al. 2007). The genes induced by CLEC-2 were also related to processes of inflammation/TREM-1 signalling and chemotaxis. Knowingly, the Syk-coupled receptor TREM-1 modulates LPS responses and is involved on endotoxic shock (Bouchon et al. 2001) and Syk signalling was also implicated on integrin activation and migration (Schymeinsky et al. 2006).

The heatmap, represented in figure 4.11B, shows a list of significantly induced genes involved on the pathway related to immune response and NFAT signalling. As shown, CLEC-2 differentially induces the expression of two NFAT members (NFATc2 and NFATc1), signalling proteins leading to NFAT activation (LAT and Rcan1) and cytokines (IL-2 and IL-10).

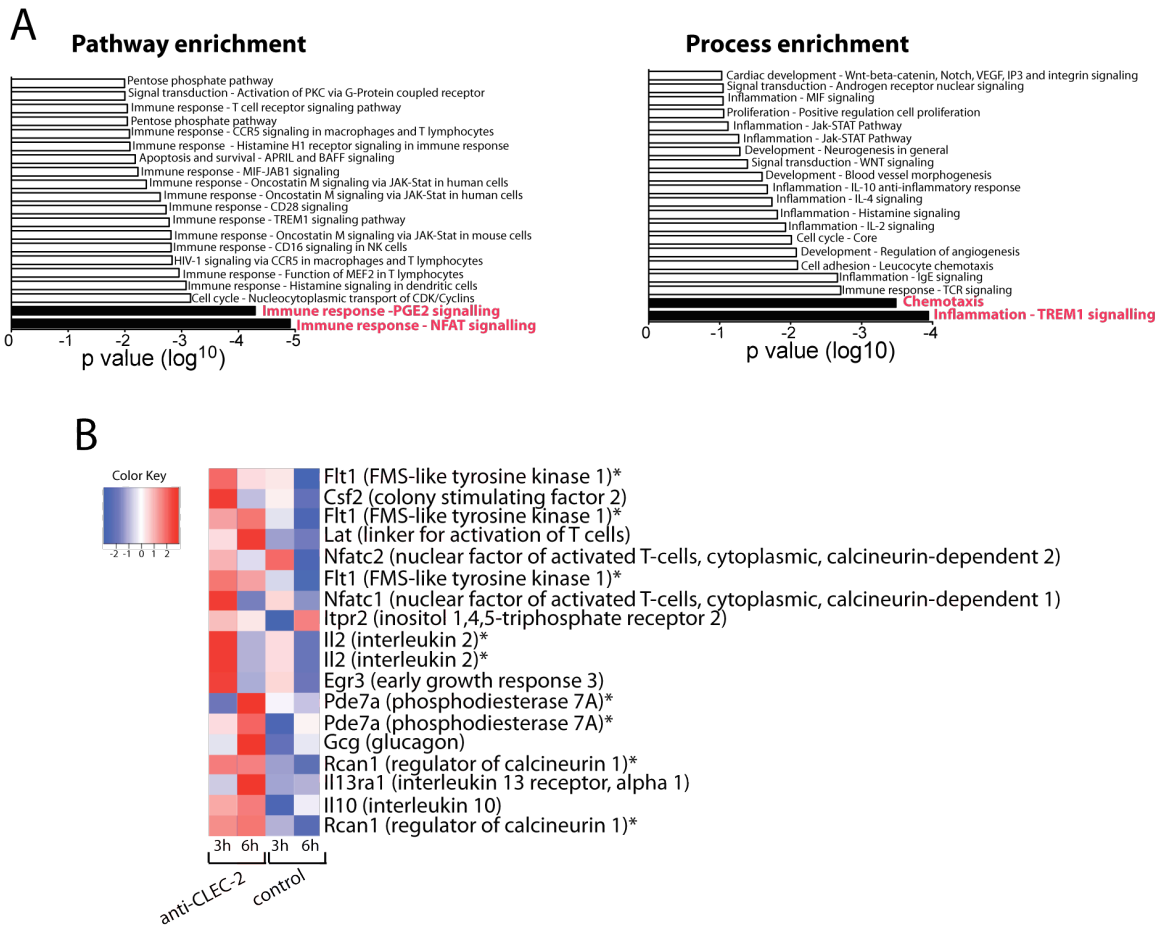

Figure 4.11 CLEC-2 modulates LPS inducing NFAT signalling
(A) The 274 genes found at least 2-folds differentially expressed upon anti-CLEC-2 Fab treatment were computationally compared with available data regarding these genes pathways and processes. Immune responses genes involved on NFAT and PGE2 signalling pathways were found statistically enriched on these lists. Inflammation and chemotaxis were found to be statistically different processes that are modulated by genes found changed by CLEC-2 triggering. (B) Heatmap of genes involved on Immune responses – NFAT signalling. * represents genes detected by different probes.

Overall, the microarray clustering gave me a glimpse of processes that CLEC-2 signalling could modulate. Besides, this opened up perspectives for more refined analysis of genes found changed by microarray. Thus I decided to investigate the ability of CLEC-2 to induce certain genes without LPS co-treatment. Therefore I cultured GMCSF BMDC for 3 hours on wells coated with control or anti-CLEC-2 Fab with or without 10ng/ml of LPS, and then obtained RNA. After quantitative PCR analysis I observed that the induction of NFAT-inducible genes *egr3*, *il2* and *il10* by anti-CLEC-2 Fab did not need LPS. However, LPS co-treatment greatly increased the induction of *il2* and *il10* (figure 4.12A and B). Interestingly, the induction of *egr3* by CLEC-2 triggering was diminished by LPS co-treatment, although the difference between control and anti-CLEC-2 Fab treated with LPS remained the same (figure 4.12B). The analysis of induction of *il33* revealed that CLEC-2 triggering induced very little amounts of *il33* mRNA, although when LPS is added together a great induction is observed (figure 4.13A). Several other genes that were not found different by microarray analysis were tested to confirm the microarray result. I found negligible changes on the induction of *tnf*, *ccl1, ccl12* and *il12b* by CLEC-2 triggering. Moreover, the addition of LPS greatly increased the induction of these genes and CLEC-2 triggering did not show any effect (figure 4.12A and B). Additionally, I selected *perp* as gene that did not show any change by microarray to further validate the assay. This comparison showed that LPS and/or CLEC-2 signalling did not affect *perp* expression (figure 4.12A).

Basically, the microarray analysis supported previous findings that CLEC-2 triggering can induce NFAT-dependent genes. Furthermore, it shows that CLEC-2 signalling can act independent of TLR signalling to induce genes and collaborate with TLR signalling to increase gene expression at mRNA level.

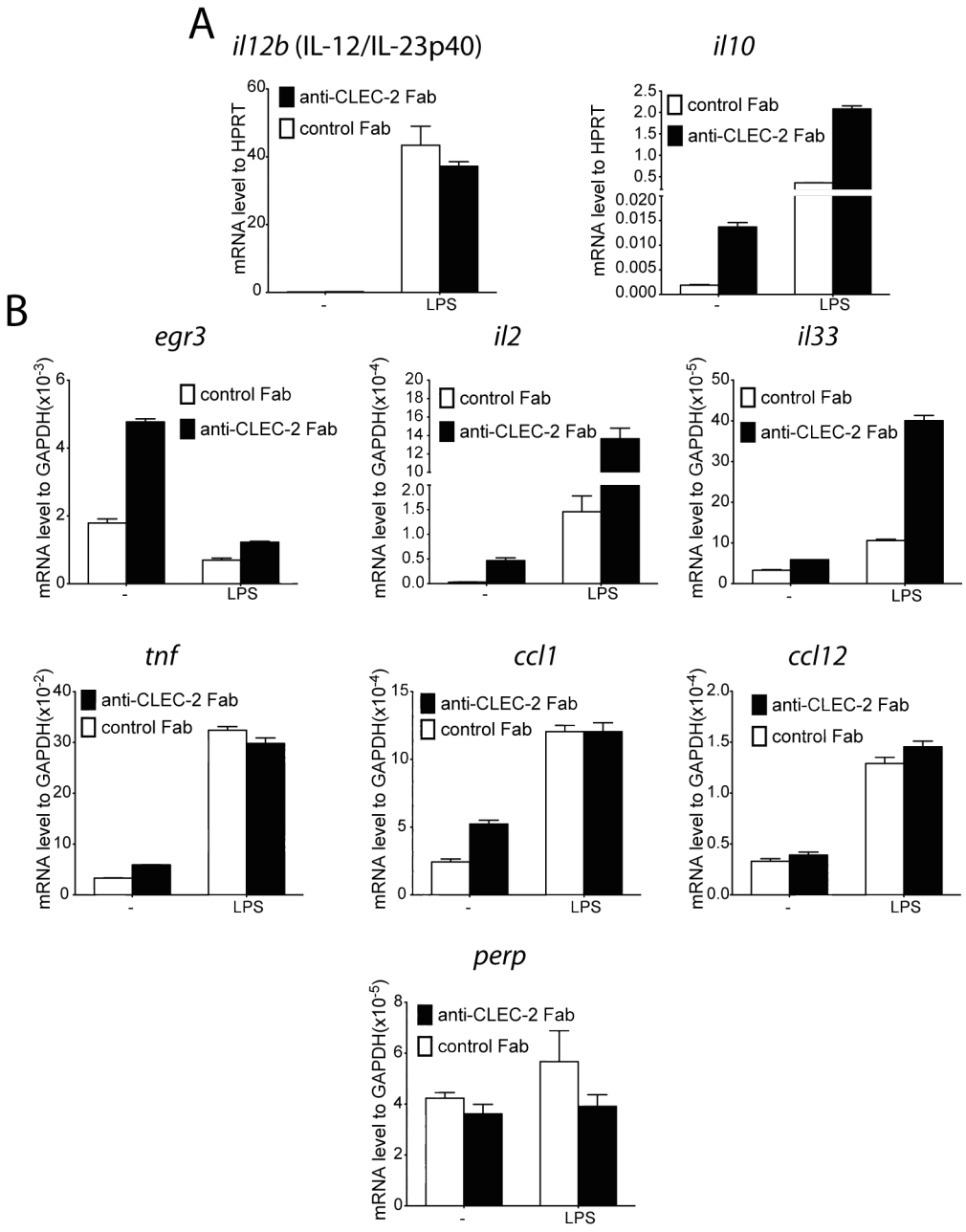

Figure 4.12 Analysis of CLEC-2 regulation of gene expression
GMCSF BMDCs were culture on wells coated with control or anti-CLEC-2 Fab on the presence or absence of 10ng/ml of LPS. After 3 hours RNA were extracted and qPCR analysis was performed to quantify gene changes. (A) *il12b* and *il10* gene changes were compared in relation to the constitutive *HPRT* expression. (B) gene changes were compared to *GAPDH* expression.

