Fakultät für Medizin

Institut für Klinische Chemie und Pathobiochemie

Generation and analysis of a Clec12a-deficient mouse model

Mercedes María Castiñeiras Vilariño

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines

Doctor of Philosophy (Ph.D.)

genehmigten Dissertation.

Vorsitzende: Univ.-Prof. Dr. Agnes Görlach

Betreuer: Univ.-Prof. Dr. Jürgen Ruland

Prüfer der Dissertation:

1. Univ.-Prof. Dr. Ulrike Protzer
2. Priv.-Doz. Dr. Philipp J. Jost

Die Dissertation wurde am 12.12.2013 bei der Fakultät für Medizin der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 17.03.2014 angenommen.

Part of this dissertation thesis has been published under:

Neumann K., **Castiñeiras-Vilariño M.**, Höckendorf U., Hannesschläger N., Lemeer S., Kupka D., Meyermann S., Lech M., Anders H.-J., Kuster B., Busch D.H., Gewies A., Naumann R., Gross O., Ruland J. (2014). "Clec12a is an inhibitory receptor for uric acid crystals that regulates inflammation in response to cell death". *Immunity*, 40(3):389-399, 13 March 2014.

Part of this work has been presented as follows:

Castiñeiras-Vilariño M. (2009). "Generation of a Clec12a deficient mouse model", oral presentation at the scientific seminar of the 3^{rd} Medical Department, Hematology and Oncology, Klinikum Rechts der Isar of the Technical University Munich.

Castiñeiras-Vilariño M., Neumann K., Gewies A., Hannesschläger N., Lemeer S., Küster B., Naumann R., Ruland J. (2012). "Analysis of Clec12a function", poster presentation at the Faculty of Medicine Council Meeting, Technical University Munich.

Contents

Abstract

The innate immune system represents the first line defence against any threat to the organism. Pathogen recognition receptors (PRRs) detect pathogen- or danger-associated molecular patterns (PAMPs or DAMPs respectively) and trigger intracellular cascades that lead to the initiation of the immune response.

Clec12a is a largely uncharacterized PRR of the C-type lectin receptor family with an immunoreceptor tyrosine-based inhibition motif. In this work, a Clec12a-deficient mouse model has been generated in order to investigate the physiological role of Clec12a. Clec12a is dispensable for myeloid and lymphoid cell development under homeostatic conditions, and for the initiation of immune responses to all tested microbial-derived PAMPs. Yet, Clec12a is required for the regulation of inflammation in response to sterile cell death and sensing of uric acid crystals. This work genetically identifies Clec12a as a regulator of non-infectious inflammation with implications for autoimmunity and inflammatory diseases.

Keywords: Clec12a, C-type lectin, pathogen recognition receptor, innate immune response, sterile inflammation.

Zusammenfassung

Das angeborene Immunsystem stellt die erste Verteidigungslinie gegen jede Bedrohung für den Organismus dar. Mustererkennungsrezeptoren (PRRs) erkennen Pathogen- oder Gefahrassoziierte molekulare Muster und lösen intrazelluläre Kaskaden aus, die zur Einleitung der Immunantwort führen.

Clec12a ist ein weitgehend uncharacterized PRR der C-Typ Lektin-Rezeptor-Familie mit einem ITIM (immunoreceptor tyrosine-based inhibition motif). In dieser Arbeit wurde ein Clec12a-defizienten Mausmodell generiert, um die physiologische Rolle von Clec12a zu untersuchen. Clec12a ist unter homöostatischen Bedingungen für die myeloide und lymphoide Zellentwicklung entbehrlich, sowohl für die Einleitung der Immunantwort auf alle getesteten mikrobiellen PAMPs. Allerdings ist Clec12a erforderlich für die Regulierung von Entzündung in Reaktion auf sterilen Zelltod und für die Erkennung von Harnsäure Krystalle. Diese Arbeit identifiziert genetisch Clec12a als Regulator von nicht-infektiöse Entzündung mit Implikationen für Autoimmun- und Entzündungserkrankungen.

Schlagwörter: Clec12a, C-Typ Lektin, Mustererkennungsrezeptor, angeborene Immunantwort, sterile Entzündung.

Chapter 1

Introduction

The human body is a system open to the surrounding environment. Through the airways, the digestive tract, the skin or wounds, the human body is exposed to threats. Immunity can be understood as the border between body and environment: the immune system is the collection of molecules, cells, tissues and organs that keeps the organism in balance and protects it from external and internal dangers. These threats can be of different nature, bacteria, viruses and fungi being the most important infecting agents, but also self molecules can signal a danger to the host (Medzhitov, 2001). In order to defend the human body from those multiple agents, immunity has developed a very complex network of mechanisms which are tightly regulated. Many of them are not yet fully understood.

1.1 The immune system

How the immune system recognizes a danger signal has been largely investigated. The self *versus* non-self model that has predominated during the last half of the 20^{th} century was the first approach to explain what could trigger an immune response: effector cells would distinguish among host and foreign molecules, the source for the latest being principally a pathogen (Burnet, 1961; Janeway, 1992). This theory however leaves certain ques-

tions unanswered, for instance why is no protective immune responses being developed against commensal bacteria. Trying to solve this dilemma, Matzinger proposed that an immune response would be triggered only when a stress, damage or non-physiological death related molecule is released and detected (figure 1.1), rather to distinguish between infectious non-self and non-infectious self, in what is called the danger model (Matzinger, 1994). What Matzinger postulated has been later on confirmed experimentally: during the last decade, several molecules related to necrotic cell death (but not apoptotic) or to cellular stress phenomena (such as heat shock proteins released after cells are exposed to elevated temperatures) can elicit immune responses in the absence of an infectious agent (Gallucci et al., 1999). However, these hypotheses could not explain autoimmunity, tumor immunity or graft rejection phenomena. Advances in these areas are giving valuable hints to understand the clinical challenges present in transplantations, cancer and other immune disorders.

Independently of the nature of the triggering agent, the first step in the activation of a defense response is the recognition of a signal. Once the immune response is initiated, effector mechanisms carry out specific functions to contain and eliminate the harm causing factor. The cellular mediators of the immune response originate from pluripotent hematopoietic stem cells found in the bone marrow. These can differentiate into myeloid or lymphoid progenitors, from which all immune cell types arise. The myeloid lineage gives raise to the main actors of the innate immune response: macrophages, mast cells, basophils, eosinophils, neutrophils and dendritic cells, which provide a first defence line characterized by an immediate response. At the same time, these cells release cytokines and other molecules that initiate a local inflammation process at the encounter site. This inflammatory response is fundamental for the subsequent adaptive immune response, which is activated with some delay during the first encounter with the triggering agent but it provides a much more efficient pathogen-specific response and it can generate lifelong immunity against that specific agent. Cellular effectors here are the