4.6.1 Summary and discussion

CLEC-2 has two reported agonists: the snake venom toxin rhodocytin and the endogenous protein podoplanin. The outcome of the interaction of CLEC-2 with these two agonists had been studied using platelets. Those studies revealed that CLEC-2 signalling via its intracellular tyrosine could couple to Syk signalling and mediated platelet aggregation (Ozaki et al. 2009). Herein, I showed that CLEC-2 signalling and function could be studied in myeloid cells by selective triggering by anti-CLEC-2. Moreover, CLEC-2 signalling induced Ca^{2+} flux and NFAT activation in a hemITAM-dependent fashion. The functional outcome of this signalling was observed in the increased production of IL-10 by DCs and macrophages co-treated with LPS. Finally, a microarray analysis showed that CLEC-2 modulation of LPS induces genes involved in NFAT signalling in immune responses.

Firstly I tried to validate the described CLEC-2 agonists. Analysing cytokine production by DCs treated with rhodocytin I observed increased cytokine production. However, this was largely Syk-independent (see figure 4.1), suggesting a CLEC-2-independent cytokine production. Rhodocytin is a tetramer that is reported to trigger multiple receptors (Suzuki-Inoue et al. 2001; Suzuki-Inoue, Fuller et al. 2006) and is purified from the snake venom through different steps and buffers (Eble et al. 2001), thus it is likely that it can get contaminated by either bacterial cell wall components (LPS and lipoproteins, TLR4 and TLR2 agonists) and/or bacterial DNA (TLR9 agonist). However, when cytokine production by Myd88-/- DCs is analysed there was still a measurable amounts of TNF and IL-12/IL-23p40, suggesting that rhodocytin might induce cytokine production by CLEC-2 and independently of TLR signalling. Accordingly, rhodocytin induced cytokine production on cell lines expressing CLEC-2 WT, but not CLEC-2 Y7F. Therefore, the cytokine measured in WT DCs might be a product of both Syk and Myd88 signalling, suggesting collaboration between CLEC-2 and TLR. In fact, other CLRs like dectin-1 (Dennehy, Ferwerda et al. 2008) and DC-SIGN (Caparrós, Munoz et al. 2006)

have been already reported to collaborate with TLR to increase cytokine production.

As my rhodocytin preparation triggered TLRs, I decided to examine the regulation of gene expression by DCs treated with more selective CLEC-2 ligands, like anti-CLEC-2 mAb and the endogenous CLEC-2 agonist PDPN. PDPN treatment of DCs did not induce cytokine production by DCs (see figures 4.2 and 4.3), while anti-CLEC-2 mAb induced different NFAT-dependent genes. This was curious, as PDPN was demonstrated to mediate platelet aggregation on a CLEC-2 dependent way (Suzuki-Inoue, Kato et al. 2007). However, platelets express higher levels of CLEC-2 than DCs. Moreover, PDPN.Fc was able to induce cytokine production on LK cells overexpressing CLEC-2, suggesting that CLEC-2 surface expression might play a role on the downstream function. However, comparison of agonistic activity of anti-CLEC-2 and PDPN.Fc on different cell lines showed that anti-CLEC-2 was better stimulator of CLEC-2 than PDPN.Fc (see figures 4.5C and 4.8A). However, my preparation of PDPN.Fc had very low amounts produced on the supernatant, thus the poor agonistic activity can be due to the degree of CLEC-2 stimulation and my inability to better stimulate CLEC-2 with PDPN.Fc.

ITAM signalling is commonly regulated by ITIM signalling (Ravetch and Lanier 2000), thus it is feasible that DCs differentially express regulatory mechanisms that does not allow PDPN to act as a CLEC-2 agonist on DCs. Intriguingly, B3Z.Syk.CLEC-2 WT cells had a higher basal activation of the NFAT reporter (figure 4.8A) and were never a homogeneous population (figure 4.8B). Appropriately, Mori and colleagues revealed that the ITIM signalling receptor G6b-B, could inhibit constitutive CLEC-2 activation on cell lines (Mori et al. 2008). It will be important to examine the expression of G6b-B on DCs and analyse if its knockdown will enable PDPN regulation of gene expression on DCs. Moreover, analysis of gene induction by PDPN on DCs deficient of tyrosine phosphatases can also give clues of DC regulatory mechanisms of CLEC-2 signalling.

Strikingly, anti-CLEC-2 mAb, compared to rhodocytin and PDPN, proved to be the best agonist of CLEC-2 regarding cytokine production and NFAT

stimulation (figure 4.5C and 4.8A). Consequently, it induces calcium flux and NFAT activation dependent of its hemITAM (figure 4.7). It will be important to evaluate the kind of calcium signalling induced by PDPN and anti-CLEC-2 on DCs. Tonic calcium signalling is proposed to be inhibitory of TLR signalling, whilst transient calcium signalling synergises with TLR signalling. Furthermore, the type of calcium signalling is related with the avidity of the ligand-receptor pair; low avidity ligands induce tonic calcium stimulation and high avidity ligands induce strong transient calcium signalling (Ivashkiv 2008). Thus, it is likely that anti-CLEC-2 mAb has higher avidity to CLEC-2 than PDPN.Fc.

CLEC-2 triggering by anti-CLEC-2 Fab, without LPS, led to induction of some NFAT-dependent genes (fig.4.12), which provided a proof of principle that signalling downstream of hemITAM-containing receptors is able to regulate gene expression. Nevertheless, to formally demonstrate a CLEC-2 selective phenomenon this assay would need to be done using Syk-/- and Myd88-/- DCs.

Undoubtedly, the most remarkable phenomenon of gene regulation by CLEC-2 is observed when anti-CLEC-2 is given with LPS. It is possible that because LPS increases CLEC-2 expression on myeloid cells, the TLR triggering would be needed prior to CLEC-2 triggering only to increase CLEC-2 expression. Accordingly, CLEC-2 over-expression on LK cells confers then ability to regulate gene expression by CLEC-2 ligation without LPS co-triggering (fig.4.5C). It will be interesting to over-express, by retroviral transduction, CLEC-2 on DCs and evaluate the need for CLEC-2 and TLR co-triggering for gene induction.

It is likely that CLEC-2 activation of NFAT and TLR activation of NFKB are simultaneously required for the gene modulation observed (see figure 4.13, for a model for CLEC-2 modulation of TLR). IL-10 gene binds to NFAT (Im et al. 2004) and its production by macrophages has been shown to be dependent of NFAT (Goodridge, Simmons et al. 2007). Nevertheless, IL-2 is also an NFAT dependent gene (Rao et al. 1997) but it was not observed in the culture supernatants of DCs stimulated by anti-CLEC-2 and LPS. Recently, Saraiva and colleagues have shown that IL-10 production by T cells requires increased ERK activation and IL-12-induced signalling. At the same time, on these

assays, T cells produced less IL-2 (Saraiva et al. 2009). Therefore, it is likely that increased NFAT activation by induced by anti-CLEC-2 together with IL-12 and ERK activation induced by LPS differentially induced IL-10 and not IL-2.

Calcineurin inhibition was shown to increase TNF production downstream of TLR signalling (Kang et al. 2007), suggesting that NFAT can modulate TLR responses. My finding points to a modulation of TLR signalling by a CLEC-2-calcium-calcineurin-NFAT activation on the regulation of IL-10 production, suggesting that the simultaneous ligation of CLEC-2 and TLR might be necessary. Considering that IL-10 can negative regulate TNF production (Gérard et al. 1993), it is possible that the increased TNF production observed upon calcineurin inhibition is due to diminished IL-10.

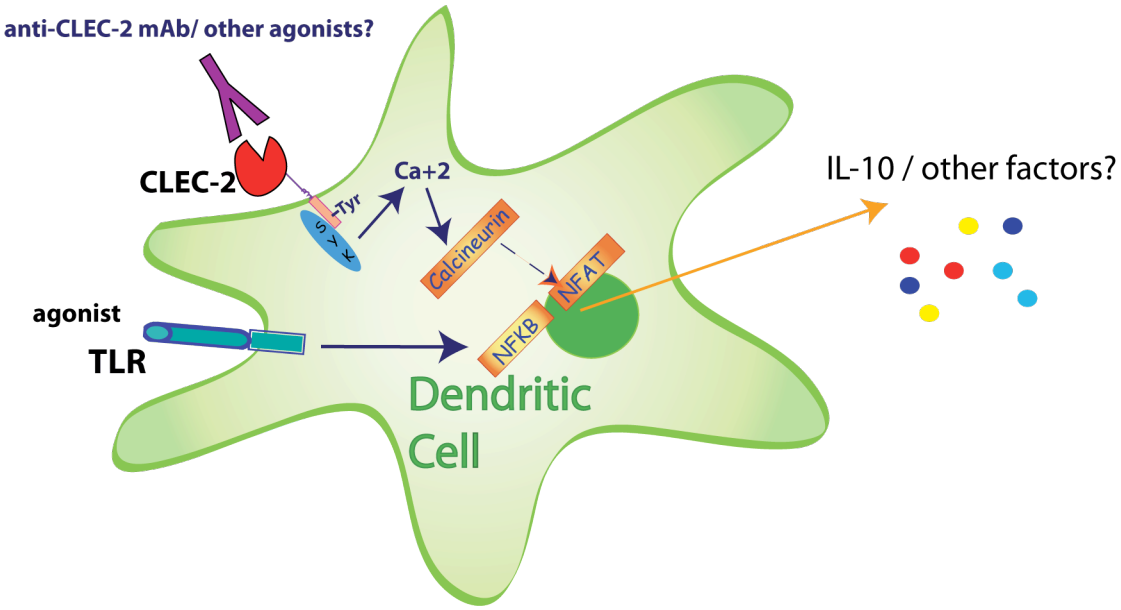

Figure 4.13 Proposed model for CLEC-2 modulation of TLR responses
NFKB activation by TLR and NFAT activation by CLEC-2 lead to augmented production of IL-10 and other NFAT-dependent genes (e.g. IL-2, egr3, glucagon, NFATc1, NFATc2) . CLEC-2 triggering by anti-CLEC-2 mAb leads to Ca^{2+} signalling, calcineurin and NFAT activation in a hemITAM-dependent manner.

Besides the similarities between dectin-1 and CLEC-2 regarding to collaboration with TLR and activation of NFAT, they appear to differ on the activation of NFκB. I failed to observe any induction of NFκB-dependent genes by selective CLEC-2 ligation with mAbs or PDPN, while dectin-1 signalling couples to NFκB activation (Leibundgut-Landmann, Groß et al. 2007). The activation of NFκB by dectin-1 was demonstrated to be via Card9-Bcl10-Malt1 (Gross, Gewies et al. 2006). Similarly, Carma1 localisation on lipid rafts couples with Bcl10 and Malt1 to activate NFκB on T and NK cells (Gaide et al. 2002; Gross et al. 2008). Recently, dectin-1 was shown to localise on lipid rafts upon triggering, which was required for optimal dectin-1 signalling and cytokine production (Xu, Huo et al. 2009). Consequently, dectin-1 ability to couples to NFκB activation might be due to the ability of its agonists to induce lipid raft localisation and thus, Card9-NFκB activation. As the induction of calcium signalling and NFAT activation does not depend on CARD9 activation I hypothesize that anti-CLEC-2 mAb does not activate NFκB due to its inability to induce efficient CLEC-2 localisation to lipid rafts and where CARD9 is localised.

Analysis of genome-wide screen for genes modulated by CLEC-2 during LPS treatment considered only genes changed 2-fold or more. Due to the limited sample replicates I considered that analysing these genes would be more robust. In fact there are many other genes that are changed less that 2-fold that might play important role in CLEC-2 induced phenomenon. The gene profile analysis pointed to an enrichment of genes involved on pathways of NFAT signalling. Besides, the processes of inflammation/TREM1 signalling and chemotaxis were found enriched on the genes modulated by CLEC-2 triggering. In line with my findings, TREM1 is a receptor that couples to Syk signalling via DAP12 (Bouchon et al. 2000) and modulates endotoxic shock responses (Bouchon, Facchetti et al. 2001). In addition, a chemotaxis gene induction profile was found enriched, which might be suggestive of a role of CLEC-2 on migratory processes in DCs. PDPN is expressed in the lungs (Ramirez et al. 2003), skin (Gomaa et al. 2007), lymphatic endothelium (Schacht, Ramirez et al. 2003) and lymph node stroma (Lee, Epardaud et al. 2007) and it is possible that CLEC-2 might allow DC migration to these tissues. Significantly, very few

DCs on lymph nodes express CLEC-2 in steady state conditions (figure 3.9B, compare isotype/PBS with anti-CLEC-2/PBS), whereas in inflammatory conditions many of these DCs express CLEC-2. It is tempting to speculate that CLEC-2 expression might define a population of migratory DCs that enter the lymph nodes after encountering PDPN on the lymphatic vessels. In fact, Syk was involved on migration, as Syk deficiency impaired neutrophils and monocytes recruitment to inflamed tissues (Schymeinsky, Sindrilaru et al. 2006).

Chapter 5. Mouse models to study CLEC-2 function

5.1 Introduction

Myeloid cells are distributed throughout tissues and perform several functions, which include cytokine production, phagocytosis of invading microorganisms and activation of adaptive immune responses. All these myeloid cell functions are mediated by signals from innate receptors, including CLRs (Robinson et al. 2006). Thus, identification of signals and innate receptors is of great importance for understanding immune responses.

The CLR family has several members that are involved on the modulation of inflammation and TLR responses, as the case of dectin-1 (Dennehy et al. 2008), DCAL-1 (CLEC12a) (Chen et al. 2006) and DC-SIGN (Gringhuis, den Dunnen et al. 2007). Previously, I showed that CLEC-2 signalling was capable of modulating TLR responses in DCs and macrophages, inducing an increased production of IL-10 via a Syk-calcineurin dependent mechanism (Chapter 4). This implied that CLEC-2 action on myeloid cells could modulate LPS-induced inflammation *in vivo*. Therefore it will be important to evaluate the capacity of CLEC-2 to act *in vivo* to modulate inflammation and to develop models to study CLEC-2 function in different cells.

In this chapter I will describe the development of mouse models to study CLEC-2 function on the modulation LPS-induced responses. Besides, I will describe the generation of a CLEC-2 (*clec1b*) conditional KO mouse.

5.2 Anti-CLEC-2 modulation of LPS-induced inflammation

Previously, I showed that cross-linking of CLEC-2 could induce modulation of TLR signalling, resulting in increased production of IL-10 by different myeloid cells (Chapter 4). Therefore, I decided to test if anti-CLEC-2 administration together with LPS could induce a similar phenomenon in mice. So, 3 hours after intra-peritoneal administration of mice with PBS, isotype-matched or anti-CLEC-2 antibody together or not with LPS, I analysed serum amounts of IL-6, IL-10 and TNF. As shown in figure 5.1A, PBS or anti-CLEC-2

did not induce any detectable levels of these cytokines. Co-administration of anti-CLEC-2 or isotype-matched control antibodies with LPS did not show differences on the serum amounts of TNF or IL-6. Strikingly, increased serum IL-10 levels were present in mice injected with anti-CLEC-2 and LPS (fig. 5.1A).

To better characterise the changes on the production of cytokines by anti-CLEC-2 and LPS administration, I decided to quantify several inflammatory mediators at the site of challenge (peritoneum) as well as systemically (serum). So, 3 hours after challenge I obtained peritoneal lavages and sera from mice challenged with LPS and anti-CLEC-2 or isotype-matched control antibodies. As observed before, IL-10 levels, but not IL-6 or TNF, are increased on the sera of mice challenged with anti-CLEC-2 and LPS compared with mice challenged with isotype-matched mAb and LPS. Also, different chemokines (MIP1β, KC, RANTES, MCP-1 and MIP1α) were quantified and no differences were observed on their production (fig.5.1B).

Analysis of peritoneal lavages revealed a similar observation: increased IL-10 in mice treated with anti-CLEC-2 and LPS compared to mice treated with isotype-matched mAb and LPS (fig.5.1C). On the other hand, there was increased TNF on the peritoneal lavages of mice treated with LPS and anti-CLEC-2 compared with mice treated with LPS and isotype-matched mAb (fig.5.1B). This differential effect on TNF in serum and peritoneum can be explained in different ways. Firstly, TNF might be differently modulated by CLEC-2 triggering on different cells. For example the triggering of peritoneum resident B cells (B1 cells) (Balabanian et al. 2002) by CLEC-2 and LPS might induce localised production of TNF. Additionally, CLEC-2 modulation can induce a differential recruitment of cells that can produce TNF when stimulated with LPS. Another possibility is that CLEC-2 might delay the production of TNF, thus production of TNF on the serum is due to the first localised production of TNF that happens later. So an increased production of TNF on the serum might be observed at later time points. On subsequent experiments I will try to examine the production of TNF on the serum on earlier and later time points to analyse this possibility.

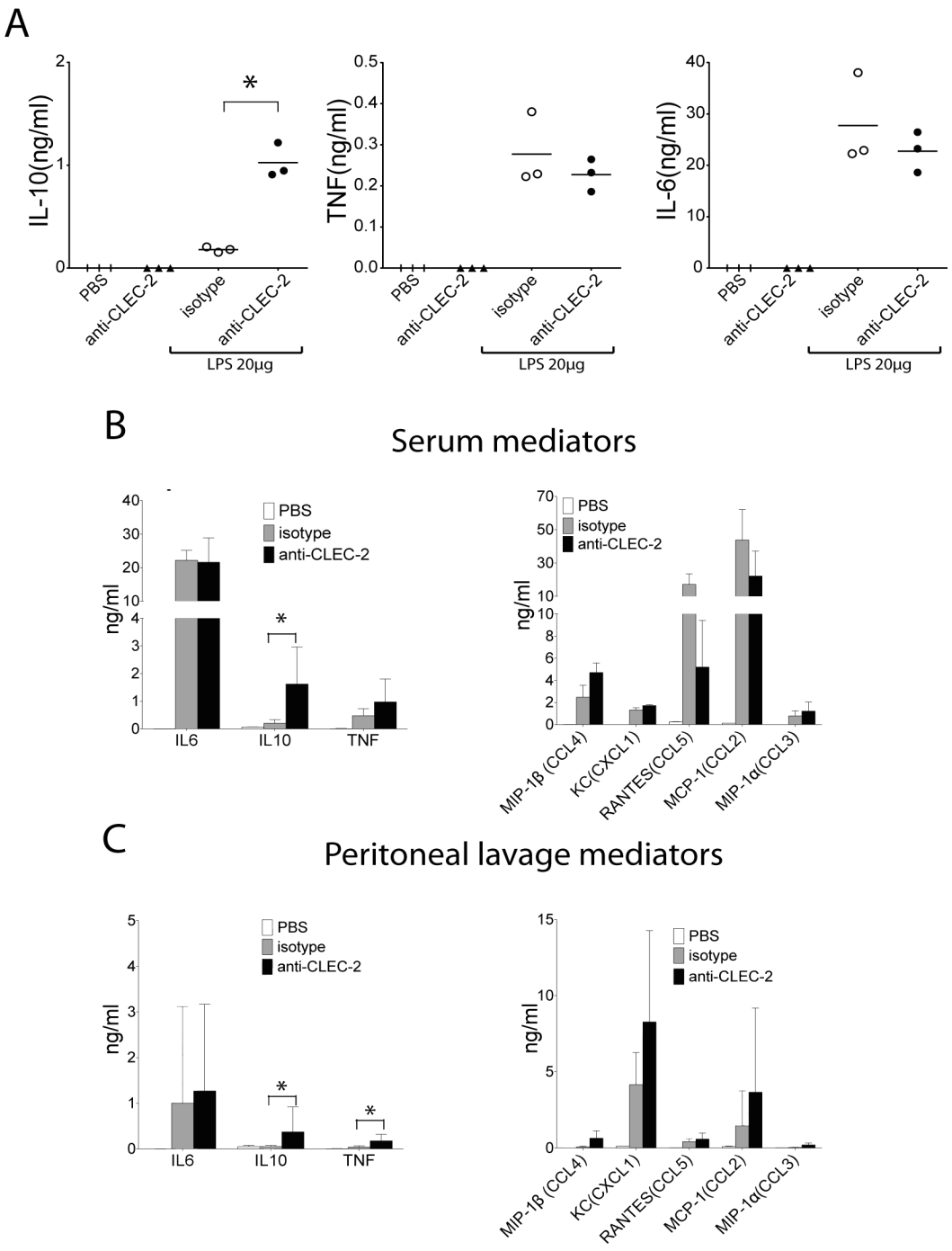

Figure 5.1 Anti-CLEC-2 co-administration increases IL-10 levels induced by LPS *in vivo*

(A) C57BL6/J mice were injected I.P with PBS, PBS with 300µg of isotype or anti-CLEC-2 mAb. 20µg of LPS were added to the indicated treatments. Mice were sacrificed 3 hours after challenge and serum was obtained. (B) (C) Mice were treated as in (A) with LPS. Mice were sacrificed 3 hours after challenge and serum and peritoneal lavage were obtained. All inflammatory mediators were quantified by cytometric bead array (CBA). Data are means ± SD of at least three mice per group in two independent assays. Statistical differences were assessed with Mann-Whitney test. *$p<0.05$

5.3 Characterisation of anti-CLEC-2 modulation of LPS responses *in vivo*

To further characterise the effect of anti-CLEC-2 *in vivo*, I tried to analyse the levels of different inflammatory mediators after challenging mice with anti-CLEC-2 or isotype-matched antibodies and different amounts of LPS. With all LPS amounts there was increased levels of IL-10 induced by anti-CLEC-2 treatment, compared to the isotype-matched treatment (fig.5.2). In contrast, at the highest and lowest LPS amounts (40μg and 10μg, respectively), an increased production of IL-6 was observed on mice challenged with anti-CLEC-2, while a diminished production of MIP-1α was observed. There was also a sharp difference on the production of TNF when compared the challenge with 40μg and 20μg of LPS. The mice challenged with 20μg of LPS and anti-CLEC-2 produced less TNF than the isotype, while on the challenge with 40μg of LPS the mice treated with anti-CLEC-2 produced more TNF when compared to isotype-treated mice (fig.5.2). There are minor contrasts between the cell culture and the mouse model experiments of CLEC-2 modulation of LPS responses, but these contrasts might be explained by the complexity of cells and systems involved on the mouse model. Nevertheless, there was a fundamental similarity between the different system: the increased production of IL-10. This differential production of IL-10 is observed regardless of the myeloid cell type used (macrophages or DCs) as well as on the mouse, strongly indicating the qualitative different response that CLEC-2 triggering can induce on the presence of LPS.