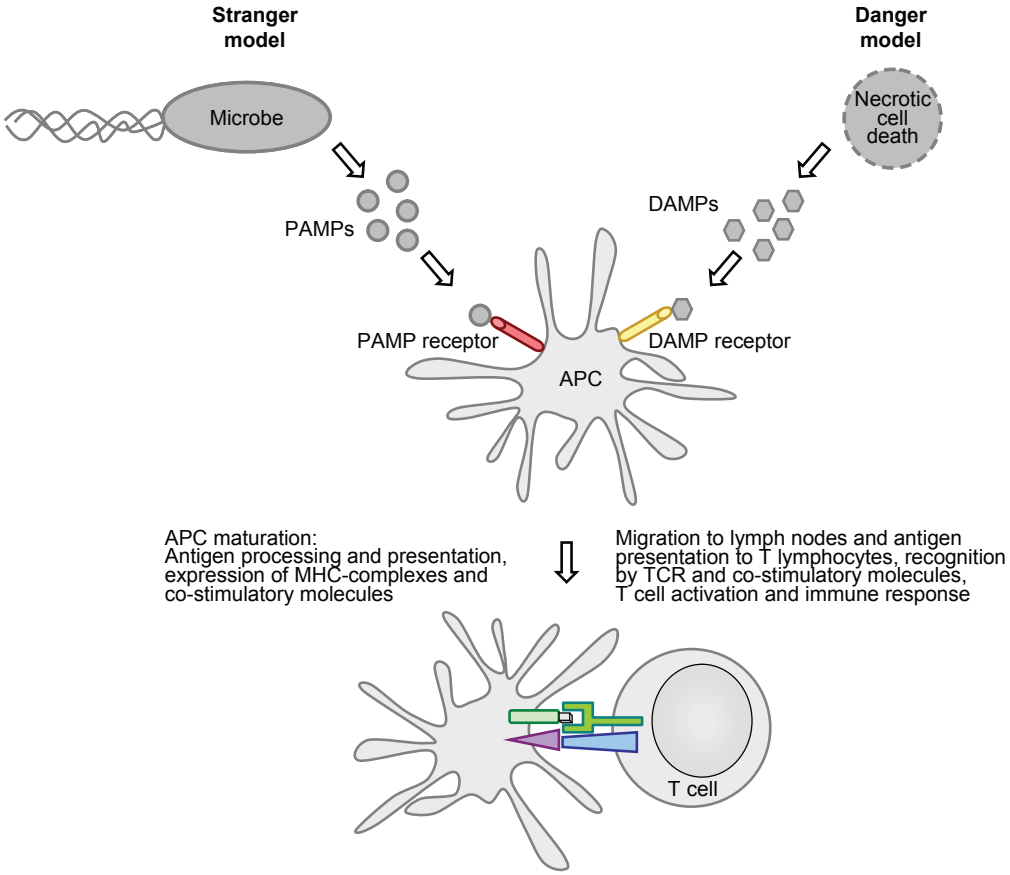

Figure 1.1: Theoretic models on immunity. Left, the stranger model: molecules present only in pathogens but not in the host are recognized by effectors of the innate immunity, leading to activation of antigen-presenting cells and the subsequent migration to peripheral immune organs for the initiation of the adaptive immune response. Right, the danger model: in a similar way, molecules released under stress conditions, damage or necrosis, lead to initiation of the immune response. According to Kono and Rock (2008).

B and T lymphocytes, derived from lymphoid progenitors. Natural killer (NK) cells also belong to the lymphoid lineage, but they are co-actors in the innate immunity together with the myeloid effector cells (Murhpy et al., 2008).

Self-regulation of the immune system is required during the whole inflammatory process so that an adequate and balanced response is produced. Once the triggering agent has been eliminated, the immune system is also responsible for terminating this inflammatory response. Therefore, the immune system can be seen as a surveillance entity in permanent activity responsible for immune homeostasis.

1.1.1 Innate immunity

The innate immunity is the front line defence of the organism. Innate immune cells recognize groups of highly conserved molecules shared by microbes that are usually essential for the survival of those organisms, but which are commonly not found in mammalian cells. These molecules are called pathogen-associated molecular patterns or PAMPs (table 1.1). Also non-infectious molecules can trigger the innate immune system: endogenous ligands that signal tissue injury, released for instance during inflammatory or autoimmune diseases, or sterile particles inhaled or ingested like asbestos or silica dioxide, are equally immunogenic (Rock et al., 2010). These molecules are called danger-associated molecular patterns or DAMPs (table 1.1).

Cells of the innate immune system scan the environment searching for foreign or danger molecules. For this purpose, these cells express a limited number of germline coded receptors (figure 1.2) that recognize PAMPs or signals of cellular damage: Fc receptors, complement receptors, integrins, lectins, etc. These receptors are collectively known as pathogen recognition receptors (PRRs). Some receptors are soluble and are mainly responsible for the opsonization of pathogens, while membrane-bound receptors can opsonize microbes or directly recognize PAMPs. Upon binding to their ligand, membrane-bound receptors are activated and signal transduction oc-

Molecule	**Origin and/or location**
HMGB1	Chromatin-associated protein (cell nucleus)
ATP	Different subcelullar compartments
DNA	Cell nucleus
HSP60	Mytochondria
Fibrinogen	Extracellular matrix
Amyloid-β	Unclear cellular origin (associated with Alzheimer's disease)
Urate crystals	Hyperuricemia (associated with inflammatory arthritis)
Cholesterol crystals	Dyslipoproteinemia (associated with atherosclerosis)
Silica dust	Marble, sandstone, soil (related to pneumoconiosis)
Asbestos	Insulation materials (related to pneumoconiosis)
Aluminium salts	Vaccine adjuvants, antacids, as antiperspirant in cosmetic products
Peptidoglycan	Bacterial cell-wall
dsRNA, Poly(I:C)	Viral RNA
LPS	Outer wall cell of gram-negative bacteria
Flagellin	Filaments of flagellated bacteria
sRNA, nucleoside analogs	Viral RNA, base analogs
Unmethylated CpG	Microbial DNA
Muramyldipeptide	Minimal bioactive peptidoglycan motif in bacteria
β-glucans	Glucose polymers in fungi cell walls
TDB	Most immunostimulatory component of *M. tuberculosis*
Hemozoin	*Plasmodium spp.*

Table 1.1: Danger- and pathogen-associated molecular patterns. A non-exhaustive list with examples of endogenous and exogenous DAMPs or PAMPs and their source.