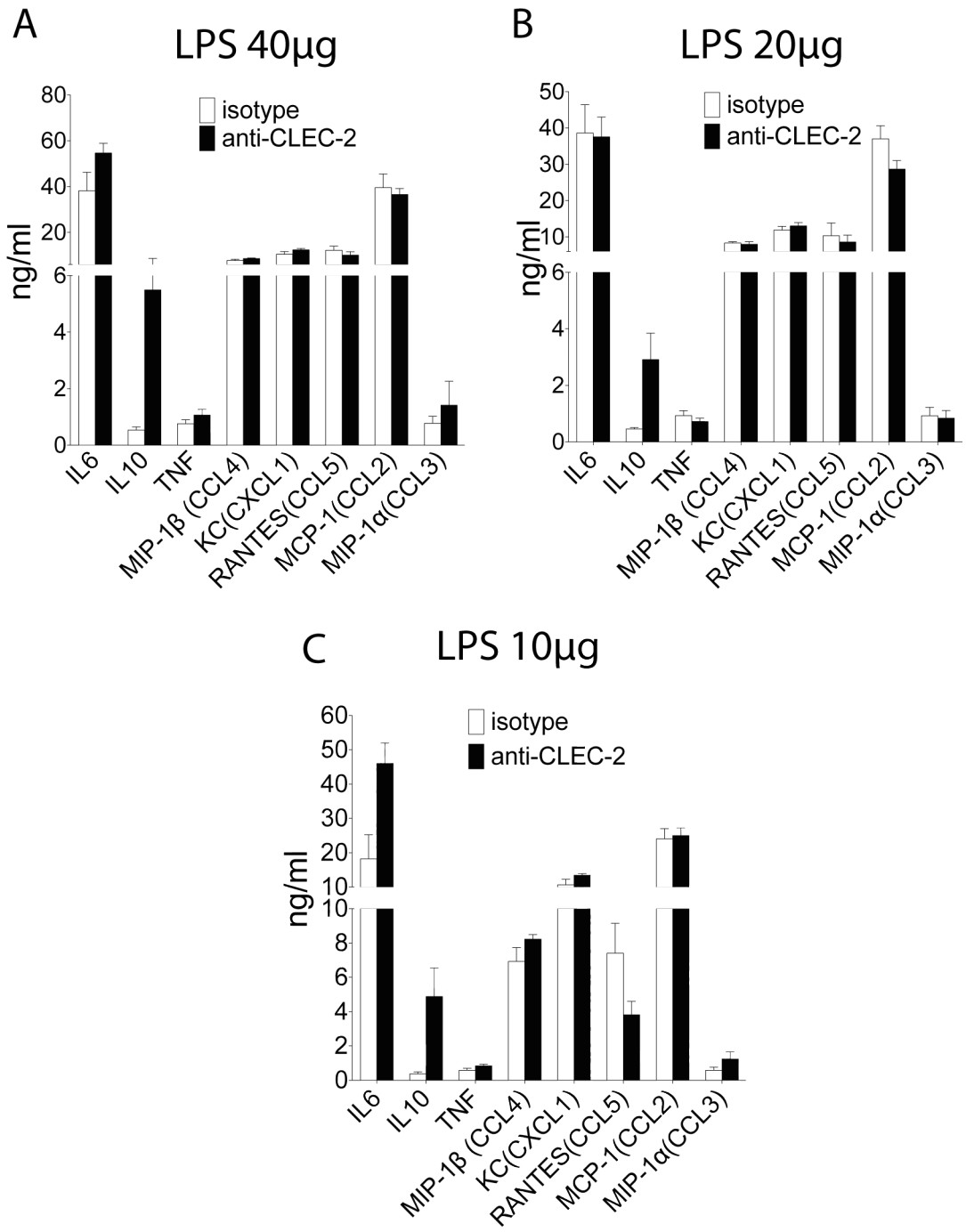

Figure 5.2 Anti-CLEC-2 modulates responses to different amounts of LPS
C57BL/6J mice were injected I.P with 300µg of isotype or anti-CLEC-2 mAb and (A) 40µg (B) 20µg and (C) 10µg of LPS. All inflammatory mediators were quantified by cytometric bead array (CBA) on serum samples. Data are means ± SD of at 4-6 mice per group in a single assay. Statistical differences were assessed with Mann-Whitney test. *$p<0.05$ **$p<0.01$.

Regardless of the differences on the production of other cytokines, the increased IL-10 level is the best read-out for CLEC-2 modulation of LPS response in this mouse model. Because of this, I decided to analyse IL-10 levels modulated by LPS and different amounts of anti-CLEC-2. Consequently, there were no differences on IL-10 in the serum of mice challenged with 20µg of LPS and different amounts of anti-CLEC-2 (300µg, 150µg, 75µg or 37.5µg) (fig.5.3A). Subsequently, I used even lower amounts of anti-CLEC-2 trying to reach a point where the increased IL-10 level was no longer observed. Hence, the challenge with 10µg of anti-CLEC-2 already showed a reduced production of IL-10, whilst the administrations of 2.5µg of anti-CLEC-2 showed no affect whatsoever on the increased IL-10 level (fig.5.3B). Overall, these assays showed a minimal amount of anti-CLEC-2 able to induce the maximal effect on induction of increased IL-10 in the serum, allowing me to further characterise the time differences on the production of different mediators.

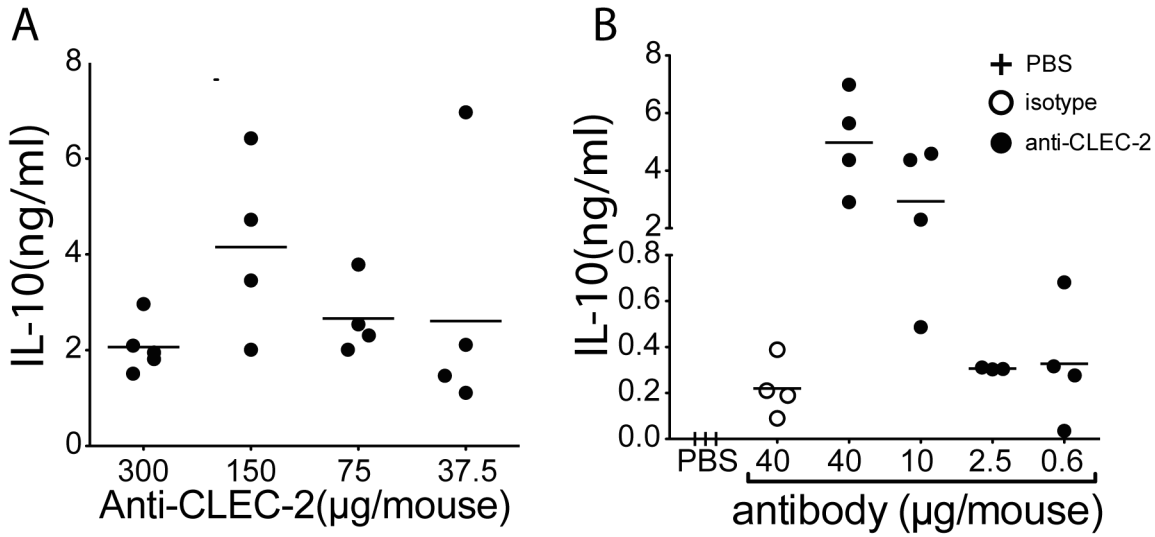

Figure 5.3 Different amounts of anti-CLEC-2 increase IL-10 on LPS-induced inflammation in mice
(A) C57BL/6J mice were injected I.P with 20µg of LPS and different amounts of anti-CLEC-2 mAb. (B) C57BL/6J mice were injected I.P with PBS, 40µg of isotype and 20 µg of LPS or different amounts of anti-CLEC-2 mAb with 20µg of LPS. IL-10 was quantified by cytometric bead array from serum samples (CBA). Each dot represents a different mouse.

As discussed above, the observed modulation of endotoxic shock by anti-CLEC-2 might change according to the time point analysed or the tissue where the inflammatory mediator is quantified. To examine the first, I challenged mice with 20μg of LPS and 40μg of anti-CLEC-2 or isotype and analysed the production of cytokines on the serum. There were no differences on the production of IL-6 at 1 hour, 3 hours or 6 hours after challenge (fig.5.C). The production of IL-10 was increased 3 hours after challenge, as shown earlier, and also at 6 hours (fig.5.4A). Strikingly, the amounts of TNF were reduced 1 hour after challenge, but not later, in mice treated with anti-CLEC-2 and LPS (fig.5.4B). Thus, the analysis at different time points revealed that CLEC-2 triggering could modulate the balance of TNF and IL-10 during endotoxic shock. This can be important, as TNF is an important mediator of mortality during endotoxemia, and IL-10 can revert TNF production and lethality (Gérard, Bruyns et al. 1993; Howard et al. 1993).