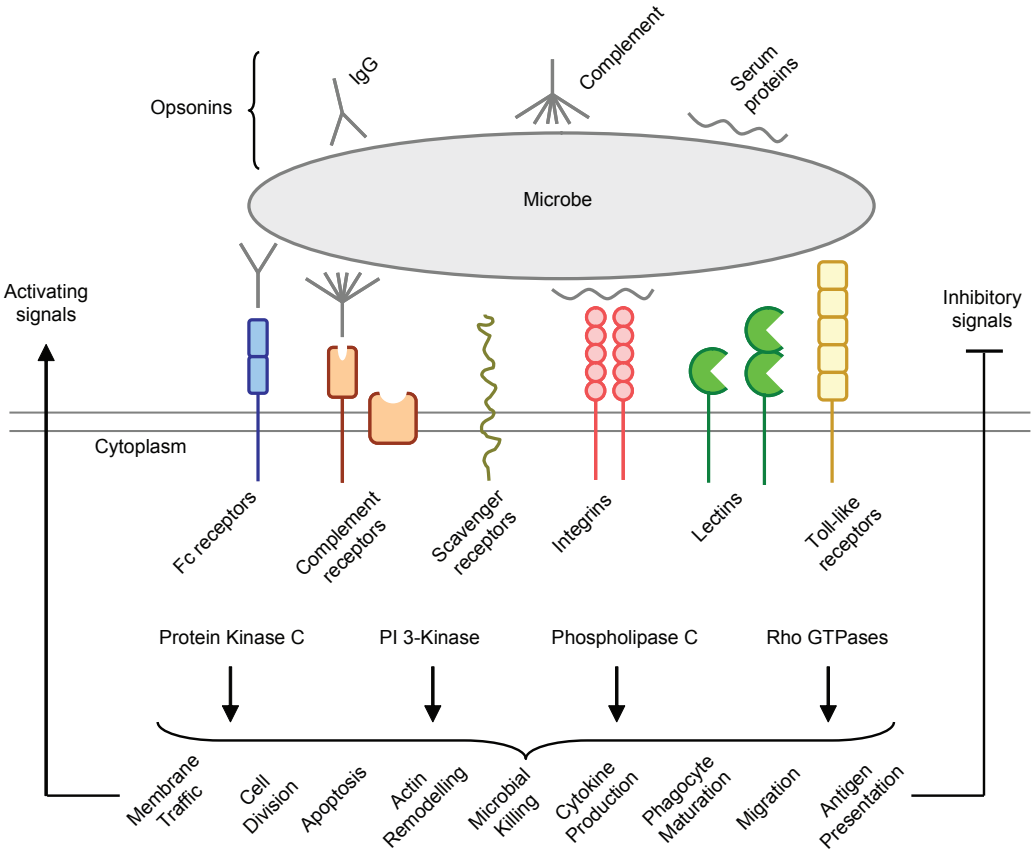

Figure 1.2: Pathogen recognition receptors. Several families of soluble and surface receptors detect pathogens and trigger intracellular signaling pathways that lead to the activation of different immunological cellular processes for eliminating the pathogenic agent. According to Underhill and Ozinsky (2002).

curs, which leads to the initiation of multiple immunological cellular processes that target the elimination of the pathogen.

Monocytes (the immature form of macrophages) circulating in the blood and tissue resident macrophages as well as dendritic cells scan the environment looking for foreign or danger-related molecules. Upon recognition these cells start producing cytokines, such as IL-1 and TNF, and chemokines that generate a gradient by which leukocytes are attracted and recruited to the site of encounter: an inflammatory response is initiated. In parallel, microbes detected by these cells are engulfed either by phagocytosis or receptor-mediated endocytosis in order to be eliminated by different mecha-

nisms. Engulfed vesicles carrying the triggering particle called phagosomes, fuse with lysosomes (vacuoles filled with lytic enzymes or reactive species like reactive superoxide), and the foreign body is degraded into smaller molecular entities for antigen presentation and eventually eliminated.

At the same time recognition of PAMPs or DAMPs by PRRs leads to the expression of molecules required for activation of the adaptive immune system. Macrophages and dendritic cells are not only responsible for scanning the environment in search for pathogens or danger molecules. They also represent the interface between the innate and adaptive immunity. The profile of secreted cytokines shapes the immune response accordingly to the intruder's nature. In this way, when a cytokine is dominant over the others, it determines whether humoral or cell-based immune responses are developed. This collaboration among innate and adaptive immunity enables an efficient defence against different classes of pathogens.

A key player in innate immunity are the natural killer (NK) cells. These are lymphocytes specialized in killing cells infected with intracellular microbes such as viruses. NK cells have a complex mechanism of activation. They express activating and inhibotry receptors that detect signals from infected and healthy cells. When a NK cell detects signals from an infected cell, it releases cytolitic molecules that alter the membrane permeability leading to cell death or induce apoptosis in the target cell. NK cells also secrete IFNγ, a cytokine which enhances the phagocytic activity of macrophages.

Another element of the innate immunity is the complement system. This group of proteins recognizes PAMPs directly or through other receptors that are bound to the patoghen. This encounter initiates an enzymatic cascade that leads to opsonization of the foreign body, chemotaxis, and agglutination. It also leads to formation of the membrane attack complex (MAC), a polymer of molecules of the complement system that forms a pore through which ions and water enter, leading to cell death.

1.1.2 Adaptive immunity

The activation of the adaptive immune system requires a primary innate immune response. Antigen presenting cells, mainly dendritic cells and macrophages, bring antigenic parts of invading microbes into contact with B or T cells using the major histocompatibility complex and other co-stimulatory molecules. B and T lymphocytes recognize a great diversity of antigens via receptors that are produced by somatic recombination of the VDJ gene segments. B cells give raise to the humoral response, called so due to the secreted antibodies that are produced upon activation of this type of lymphocytes. In contrast, T cells are responsible for the cellular response. Cytotoxic T lymphocytes directly kill virus-infected cells, while several subsets of T helper cells are specialized in combating intracellular bacterial infections and the activation of B cells.

B lymphocytes

Antibodies found in blood, lymph and several mucosal tissues are the product of B cells. Soluble antibodies can participate in the host defence by neutralizing toxins, taking part in the opsonization of extracellular microbes and activating the complement system. These three ways result in enhanced ingestion by phagocytes and the consequent lysis of microbes or their products.

B lymphocytes can also recognize encapsulated pathogens and toxins of sugar and lipidic nature *per se* via membrane-bound antibodies or via Toll-like receptors (Batista and Harwood, 2009; Browne, 2012). Alternatively, B cells can recognize microbes opsonized with antibodies or coated with the C3d complement molecule: for this, B lymphocytes express receptors against the non-antigen specific Fc fragment of immunoglobulins or the CD21 complement receptor, respectively. The B cell receptor (BCR) comprises one membrane-bound antibody and the CD79 molecule, a heterodimer formed by two immunoglobulin chains which contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their intracytoplasmic regions. Once antigens are

recognized simultaneously by several proximate BCR complexes, the ITAMs present in the non-covalently attached immunoglobulin chains are phosphorylated by kynases and intracellular signal transduction occurs (Batista and Harwood, 2009). The final consequence is the activation of transcription factors like NFκB, which in turn lead to transcription of genes responsible for B cell proliferation (Ruland and Mak, 2003). This clonal expansion is accompained by a differentiation of the B cells into antibody-secreting cells, which further enhances the humoral response.

In the case of proteic antigens, B cells require the help of T cells to get fully activated. After binding of the BCR complex to an antigenic molecule takes place, this is endocytosed and processed to be displayed via MHC class II molecules to T cells. Co-stimulatory molecules play an important role in this B-T cell interphase. CD80 and CD86 expressed in the surface of the B lymphocyte interact with CD28 in the T cell. After this contact occurs, B cells get activated and start overexpressing the CD40 molecule, which binds to the CD40 ligand (CD40L) from the T cells. This contact is required to initiate heavy class switching and affinity maturation processes in B lymphocytes (Batista and Harwood, 2009).

T lymphocytes

T cells contribute to the elimination of intracellular pathogens, virus-infected cells, fungi, protozoans and even cancer cells using different mechanisms. First, in order to achive T cell responses, the T lymphocyte must recognize the MHC-associated antigen through the T cell receptor (TCR) and an additional molecule responsible for adhesion and signal transduction: CD4 or CD8. The CD4 molecule displayed in the T cell surface recognizes the MHC class II molecule of the antigen-presenting cell, while CD8 binds to MHC class I molecules. The TCR complex is furthermore associated to CD3 and δ chains that serve as signal transductors. Once a T cell gets activated, it rapidly secretes different cytokines, according to the T cell type, which in turn enhance different mechanisms of immune response.

$CD4^+$ T cells become helper T cells. These cells are required for triggering humoral immune responses by activating B lymphocytes. T helper cells can differentiate into T_h1 or T_h2 cells. T_h1 cells mainly stimulate phagocytic activity of macrophages by secreting IFNγ. T_h2 cells produce IL-4 and IL-5, cytokines that stimulate immunoglobulin switch class to IgE production in B cells and activate eosinophils respectively. This response is especially effective against helminths. In parallel, $CD4^+$ T lymphocytes release IL-2 soon after activation, a cytokine that initiates the clonal expansion.

$CD8^+$ T cells in contrast are the cellular effectors of cytolytic mechanisms, therefore they are also called cytolytic T lymphocytes (CTLs). The main function of CTLs is to directly eliminate infected cells. CTLs identify these cells through the MHC class I molecule, which carries antigenic particles of the microbe. The activation of $CD8^+$ T cells leads to the secretion of perforins and granzyme. Perforin is required to form pores in the membrane of the infected cells, and granzymes (caspase-activating enzymes that lead to apoptotic cell death) are delivered through these pores into the target cell.

The final consequence after $CD4^+$ or $CD8^+$ T cell activation is the elimination of the infectious agent in a rapid manner according to the infection type. This adaptive response is more specific to the type of pathogen invading the host, in contrast to the initial innate immune response, but it also needs more time to develop.

Further subsets of T cells are involved in different mechanisms of immune response and homeostasis. The T_h17 cells for instance are involved in the clearance of fungi and bacteria by recruiting neutrophils and through the induction of a massive inflammatory response (Steinman, 2007). These cells secrete IL-17 (hence the name T helper 17) and have been involved in tissue damage linked to autoimmune diseases (Korn et al., 2009), but their mechanisms of action in health and disease remain yet poorly understood.

A fourth subset is comprised by the T regulatory cells. These T_{Reg} cells are crucial for limitating collateral tissue damage during inflammatory processes. T_{Reg} cells secrete inhibitory cytokines such as IL-10 and TGFβ.

However, by limiting the immune response, T_{Reg} cells might also contribute to pathogen survival and chronic infections (Belkaid, 2007).

1.1.3 Homeostasis in the immune system

An immune response is inherently linked to an inflammatory component, which exacerbated can lead to tissue damage. The immune system is therefore also responsible for keeping a harmonized environment and terminating the immune response once the hazard has been cleared. Although the mechanisms that lead to termination of the inflammatory response are not fully understood, it is known that inhibitory signals are required to bring the immune system back to balance (e Sousa, 2004).

The immune system is also involved in the homeostasis of the organism. A good example is the intestinal tract, where an equilibrium is kept among comensal bacteria and lymphoid tissue draining the gut. Here, the action of both innate and adaptive cellular effectors contributes to a balance between the comensal bacteria that are beneficial for digestive processes, while protecting against pathogenic bacteria and other harmful ingested particles. This tolerance occurs mainly due to the action of the T_{Reg} cell subset and its production of the anti-inflammatory cytokines IL-10 and TGFβ (Garrett et al., 2010). However, the mechanisms by which the immune system differentiate among commensal and pathogenic bacteria remain unclear.

Autoimmune diseases represent also a good example of loss of the immune homeostatic equilibrium. Self-molecules, predominantly secreted proteins and components of the extracellular matrix, are constantly presented by APCs via MHC class II molecules to cells of the adaptive immune system. For instance, T lymphocytes that interact too strongly with self-antigens receive a signal that leads to apoptosis. Some other of these T lymphocytes become T_{Reg} cells. This permits to induce tolerance against self-molecules present in the host, so that immune cells that are constantly scanning the organism do not get activated. In the case of autoimmune diseases, an abnormal and deleterious immune response is developed against self-molecules.

1.2 Pathogen recognition receptors

As described in section 1.1.1, cells of the innate immunity scan the environment for pathogen- or danger-associated molecules. For this purpose, cells use pathogen recognition receptors (PRRs) displayed in the cellular surface, localized in the cytoplasm or secreted into tissue fluids or the circulation (Janeway and Medzhitov, 2002). It is necessary to mention that the term PRR refers strictly speaking to receptors that bind pathogens, but PRRs might also bind ligands related to danger signals. The actions of PRRs are manifold. These receptors are not limited to binding or opsonizing pathogens, but they might also be involved in phagocytosis or in signal transduction after ligand recognition (Medzhitov, 2001).

A miscellanea of protein families have been involved in the recognition of pathogen and danger signals by cells of the immune system. The localization of these receptors is related to the nature of the ligands: they can be secreted, they can be bound to cytoplasmic or intracellular membranes, or they can be found within the cell surveilling the cytoplasm.

1.2.1 Secreted pathogen recognition receptors

The group of soluble PRRs is vast and very heterogeneous. According to their structural classification, several families of soluble proteins have been involved in pathogen recognition. These include the collectins (collagen-containing C-type lectins), pentraxins, ficolins, peptidoglycan recognition proteins and complement receptors, among others.

Two of the best characterized receptors of this class are the mannan-binding lectin (MBL, a collectin protein) and the C-reactive protein (CRP, a pentraxin). Both are proteins released during the acute phases of infection: CRP is indeed an standard clinical marker of early inflammation. These acute-phase proteins can activate the complement pathway after recognition of PAMPs, leading to opsonization of microbes. They can also induce the

physiological clearance of apoptotic cells through phagocytes (Stuart et al., 2005).

1.2.2 Cytoplasmic receptors: NLRs, RLHs

Intracellular pathogens or bacterial constituents resulting from intracellular lytic processes can be detected within the cytoplasm through two big families of soluble PRRs: the NOD-like receptors and the RIG-like helicases.