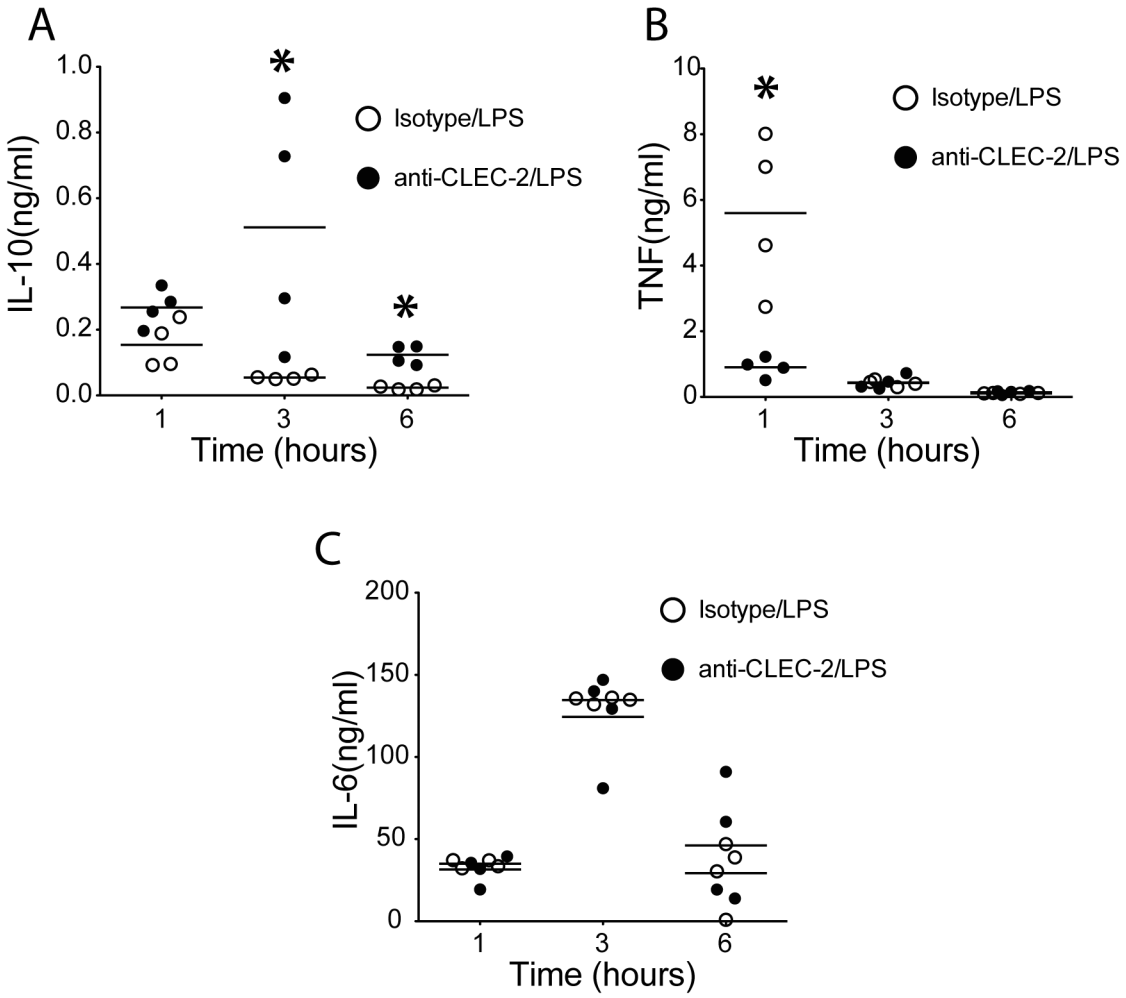

Figure 5.4 Kinetics of inflammatory mediators modulated by anti-CLEC-2 and LPS

C57BL/6J mice were injected I.P with 20µg of LPS and 40µg of isotype or anti-CLEC-2 mAb. Serum samples were obtained 1, 3 or 6 hours after injections. (A) IL-10, (B) TNF and (C) IL-6 were quantified by CBA. Data is representative of two independent assays. Each dot represents a different mouse. Statistical differences were assessed with Mann-Whitney test. *$p<0.05$.

5.4 Involvement of myeloid cells in CLEC-2 modulation of LPS responses

Many cell types were demonstrated to produce IL-10, including NK cells, B cells, neutrophils, macrophages and DCs (Saraiva and O'Garra 2010). Considering a broad expression of CLEC-2 (Chapter 3), and that the LPS receptor TLR4 is widely expressed in immune cells as well as on epithelial cells, it is conceivable that many cells are involved in the increased production of IL-10 during CLEC-2 modulation of LPS responses *in vivo*.

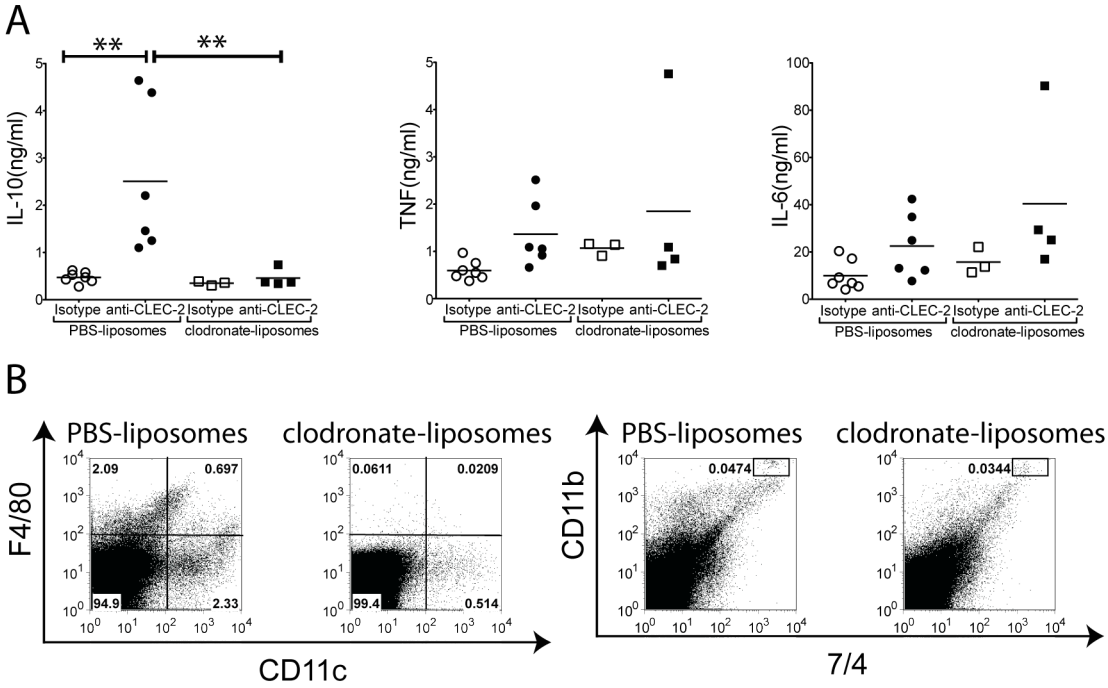

Figure 5.5 DCs and macrophages are involved in the potentiation of IL-10 induced by anti-CLEC-2 and LPS
(A) C57BL/6J mice were injected I.P with 300µl of PBS- or clodronate-liposomes. After 4 days the mice were challenged for 3 hours with 20µg of LPS and 40µg of isotype or anti-CLEC-2 mAb. Mice were sacrificed and spleen and serum samples were obtained. (B) Splenocytes were stained with anti-F4/80, anti-CD11c, anti-CD11b and 7/4 and analysed by FACS. IL-10, TNF and IL-6 were measured by CBA. Data is representative of two independent assays. Each dot represents a different mouse. Statistical differences were assessed with Mann-Whitney test. **p<0.01.

To analyse the involvement of myeloid cells in the modulation of IL-10 responses to LPS by anti-CLEC-2 I decided to try depletion assays. I made use of clodronate-loaded liposomes that induce depletion of phagocytic cells (Van Rooijen and Sanders 1994). The most affected cells are the phagocytes that have a long half-life, like DCs and macrophages. Thus, I treated mice with PBS- or clodronate-liposomes and 4 days later I challenged them with LPS and isotype or anti-CLEC-2 antibody. Remarkably, clodronate-liposome treatment of mice completely prevented anti-CLEC-2 modulation of IL-10 production (fig.5.5A, left). No effect of clodronate-liposome treatment was observed on the serum levels of TNF or IL-6 (fig.5.5A). Furthermore, all mice challenged were sacrificed and their spleen were analysed for the presence of myeloid cells. As shown on figure 5.5B, the clodronate-liposome treatment induced the depletion of DCs and monocytes, but not neutrophils.

The clodronate-liposomes depleted both monocytes/macrophages and DCs, making it impossible to evaluate the contribution of each cell type. To further discriminate these two cell types I made use of a more specific depletion, the transgenic mouse line expressing the diphtheria toxin receptor under the control of the CD11c promoter (CD11c-DTR-GFP). These mice have been demonstrated to deplete DCs after a single IP injection of diphtheria toxin (DT), while the monocytes population remains intact (Jung, Unutmaz et al. 2002). Thus the specific depletion of DCs can help discriminate between the role of DCs and monocytes on CLEC-2 modulation of LPS responses *in vivo*. For this end, I generated bone marrow chimeras by irradiating C57Bl/6 mice (WT) and transferring bone marrow cells from WT or CD11c-DTR mice. After 12 weeks I analysed the splenic DCs of these chimeras and observed that the great majority of DCs were derived from the CD11c-DTR-GFP mice, as the CD11c cells were expressing GFP, while the WT DCs were GFP negative (fig.5.6B, PBS column). Thus, I treated mice with 100ng of DT and 18 hours later I challenged the mice with LPS and isotype-matched or anti-CLEC-2 antibody. Notably, DC-depleted mice showed reduced IL-10 levels compared to

DC-sufficient mice, while the TNF and IL-6 levels were unchanged (fig.5.6A). In line with that, the analysis of splenic DCs on these mice showed that DT treatment depleted DCs only in CD11c-DTR-GFP and not in WT chimeric mice (fig.5.6B, lower panel).

On the whole, these data show that DCs and monocytes/macrophages are important targets of CLEC-2 modulation of endotoxic shock. Importantly, the depletion of DCs and monocytes/macrophages only affected the production of IL-10 and not of other mediators (TNF and IL-6), emphasizing the role of CLEC-2 in the modulation of IL-10 production by TLR stimulation of myeloid cells.