NOD-like receptors

The first cytoplasmic PAMP receptors to be discovered were the nucleotide-binding oligomerization domain-containing proteins NOD1 and NOD2. Nowadays the NOD-like family of receptors comprises over 20 members (i.e. NALP3, IPAF, NAIP, etc.), but the physiological role of most of them remains unknown. Structurally, most of these proteins contain a leucin-rich repeat domain responsible for ligand binding, and a NACHT domain involved in self-oligomerization. It is not clear whether these receptors recognize their ligands directly or through complementary molecules. Activation after ligand recognition induces a conformational change that leads to oligomerization and the consequent formation of the inflammasome (a multiprotein complex that activates caspases and transcription factors involved in inflammatory processes).

NLRs detect bacterial peptidoglycans (PGNs), which are units of the bacterial cell wall that are released into the cytosol after degradation of microbes in lytic vesicles. NLRs have been related to the production of the potent proinflammatory cytokines IL-1β and IL-18 (Meylan et al., 2006). These cytokines are responsible for rapid recruitment of neutrophils to inflammatory sites, induction of IFN-γ produced by T and NK cells, the febrile response, and the stimulation of T_h17 immune responses (Martinon et al., 2009). Indeed, mutations that lead to a suspected constitutive activation of NALP3 have been found in experimental autoimmune encephalomyelitis (Gris et al., 2010). Mutations in NOD2 that lead to a diminished production

of the inhibitory cytokine IL-10 have been found in patients suffering from Crohn's disease (Noguchi et al., 2009).

In the recent years it has been shown that NLRs detect other bacterial components than PGNs, such as bacterial RNA and synthetic base analogues (Kanneganti et al., 2006). Also DAMPs can trigger the inflammasome formation through NLR activation. For instance, low intracellular potassium levels, a sign of acute cellular stress, or crystallized monosodium urate from necrotic cell lysates lead to caspase-1 activation through the NALP3 inflammasome (Meylan et al., 2006).

RIG-like helicases

Retinoic-acid-inducible gene (RIG) I-like helicases are responsible for the detection of viral nucleic acids in the cytoplasm. RLHs are a group of 3 proteins: RIG-I, MDA5 and LGP2. RIG-I detects dsRNA from paramyxoviruses, influenza virus and Japanese encephalitis virus, while MDA5 senses dsRNA from picornavirus (Kato et al., 2006). Both RIG-I and MDA5 trigger antiviral responses after activation, in contrast to LGP2, which might sequestrate dsRNA preventing activation of RIG-I and MDA5 (Yoneyama et al., 2005).

The activation of RIG-I and MDA5 leads to activation of the NFκB transcription factor and IRFs, which cooperate to induce IFN-mediated antiviral responses (Yoneyama et al., 2004). This cytokine is secreted by infected cells and acts by paracrine signalling in neighbouring cells. It leads to protein kinase R and RNAse L secretion, which in turn diminish both viral and host protein synthesis within the cells, to avoid extension of the infection. Among other functions, IFN also increases the expression of MHC molecules, therefore contributing to prime cytolitic $CD8^+$ T cells and T helper responses (Takeuchi and Akira, 2008).

1.2.3 Membrane-bound receptors: TLRs, CLRs

Membrane-bound pathogen recognition receptors might be present in the cytoplasmic membrane or in the membrane of intracellular compartments.

There are several families of membrane-bound receptors involved in innate immunity. Scavenger receptors, Toll-like receptors and C-type lectins are the best characterized. Due to the relationship with our protein of interest only the last two groups will be described in this work.

Toll-like receptors

Mammalian Toll-like receptors are related to the Toll receptor discovered in *Drosophyla melanogaster*. This receptor was first shown to result essential for embryogenesis in insects, and later on a role in the defense against fungal infection was demonstrated (Hashimoto et al., 1988; Lemaitre et al., 1996). Up to now over 10 TLRs have been characterized in mammals, which recognize a broad spectrum of PAMPs. All of them share a very similar structure: they are transmembrane proteins with an extracellular leucine-rich repeat (LRR) domain, responsible for ligand recognition, and an intracellular Toll/IL-1 receptor (TIR) domain responsible for signal transduction (O'Neill et al., 2013).

TLRs can be found in the cytoplasmatic membrane or intracellularly in phagosomes, according to the nature of their ligand. TLR3, TLR7, TLR8 and TLR13 are localized in the membranes of endosomes and detect viral nucleic acids (Alexopoulou et al., 2001; Hemmi et al., 2002; Jurk et al., 2002; Shi et al., 2011). TLR9 is expressed also in the lipidic membrane of intracellular compartments and it detects unmethylated DNA, which is restricted to bacteria (Hemmi et al., 2000). In contrast TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed in the cytoplasmic membrane. TLR2 forms heterodimers with TLR1 or TLR6 to detect different forms of lipopeptides found in the microbial cell wall (Schwandner et al., 1999; Takeda et al., 2002). TLR4 is the receptor for lipopolysaccharide (LPS), the major structural component of Gram-negative bacteria outer wall (Hoshino et al., 1999). TLR5 detects flagellin, a protein that builds the bacterial flagelli (Hayashi et al., 2001), and TLR11 senses uropathogenic bacteria such as *Escherichia coli* (Zhang et al., 2004).

TLRs might detect their ligands directly or via adaptor molecules. For instance, TLR4 uses several adaptors for ligand recognition. LPS is bound to LPS binding protein (LBP) in serum, and LBP binds to CD14, which brings LPS in contact with TLR4 aided by a third molecule, MD-2 (Coats et al., 2005; Remer et al., 2003; Shimazu et al., 1999). Recognition of PAMPs through TLRs leads to the recruitment of adaptor molecules such as MyD88, TIRAP, TRAM or TRIF, through the TIR domain. These in turn are responsible for the activation of kinases, which in final steps of the signaling cascade mediate translocation of the transcription factors NFκB, IRFs, AP-1 and CREB to the nucleus (Hu et al., 2007; Ruland, 2008; Takeda and Akira, 2005). These transcription factors are involved in the expression of genes which are involved in the production of pro- and anti-inflammatory cytokines during the initial innate immune response and other molecules which also guide the secondary adaptive immune response (Medzhitov and Janeway, 2000).

C-type lectins

C-type lectin receptors (CLRs) belong to a broad subfamily of animal lectins. The term C-type makes reference to Ca^{+2}-dependent carbohydrate binding properties of this lectins. However, far from the original concept that CTLs bind only sugars, it is known that CTLs can bind also proteins, lipids or anorganic molecules (i.e. calcium carbonate) through their C-type lectin-like domain (Drickamer, 1999). CLRs are mainly type II single-pass transmembrane proteins, with a cytoplasmic N-terminus and an extracellular C-terminus. Some CLRs however have soluble forms and can be found in the serum, such as collectins and the mannose-binding lectin (Weis et al., 1998). All CLRs are characterized by the presence of at least one C-type lectin-like domain (CTLD) responsible for ligand binding activity, and they are divided in 17 groups according to their domain architecture (Zelensky and Gready, 2005).

C-type lectins are expressed in a variety of cell types and their functions are multiple. They have been involved for instance in cell adhesion, migration, differentiation, tissue remodeling or NK cell regulation (Engering et al., 2002; Osorio and e Sousa, 2011). CLRs are well known receptors for glycoproteins found in the host. Their endocytic function might serve to present self-antigens via MHC class II molecules and induce tolerance. However the identification of exogenous ligands has given a new perspective to the CLR family in the last decade. C-type lectins expressed in cells of the innate immunity can act as pathogen recognition receptors too (table 1.2. Apparently several CLRs do not have only endocytic activity after binding to their ligands, but they can also trigger innate immune responses and even shape adaptive immune responses (Geijtenbeek and Gringhuis, 2009; Iwasaki and Medzhitov, 2010). Unfortunately the property of several C-type lectins to induce tolerance can be used by several pathogens to escape immunity (Engering et al., 2002; van Kooyk and Geijtenbeek, 2003).

1.3 Myeloid C-type lectins

Several genes coding for myeloid C-type lectins are found in closest proximity to the NK gene cluster (figure 1.3). This cluster contains activating and inhibitory receptors expressed by natural killer cells. In NK cells, these receptors cross-talk to regulate the cytolytic activity of NK cells. Since the neighbouring C-type lectins are also activating and inhibitory receptors, it has been speculated that CTLs might work in a similar way to NK receptors.

Proteins coded by genes of the Dectin-1 cluster include Clec12a, Clec2, Clec9a, Clec12b, Clec1, Dectin-1 and LOX1. These C-type lectins belong to the group V (NK receptors), which comprehends proteins with a short cytoplasmic tail, a transmembrane domain, an extracellular stalk region, and a C-type lectin-like domain (Zelensky and Gready, 2005). The stalk region contains aminoacids needed for oligomerization and its length varies greatly among the different members. This group of myeloid CTLs is gain-

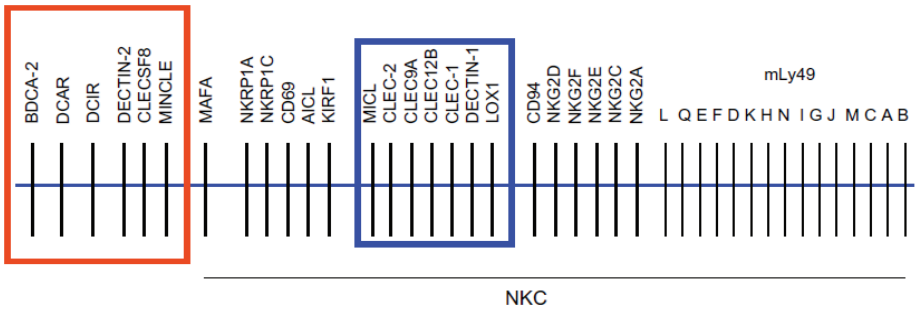

Figure 1.3: C-type lectin genes neighbouring the natural killer cluster. The Dectin-2 cluster (red box) and the Dectin-1 cluster (blue box) are situated in the telomeric region of the natural killer cluster. From Graham and Brown (2009).

ing importance in the area of innate immunity, not only for their role as recognition receptors for PAMPS but also DAMPs. For instance, LOX1 is a CLR that responds to oxidized low-density lipoprotein and oxidized lipids found in aged or apoptotic cells (Oka et al., 1998). Clec9a-expressing reporter cell lines triggered immune responses in the presence of ultraviolet-irradiated mouse embryonic fibroblasts, and Clec9a has been shown to trigger potent cytotoxic cellular responses upon necrotic cell death *in vivo* (Sancho et al., 2009). Clec2 binds rhodocytin, a snake venom, and podoplanin, an endogenous protein found in several cell types. This shows that one C-type lectin can also bind ligands of different nature, possibly having different actions for each of them (Suzuki-Inoue et al., 2006). Dectin-1, in contrast, binds the β-glucans, polysaccharides found in the cell wall of yeasts and certain fungi (Brown and Gordon, 2001). Ligands for Clec12a and Clec12b have not been yet identified, although there is evidence that Clec12a recognizes an endogenous ligand (Pyz et al., 2008).

Many other C-type lectins outside this cluster expressed in myeloid cells and playing a role in innate immunity have been well characterized. For instance, DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin) binds not only endogenous molecules such as ICAM-2 or ICAM-3, but also PAMPs present in viruses such as HIV (Geijtenbeek

et al., 2000) or HCV, in bacteria, fungi or even parasites (Cambi et al., 2008; Colmenares et al., 2002).

Some CTLs might form oligomers in order to become active. Their actions can be very different according to the signaling motifs present in the protein. While killer activation receptors expressed by NK cells possess ITAMs with 2 tyrosines available for phosphorylation, other CTLs, like Dectin-1, possess only 1 tyrosine, what by some groups is called a hemITAM sequence. Other CTLs have an ITIM, which in principle has the opposite action as an ITAM and therefore they act as inhibitors: they recruit phosphatases that contribute to the dephosphorilation of other proteins. Some CTLs do not possess any signaling motif but possess positively charged residues in the transmembrane region that recruit adaptor molecules such as Fcγ, DAP10 or DAP12, which in turn signal through their intracellular motifs.

1.3.1 Signaling pathways of CLRs

CLRs expressed in myeloid cells are mainly involved in endocytosis of ligands, but only a few can directy regulate gene expression in immune responses (Weis et al., 1998). Leucine repeats, tri-acidic clusters and tyrosine-based motifs found in CLRs are required for endocytosis of the ligands and its delivery to endosomal and lysosomal compartments.

Those CLRs that can activate signaling cascades for regulation of gene expression act through three possible signaling motifs. CLRs might use ITAMs to recruit kinases, directly or via adaptor molecules. A third subset is comprised by CLRs carrying an ITIM in their intracellular region, which are suspected to play an inhibitory role, possibly terminating immune responses or collaborating to immune tolerance. The signaling motifs found in different myeloid CLRs are depicted in figure 1.4.