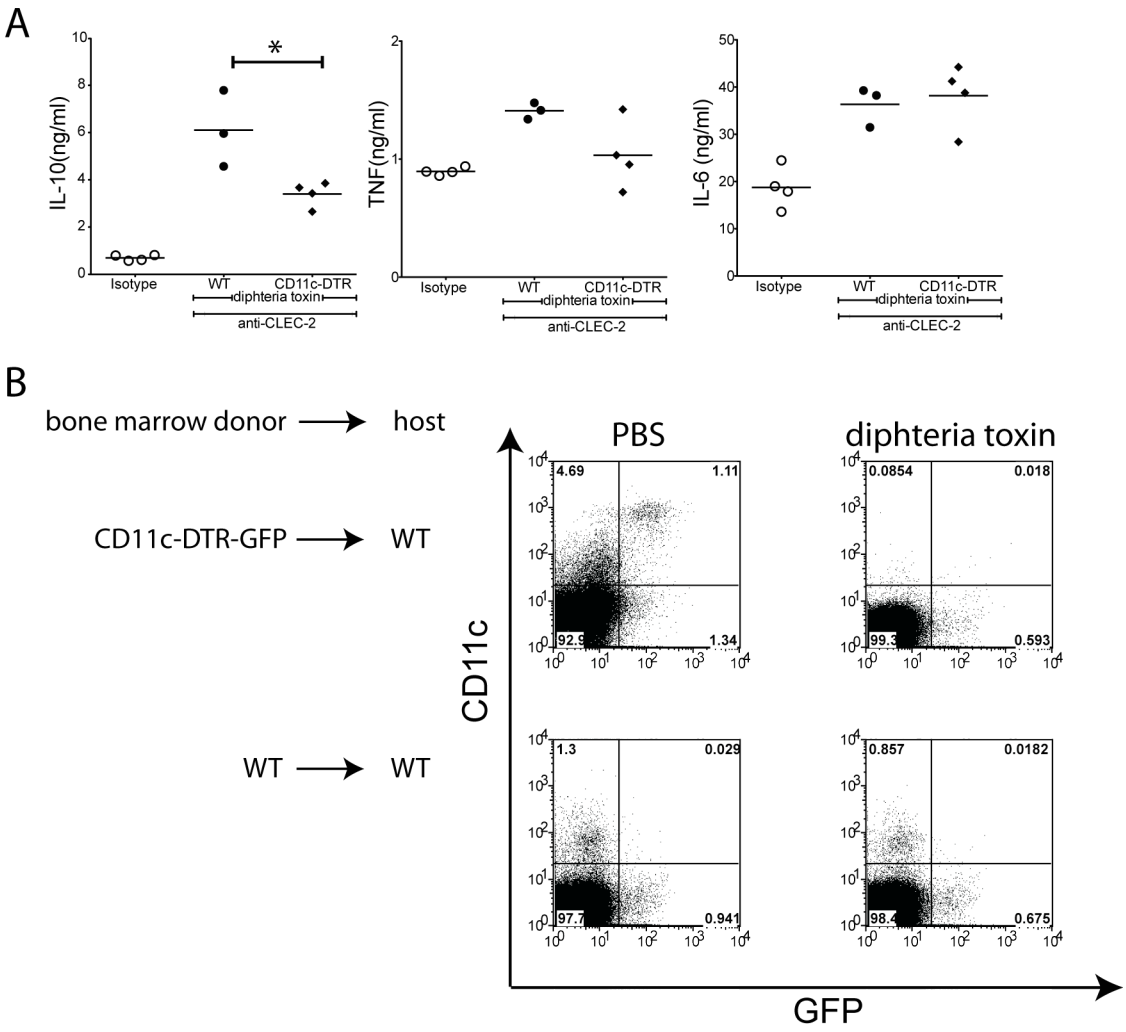

Figure 5.6 Involvement of DCs on CLEC-2 modulation of LPS-induced responses (A) CD11c-DTR-GFP or C57BL/6J(WT) bone marrow cells were used to reconstitute lethal irradiated WT mice. These mice were injected with PBS or 100ng of diphtheria toxin I.P and 18 hours later challenged with 20µg of LPS and 40µg of isotype or anti-CLEC-2 mAb. 3 hours after the challenge the mice were sacrificed and spleen and serum samples were obtained. IL-10, TNF and IL-6 were measured on the serum by CBA. (B) Splenocytes were stained with anti-CD11c antibody and analysed by FACS. Data is representative of two independent assays. Each dot represents a different mouse. Statistical differences were assessed with Mann-Whitney test. *$p<0.05$.

5.5 CLEC-2 conditional knockout mouse

The discovery of genetic manipulation of embryonic stem (ES) cells allowed generation of mouse lines with targeted mutations (Koller and Smithies 1992). More recently, the CRE-loxP recombinase system has allowed scientists to generate mouse lines with genetic deletions conditioned by CRE expression, generating inducible or conditional KO mice (Rajewsky et al. 1996). So, based on my previous findings showing wide expression of CLEC-2 in haematopoietic cells and the fact that *Pdpn*-/- mice were not viable and showed a similar phenotype to Syk-/- mice (Schacht, Ramirez et al. 2003), I decided to generate a mouse line carrying a conditional deletion of the CLEC-2 gene (*clec1b*) that will allow me to impair CLEC-2 expression on certain cell types and/or conditions.

The way I decided to do this was to insert one loxP site between exons 1 and 2 and another loxP between exons 4 and 5. The *clec1b* gene is composed of 6 exons, with the exon 1 corresponding to the N-terminus, while the C-terminus is encoded by the exon 6 (see 5.7A). The insertion of loxP sites between exons 1 and 2 and exons 4 and 5 allows the deletion of exons 2, 3 and 4 encoding the transmembrane, neck and part of CTLD. Furthermore, exon1 does not contain full codon sequences and with two nucleotides from exon 2 it will form a correct codon sequence (see figure 5.7B). Thus the deletion of exon 2 will generate a frame shift that would impair CLEC-2 expression.

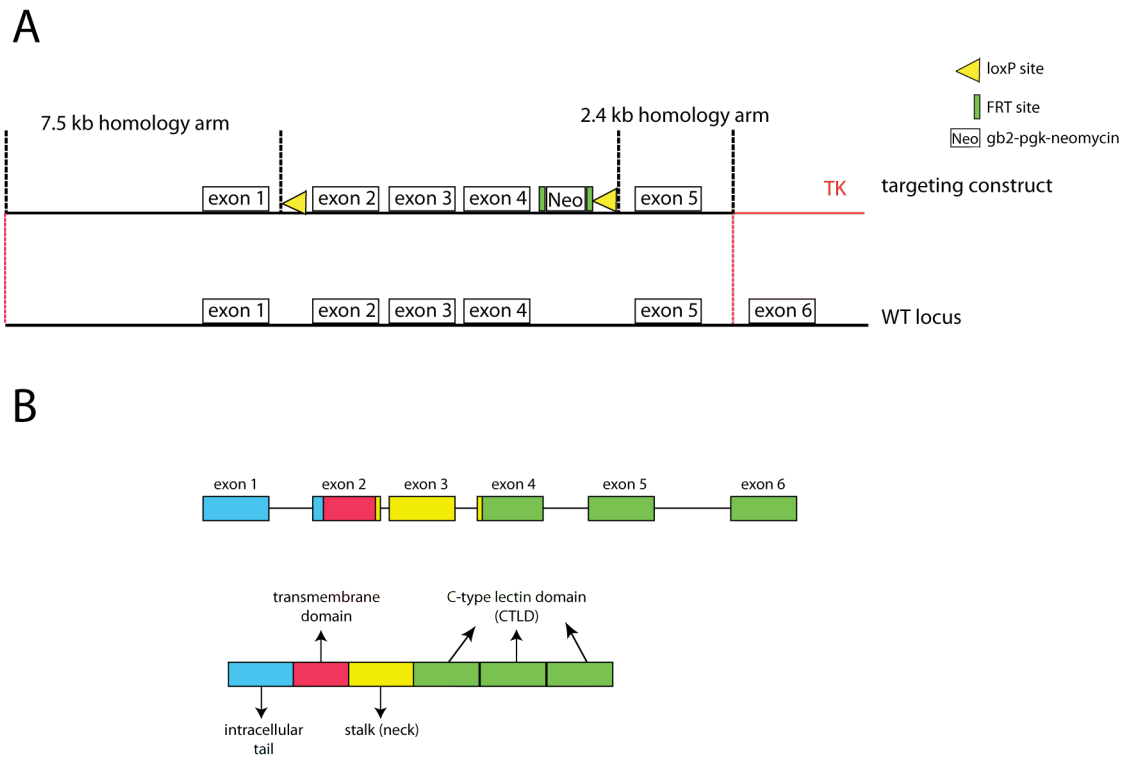

Figure 5.7 Schematic representation of *clec1b* targeted disruption
(A) Schematic representation of targeting construct used to insert neomycin gene and loxP and FRT site on the *clec1b* (CLEC-2) gene locus on C57BL/6 embryonic stem (ES) cells. (B) Representation of aminoacids and domains encoded by each *clec1b* (CLEC-2) exon.

As a part of cloning and selection, I inserted a resistance gene in the DNA vector used to target the ES cell genome. The resistance gene that I chose was the neomycin (NEO) gene being regulated by two different promoters, the gb2 prokaryotic promoter and the pgk mammalian promoter. With these two promoters, the targeting construct can be selected on bacteria and ES cells. As a part of the strategy to generate CLEC-2 conditional and null mice, I decided to insert this NEO gene together with a loxP site between the exons 4 and 5. By using this approach I expect to generate CLEC-2 null mice when both CLEC-2 alleles have the NEO insertion, as this NEO gene between CLEC-2 exons with a strong promoter and poly A tail is likely to impair the expression of CLEC-2 as demonstrated previously (Ohno et al. 1994). The NEO gene is flanked by FRT recombination sites, thus making it possible to excise the NEO cassette by expression of the Flp recombinase and this way generating the final conditional CLEC-2 KO mouse.

After successful cloning and insertion of modifications on *clec1b* gene, we (myself and the London Research Institute Transgenic lab) successfully generated C57Bl/6 ES cells carrying mutated *clec1b*. These 3 corrected targeted ES cells were injected into 8-cell stage white fur embryos and then into pseudo-pregnant female mice. This strategy generated mice that was had 80% of its coat black, indicating that its genome was partially from the C57Bl/6 ES cell. Only 2 of the 3 ES cell clones could give rise to chimeric mice, and among these 2 chimeric mice only one transmitted the mutated *clec1b* gene to its litter, thus giving rise to *clec1b* loxP FRT-NEO-FRT loxP mouse, from now on referred as CLEC-2 NEO.

As for PDPN -/- mice (Schacht, Ramirez et al. 2003), CLEC-2 deficient mice are not viable and show blood inside the lymphatics (Bertozzi et al. 2010; Suzuki-Inoue et al. 2010; Tang, Li et al. 2010). I decided to analyse CLEC-2 deficient embryos. To do so, I mated two heterozygous wt/CLEC-2 NEO mice and obtained the embryos at embryonic day 15.5. I genotyped the embryos for the presence of inserted 5' loxP site in the CLEC-2 gene locus. Among nine embryos genotyped, three embryos showed only amplification of the WT allele, five embryos showed amplification of both WT and targeted allele (with loxP insertion) and one showed only amplification of the targeted allele (figure 5.8A). Amongst these embryos, the one genotyped as homozygous for the targeted allele showed a localised bleeding on the area of development of the lymph sac (figure 5.8B, arrow). Accordingly, analysis of CLEC-2 expression obtained from foetal liver showed impaired CLEC-2 staining on the surface of CD41+ cells from embryo homozygous for the targeted allele, whilst WT and heterozygous embryos showed similar CLEC-2 surface expression (figure 5.8B, lower panel). Therefore, it implies that targeting CLEC-2 gene locus by insertion of a NEO cassette impairs CLEC-2 expression. Importantly, no mouse homozygous for CLEC-2 NEO was observed among more than one hundred mice born from the cross of two heterozygous wt/CLEC-2 NEO mice. Even though the bleeding of CLEC-2 NEO homozygous mice is localised and not as remarkable as the bleeding observed in Syk-deficient mice, this shows the importance of normal CLEC-2 expression for normal development of mice.