The consensus for the classical ITAM motif sequence is YxxL/Ix$_{6-8}$YxxL/I (Cambier, 1995). This signaling motif is found in Fc receptors, TREMs and Dectin-2 among other molecules, while several CLRs possess only one tyrosine available (YxxL/I) for phosphorylation in this signaling domain, hence

CLR	**Ligand (PAMP/DAMP)**	**Motif or Adaptor**	**Outcome**
BDCA2	HIV-1 (gp120)	FcRγ	Inhibition of TLR-9, type I IFN, TNF and IL-6 production
CLEC2	HIV-1, rhodocytin, podoplanin	hemITAM	ND
CLEC5A	Dengue virus	DAP10 and DAP12	TNF production
CLEC9A	F-actin	hemITAM	TNF production, Ag cross-presentation
CLEC12A	Endogenous ligand in several tissues	ITIM	Inhibitory (ND), endocytic activity
CLEC12B	ND	ITIM	ND
DCAR	ND	FcRγ	Activating (ND)
DCIR	HIV-1	ITIM	Inhibition of IL-12, TNFα and IFNα production by TLR-activated DCs
DC-SIGN	*M. tuberculosis*, *M. leprae*, BCG, *H. pilori*, HIV-1, dengue virus, SARS coronavirus, *C. albicans*, Leishmania, *S. mansoni*	None	IL-10 production, induction of Th1, Th2, Th17 and Treg differentiation
Dectin-1	*M. tuberculosis*, *C. albicans*, *A. fumigatus*, *P. carinii*	hemITAM	Induction of Th1 and Th17 differentiation, TNF production, phagocytosis
Dectin-2	*M. tuberculosis*, *C. albicans*, *A. fumigatus*, *D. pteronyssinus*	FcRγ	TNF and IL-6 production
Langerin	*M. leprae*, HIV-1	Proline-rich domain	Endocytosis and Ag presentation
Mincle	Mannose, glycolipids; SAP-130	FcRγ	*M. tuberculosis, C. albicans, Malassezia spp.*; dead cells.
MGL	Filoviruses, *S. mansoni*	ND	Inhibits T cells by CD45 binding

Table 1.2: C-type lectin receptors. Different C-type lectin receptors are listed with their exogenous and/or endogenous ligands, their signalling motifs or adaptor molecules and immunological responses after stimulation. ND, not determined. Adapted from Geijtenbeek and Gringhuis (2009) and Sancho and e Sousa (2012).

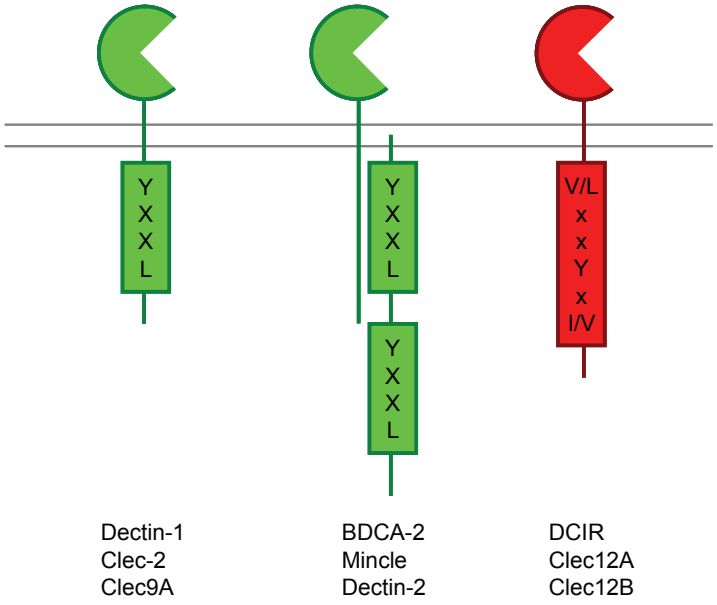

Figure 1.4: Myeloid CLRs and their signaling motifs. Left figure: a signaling motif resembling the ITAM consensus but with a single tyrosine, therefore also called hemITAM, recruits the kinase Syk. Middle figure: myeloid CLRs that require an ITAM-bearing adaptor (i.e. FcRγ chain or DAP12). Right figure: inhibitory myeloid CLRs recruit the phosphatases SHP-1 and SHP-2 through their ITIM motifs. Adapted from Robinson et al. (2006) and Sancho and e Sousa (2012).

called hemITAM. This motif can contribute to the initiation of cytoskeleton reorganization for endocytic processes upon activation, but it also mediates activation of the kinase Syk. For instance, Dectin-1 dimerizes after binding its ligand and the tyrosine of the hemITAM is phosphorylated by Src kinases. This event leads to recruitment of Syk, followed by coupling of Card9-Bcl10-Malt1 molecules that derive in NFκB and NFAT translocation to the nucleus at final steps of the signaling cascade (Ruland, 2008). In a similar way, CLRs that do not possess a signaling motif recruit ITAM-bearing adaptor molecules, such as FcRγ chain or DAP12, which in turn follow the same signaling cascade (Osorio and e Sousa, 2011).

C-type lectins bearing an ITIM in their cytoplasmic tails are supposed to work in an inhibitory manner. The consensus sequence for the ITIM motifs is (I/V/L)xYxx(L/V). The tyrosine residue here is phosphorylated as well by Src kinases, but in contrast it does recruit SHP and SHIP phosphatases. These proteins in turn dephosphorylate other tyrosyl-phopshorylated molecules, counteracting activated receptors. Which molecules are the exact substrate of these phosphatases remains unclear and could be Src as well as Syk kinases, among others (Daëron et al., 2008).

However, it is important to mention that cross-talk among several ITAM- and ITIM-bearing receptors has demonstrated that this relationship might not work in such a unidirectional manner. That ITAM-bearing receptors can counteract the pro-inflammatory response triggered after activation of pathogen recognition receptors indicates that ITAM signaling might be more complex than expected. Both ITAM and ITIM signaling can derive in cellular activation and inhibition. This dual role of ITAM- and ITIM-bearing receptors has been seen not only in C-type lectins but also in Fc receptors and remains being investigated (Barrow and Trowsdale, 2006; Isakov, 1997; Lowell, 2011).

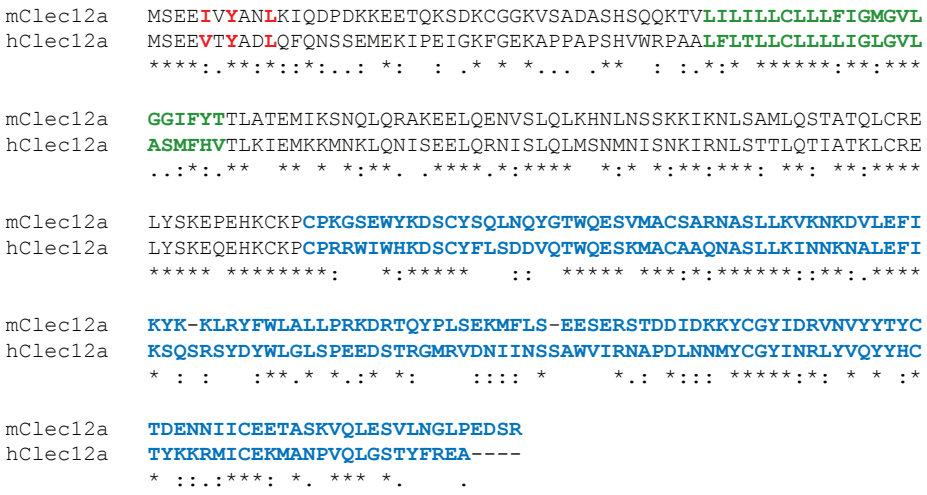

Figure 1.5: Alignment of murine and human Clec12a protein sequences. The sequences of human (hClec12a) and murine Clec12a (mClec12a) were obtained from the accession numbers NCBI NP_612210.4 and NP_808354.1 respectively. Both sequences were aligned using ClustalW2 software. Asterisks represent identical residues, conserved substitutions are indicated with colons (**:**) and periods (**.**) mean semi-conserved substitutions. Red text indicates the ITIM sequence, green corresponds to the predicted transmembrane hydrophobic region and blue is the expected carbohydrate recognition domain.