This single embryo analysed so far suggests that CLEC-2 deficiency led to a bleeding at the site where the lymphatics start to develop. It is worth point out that this suggests that my strategy to impair CLEC-2 expression in mouse has worked and, similarly to other reports led to bleeding on embryos (Bertozzi, Schmaier et al. 2010; Suzuki-Inoue, Inoue et al. 2010). It will be important to further examine more of these embryos. Additionally, it will be even more important to analyse embryos where CLEC-2 deficiency will be generated deletion of *clec1b* exons, for example by crossing to constitutive CRE-expressing mice. Finally, the crossing to selective CRE lines will allow analysis of CLEC-2 function in different cell types.

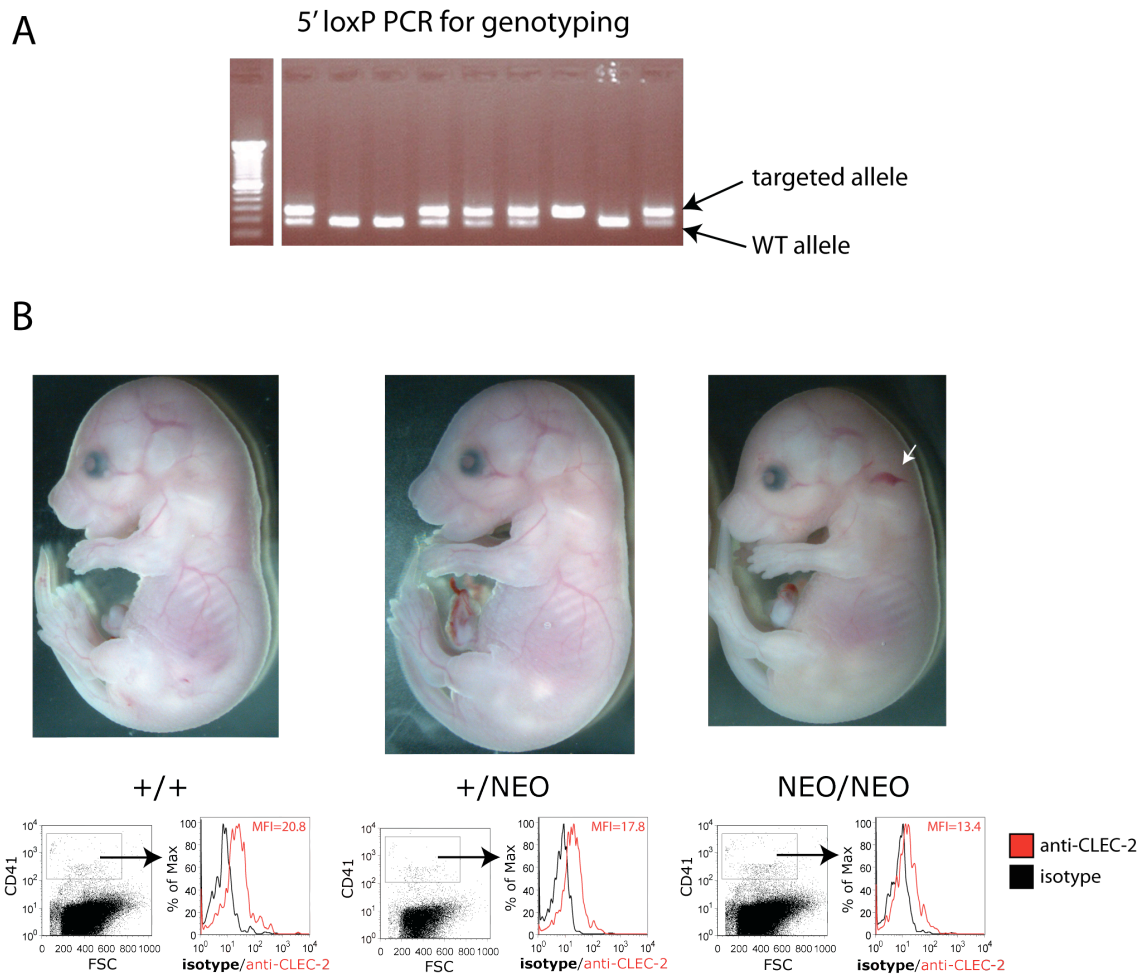

Figure 5.8 NEO insertion on *clec1b* locus impair CLEC-2 expression and leads to bleeding on embryos

Embryos were acquired at embryonic day 15.5 from breeding of heterozygous C57BL6J (+)/CLEC-2 NEO (NEO). (A) DNA was extracted from tails and analysed by PCR. Primers and genotyping strategy is represented in figure 2.4. (B) Embryos were analysed for gross bleeding defects on a low magnification upright microscope with external light source. Arrow indicates localised bleeding observed. Foetal livers cell suspensions were obtained and stained with anti-CD41-FITC and isotype-Alexa647 or anti-CLEC-2-Alexa647 antibodies.

5.5.1 Summary and discussion

Beside the great data available regarding CLEC-2 signalling and function in platelets, there is a lack of reports analysing CLEC-2 functions *in vivo*. In this chapter I characterised some *in vivo* functions of CLEC-2 in myeloid cells. I described that CLEC-2 triggering induces increased serum levels of IL-10 in a mouse model on LPS-induced inflammation. This increased IL-10 levels were dependent on the presence of macrophages and DCs. Finally, I generated a conditional CLEC-2 KO mouse that will be used to further characterise CLEC-2 function in different cell types.

The involvement of CLEC-2 on modulation of TLR responses on macrophage and DCs culture led me to investigate the effect of CLEC-2 triggering in mouse model LPS-induced inflammation. In this model, anti-CLEC-2 mAb administration simultaneously with LPS increased IL-10 levels in the serum and peritoneum, similar to the cell culture observations. However, it is conceivable that the *in vivo* requirements for CLEC-2 modulation of LPS responses are different, and the anti-CLEC-2 treatment of mice is in fact blocking CLEC-2 function. It will be important to determine Syk activation on myeloid cells followed anti-CLEC-2 mAb administration.

Based on the fact that anti-CLEC-2 administration might be acting as a CLEC-2 agonist in mice, I measured different inflammatory mediators to evaluate the modulation of LPS responses. The CLEC-2 triggering during endotoxemia increased IL-10 systemically (serum) and locally (peritoneal lavages). However, the levels of TNF were increased by anti-CLEC-2 mAb in the peritoneum, but not in the serum. Considering that LPS induces recruitment of cells to the peritoneum, CLEC-2 triggering might modulate this recruitment by increasing the recruitment of IL-10- and TNF-producing cells. Another attractive hypothesis is the recent find that engagement of integrins can modulate TLR responses inducing IL-10 via Syk signalling (Wang et al. 2010). Thus, modulation of expression/activation of integrins by CLEC-2 could account the increased IL-10 production observed. It also suggests that LPS-activated DCs migrating on lymphatics could potentially activate Syk via CLEC-2-PDPN

interaction, leading to increased IL-10 production and dampening of inflammation. The diminished TNF levels observed in the serum 1 hour after challenge might be due to a delayed activation of TNF-producing cells. This delayed activation might be due to increased IL-10 that is not observed because of consumption of this cytokine or to an impaired recruitment of TNF-producing cells to the local inflammatory site (peritoneum).

Myeloid cells migrate to inflammatory sites and produce different inflammatory mediators, thus these cell types were likely to be involved on the increased IL-10 production observed by anti-CLEC-2 and LPS *in vivo*. Accordingly, depletion of monocytes and DCs greatly impaired the increased IL-10 production observed. Furthermore, the depletion of these cell types did not affect the LPS-induced production of IL-10, TNF or IL-6. This suggests that that monocytes and DCs are not the primary source of these cytokines *in vivo*, but CLEC-2 triggering can turn DCs and macrophages into IL-10 producers. Interestingly, CLR and TLR co-activation on neutrophils were shown to promote increased production of IL-10 (Zhang et al. 2009). Collectively my data shows that CLEC-2 stimulation during endotoxic shock induces an increased IL-10 production and a transient diminishment of TNF production by acting on myeloid cells, constituting a potential therapy for different inflammatory conditions.

To better study CLEC-2 function *in vivo*, I generated a mouse line carrying a NEO gene on the middle of CLEC-2 gene. This approach led to impaired CLEC-2 expression on cells and bleeding on embryos (figure 5.8A). Nevertheless, I observed a bleeding localised on the site of lymph sac development, different from reported data where the bleeding was observed on different sites (Bertozzi, Schmaier et al. 2010; Suzuki-Inoue, Inoue et al. 2010). Among the hypothesis to conciliate my findings with the reported data there is the fact that this embryo have another gene inserted on the middle of CLEC-2 and this besides affecting CLEC-2 expression might affect other genes. Moreover, this is a single embryo analysed and further embryos shall be studied to draw further conclusions.

My ultimate goal is to generate mouse lines deficient of CLEC-2 by the excision of CLEC-2 exons by CRE-expression, thus excluding the potential effect of the NEO insertion on the mouse genome. So, CLEC-2 NEO mice will have the NEO insertion deleted by crossing with a mouse line that carries the Flp recombinase under the control of a constitutive expressed promoter (hAct-Flpe, or human actin Flp mice). This will generate a mouse that carries a CLEC-2 floxed gene that should show a normal CLEC-2 expression. The CLEC-2 floxed mice will then be crossed to different CRE-expressing mice to generation of CLEC-2 conditional knockout mice. An attractive mouse line will be the $DC^{CLEC\text{-}2\text{-}/\text{-}}$ that will be generated by crossing CLEC-2 floxed mice with CD11c-CRE mice. These mice will be assessed by their production of IL-10 during anti-CLEC-2 mAb and LPS challenge, formally demonstrating the role of CLEC-2 on DCs on the induction of IL-10.

Many CLRs play important role on the recognition of mislocalised proteins, as the case of DNGR-1 (CLEC9a) that recognises a pre-formed ligand only exposed upon loss of membrane permeability (Sancho et al. 2009). In line with that, CLEC-2 recognises the endogenous glycoprotein PDPN that has a restricted expression pattern. Therefore it is possible that induced PDPN expression on inflamed tissues can trigger CLEC-2 and modulate TLR responses like the Syk-coupled receptor TREM-1 (Bouchon, Facchetti et al. 2001). Thus, ablation of CLEC-2 expression on mice by crossing CLEC-2 floxed mice with different myeloid specific CRE (CD11c-CRE for DCs, LysM-CRE for monocytes, MRP8-CRE for neutrophil) or a hematopoietic CRE (Vav-iCRE) will generate mice that will be tested for their role on the modulation of different inflammatory conditions, such as infection and cancer.

As mentioned above, CLEC-2 -/- mouse line was very recently reported. These CLEC-2-/- mice showed developmental defects on the formation of lymphatics (Bertozzi, Schmaier et al. 2010; Suzuki-Inoue, Inoue et al. 2010) similar to the ones observed on Syk-/-, SLP76-/- and PDPN-/- mice. It is proposed that CLEC-2-expressing platelets aggregate upon encountering PDPN on the developing lymphatics, with this CLEC-2-induced platelet aggregation being required for the separation of the blood and lymphatic

vessels. This proposed model is likely by the fact that CLEC-2 signals via SLP76 (Suzuki-Inoue, Kato et al. 2007), and platelets defective on SLP76 show a similar phenotype to CLEC-2-/- mice (Bertozzi, Schmaier et al. 2010). However, Böhmer and colleagues identified by fate mapping of Syk that a myeloid cell population was responsible for the correct lymphatic development (Böhmer et al. 2010). This indicates that CLEC-2 expression on macrophages can also be responsible for the lymphatic development. On this lines, the ablation of CLEC-2 expression on platelets (by crossing CLEC-2 floxed with PF4-CRE) or different myeloid cells will help identify a role of a given cell type expressing CLEC-2 on the vascular development.

Chapter 6. Discussion

Inflammation is a response of multicellular organisms to changes in tissue homeostasis. It has mechanisms of detection of tissue damage, aggressors and metabolic changes and, as consequence, initiates a process of restoration of tissue homeostasis. One way applied to survey tissue homeostasis is to express receptor in myeloid cells (DCs, monocytes and neutrophils) that recognises homeostatic changes. These cells are capable of migrate to tissues, phagocyte and produce several inflammatory mediators upon detection of inflammatory triggers. Furthermore, in mammals DCs have an increased capacity to emigrate from inflamed tissue via lymphatics and activate adaptive immune responses.

The study of innate receptors that signals to gene induction and production of inflammatory mediators had greatly developed in the past decade. The great advance was made when Ruslan Medzhitov and Charles Janeway identified the TLR signalling in myeloid cells controlled cytokine production and activation of adaptive immunity (Medzhitov et al. 1997). This provided the first molecular evidences that the innate immune system could influence production of inflammatory mediators and activation of adaptive immune response.

Subsequently, several reports showed that TLRs recognise different endogenous and microbial-derived molecules, which developed the concept that innate immunity recognises and signals to different homeostatic changes in the organisms. Following that, the NLRs and RLRs were also shown to play directly role on this recognition and activation of inflammatory responses.

More recently, the CLRs joined the other three family of receptor and were shown to signal to initiation of inflammation by recognition of microbes. The CLRs also interacts with endogenous molecules, which leads to different responses.

At the beginning of my studies, my first hypothesis was that CLEC-2 would act as dectin-1 and detect microbes. I was unable to confirm this hypothesis and the endogenous protein PDPN was shown to be a CLEC-2

ligand (Suzuki-Inoue, Kato et al. 2007). Nevertheless, in cell culture assays virus budding from PDPN-expressing cells carries the CLEC-2 ligand allowing virus interaction with CLEC-2 (Chaipan, Soilleux et al. 2006). Therefore, it is possible that CLEC-2 plays a role on the detection of viruses that infect cells/tissues were PDPN is expressed. This scenario might constitute a condition where myeloid cells are co-triggered by signals derived from CLEC-2 and TLRs present on those viruses.

Tissue damage products were shown to be detected by myeloid innate receptors and trigger inflammation. CLEC-2 expression on myeloid cells might constitute a surveillance mechanism to detect PDPN misexpression on different sites and detect homeostatic changes. In line with that, PDPN is expressed in carcinogenic skin and metastatic cancer cells (Breiteneder-Geleff, Soleiman et al. 1999; Durchdewald, Guinea-Viniegra et al. 2008), two conditions intrinsically linked to changes in tissue homeostasis.

I showed that PDPN was expressed on peritoneal cells only upon LPS triggering (figure 3.6A). This suggests that inflammatory triggers, such as LPS, can induce the CLEC-2 agonist constituting a model where both CLEC-2 and TLR agonists are present inducing a cross talk between resident peritoneal cells and myeloid cells. Accordingly, DC-SIGN was shown to mediate DC maturation on a process dependent on ligation by MAC-1 on neutrophils (van Gisbergen et al. 2005).

The studies of CLEC-2 function and signalling in myeloid cells focused on the premise that signals derived from hemITAM containing receptor can initiate inflammatory responses. A recent report showed that selective CLEC-2 signalling could lead to initiation of inflammation. Kerrigan and colleagues showed that a chimeric receptor consisting of dectin-1 CTLD and CLEC-2 hemITAM can induce TNF production by triggering with zymosan, implying that CLEC-2 signalling can lead to initiation on inflammation (Kerrigan, Dennehy et al. 2009). Here, I used mAbs and PDPN.Fc to selective trigger CLEC-2 signalling in DCs and failed to observe any cytokine production. In fact, dectin-1 triggering by B-glucan micro-particles also fails to induce cytokine production, whilst curdlan proved to be a strong dectin-1 agonist (LeibundGut-Landmann,

Gross et al. 2007; Hernanz-Falcón, Joffre et al. 2009). This suggests that the nature of CLEC-2 agonists might play a role on the outcome of its signalling.

Despite the failure to confirm whether CLEC-2 signalling can initiate inflammatory responses, I showed that this signalling could modulate LPS-induced inflammation increasing IL-10 levels. In a mouse model of LPS-induced inflammation, DCs and macrophages play crucial part in the IL-10 increase. IL-10 is a potent anti-inflammatory cytokine produced by different cell types, including myeloid cells (Saraiva and O'Garra 2010). Therefore, it is likely that CLEC-2 might cooperate to control exacerbated inflammation.

NFAT activation by CLRs is a sharp difference between the TLR and the CLR family. CLEC-2 modulation of TLR response is dependent of calcineurin and, probably, of NFAT activation. The production of PGE2, an important vessel dilation mediator, is greatly dependent of NFAT upon dectin-1 triggering (Goodridge, Simmons et al. 2007), suggestive of another role for CLRs in the control of inflammation. Recently, calcineurin and NFAT were also implicated in the resistance against fungal infection (Greenblatt et al. 2010), emphasizing the importance of this pathway for immunity. Consequently, CLEC-2 cooperation with TLRs might constitute a mechanism to induce NFAT activation during TLR triggering allowing a proper control of the inflammatory process. Recently, the LPS co-receptor CD14 was shown to activate NFAT and contribute to IL-2 production and induction of DC death upon LPS challenge (Zanoni et al. 2009). This report suggests that NFAT activation during inflammation might be important to maintenance of DC population.

The efficient control of inflammatory responses requires proper balance of effector cells and mediators. Dcir, a ITIM containing CLR, was shown to be important to maintain the homeostatic pool of DCs. Deficiency of this molecule leads to increased numbers of DCs and autoimmune diseases (Fujikado et al. 2008). This suggests that unopposed tyrosine kinase signalling in DCs is involved on the increased DC numbers. A constitutive CLEC-2 signalling, triggered by PDPN or lack of regulatory mechanisms, might be involved in the increased DC population or even DC differentiation. In B lymphocytes, Syk

signalling is important for generation and maintenance of these cells (Turner, Mee et al. 1995; Turner et al. 1997).

Inflammatory responses differ on many ways, which depends on the tissue and inflammatory triggers. A recent example is the different outcome of immune responses upon intravenous or intranasal *Listeria monocytogenes* infection (Pepper et al. 2010). PDPN restricted expression in certain tissues might be indicative of a differential localised function of CLEC-2. PDPN might influence a differential response in these tissues, as a required step for the specific inflammatory response on the given site.

Another way that PDPN restricted expression might control inflammatory responses is through migration of myeloid cells to these sites. DC-SIGN, another CLR, had been implicated in the mediation of DC trafficking by interaction with ICAM-2 (Geijtenbeek et al. 2000). CLEC-2 interaction with PDPN can possibly mediate a similar phenomenon, providing adhesion of myeloid cells when these migrate to lung, kidney or lymphatics.

Studies in cell mobilisation commonly focused in the immigration and extravasation of cells from blood to tissues. This is due to the fact that the majority of cells do not emigrate from tissues to vessels. Nevertheless, DCs have an increased ability to emigrate, from tissues to secondary lymphoid organs, carrying antigens to activate adaptive immunity. Recently, Pflicke & Sixt showed that DCs uses preformed portal in the lymphatic collectors to emigrate from tissues. These lymphatic collectors are blunt end lymphatic vessels characterised by increased PDPN expression. Moreover, Pflicke & Sixt showed that DC emigration is completely independent of integrins-mediated adhesion or movement (Pflicke and Sixt 2009). Therefore, up-regulation of CLEC-2 triggered by inflammation might allow increased DC entry into lymphatics by mediating adhesion and movement of DCs on PDPN surfaces.

It is very clear nowadays that CLRs are able to recognise and signal to start the inflammatory process. However, this family is much more diverse and contains many different signalling motifs and members (as seen on table 4). Furthermore, the study of CLRs is still on its infancy and there are many aspects yet to be described.

It is still unclear if all CLRs can in fact couple to signalling to downstream function, which is the case of mannose receptor, mouse DCAR2 and DEC205. In addition, several other members are still to have ligands and agonists described. As it stands, the manner that these receptors interact with their ligands/agonists is a crucial step for the outcome of the downstream signalling. Therefore, the identification of CLRs ligands/agonists will greatly benefit the identification of the function of these receptors.

In this thesis, I developed several tools and models that helped on the study of CLEC-2 function. The generation of anti-CLEC-2 mAbs greatly helped the identification of cell types that express this receptor (Kerrigan, Dennehy et al. 2009). I also showed that these mAbs could be used as selective CLEC-2 agonists, which was confirmed by others (Hughes et al. 2010). Moreover, the identification of CLEC-2 signalling via a Syk-Ca^{2+}-calcineurin-NFAT pathway to modulate TLR responses by macrophages and DCs will greatly benefit future studies about the role of this receptor in the initiation of inflammation. The discovery that CLEC-2 can modulate LPS-induced inflammation *in vivo* dependently of myeloid cells will certainly draw more attention to the role of CLEC-2 in these cell types. Additionally, the generation of a conditional CLEC-2 KO mouse line will significantly help the identification of *in vivo* functions of CLEC-2 on different cell types.

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