1.3.2 Clec12a

Genes for Clec12a are located in the chromosome 6F3 in *Mus musculus* and 12p13 in *Homo sapiens*. They are located within the natural killer (NK)-gene cluster. Both genes contain 6 exons and share 72% homology. Other designations are CLL-1 (C-ytpe lectin-like molecule 1), DCAL-2 (dendritic cell-associated lectin-2) and MICL (myeloid inhibitory C-type lectin-like receptor).

Clec12a is a type II transmembrane C-type lectin. It has an extracellular region with a CTLD recognition domain followed by a stalk region. It has an hydrophobic transmembrane region followed by an intracytoplasmatic tail containing an immunoreceptor tyrosine-based inhibitory motif (Chen et al., 2006; Marshall et al., 2004; Pyz et al., 2008). Human and murine Clec12a proteins share 65% homology, and both conserve the ITIM motif (figure 1.5).

Clec12a has been identified as a marker for leukemic cells in cases of acute myeloid leukemia (van Rhenen et al., 2007). However its function in homeostasic conditions or in diseases remains unknown. It has been shown that Clec12a can recruit the inhibitory phosphatases SHP-1 and SHP-2 upon activation (Han et al., 2004; Marshall et al., 2004). Other groups however have delivered contradictory results on the effect of Clec12a in cellular activation. While Chen et al. (2006) describe an increase in IL-6 and IL-10 production by dendritic cells upon activation, Lahoud et al. (2009) could not confirm these data. However all these results are based on antibody cross-linking experiments, which can differ from stimulations with the real agonists of the receptor. Indeed, the ligand of Clec12a has not been identified yet, but there is evidence for an endogenous ligand predominantly expressed in kidney and, to a lesser extent, in liver, lung, heart and spleen (Pyz et al., 2008). As well as the nature of these ligands, the function of Clec12a *in vivo* remains completely unknown.

1.3.3 Investigative approach and objectives

The objective of this work is to identify the physiological role of Clec12a *in vivo*. Knockout animal models are genetically engineered animals where a gene has been inactivaded by replacing it with a sequence of DNA generated artificially. This replacement takes place due to homologous recombination, a phenomenon that occurs naturally during meiosis in eukaryotic cells and is responsible for genetic diversity in the offspring (Radding, 1973). Due to the extensive use of laboratory mice (*Mus musculus*) in immunological studies, a *Clec12a*-deficient mouse model was generated in order to investigate the roles of Clec12a in the development and function of the immune system *in vivo*, focusing on the inhibitory properties of this receptor (figure 1.6).

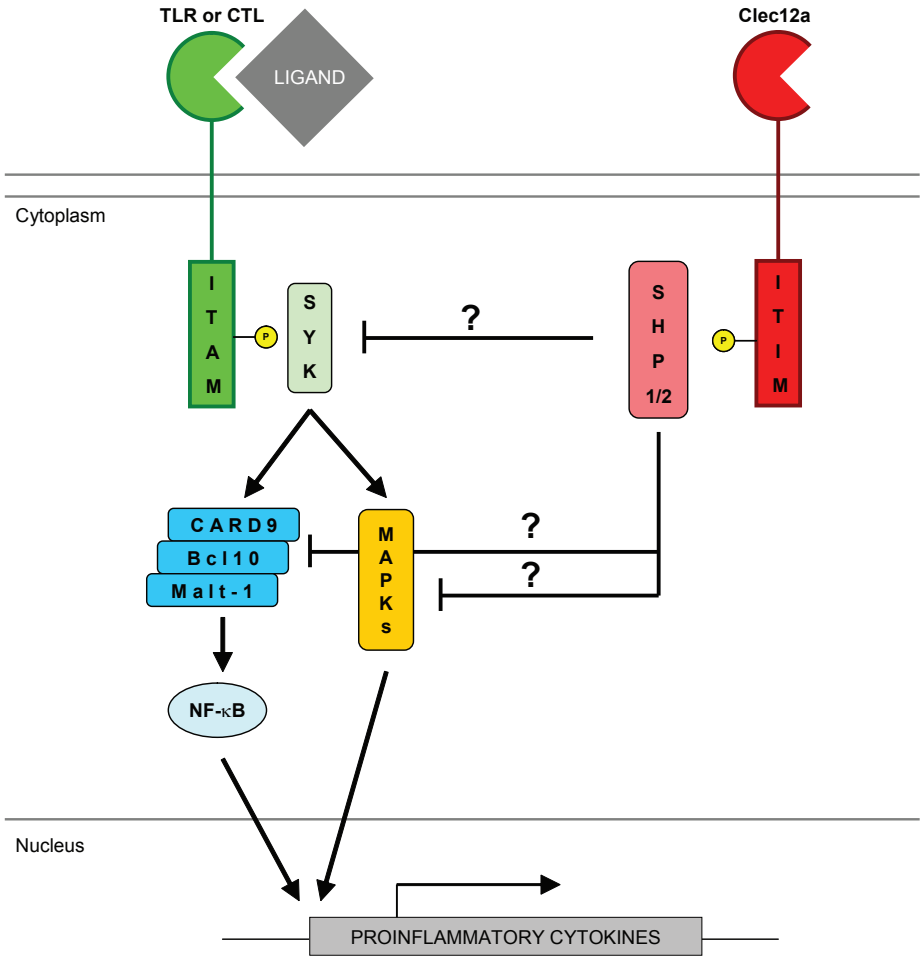

Figure 1.6: Hypothetic model of Clec12a physiological role. Clec12a might work as an inhibitory receptor by recruiting SHP phosphatases and therefore down-regulating signals from ITAM-bearing receptors in homeostatic conditions or under CLR or TLR stimulation.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Reagents

Except otherwise indicated, all cell culture reagents were from Invitrogen, chemicals were from Sigma Aldrich and flow cytometry reagents were from Becton Dickinson.

2.1.2 Cell culture

Cell culture dishes were from Techno Plastic Products. Disposals such as pipettes and tissue culture tubes were from Becton Dickinson Falcon. Incubators were from Heraeus (Thermo Scientific).

2.1.3 Animals

C57BL/6JOlaHsd mice were purchased from Harlan Laboratories, Inc. All animals were kept in individually ventilated cages according to European Union regulations. Health monitoring and animal experiments were performed following national and institutional guidelines.

2.2 Methods

2.2.1 Agarose gel electrophoresis

Separation of nucleic acids by size can be achieved in an agarose three-dimensional matrix using their negative charge. For this, a 1% agarose (Biozym) gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 0.5 M EDTA) and 1 drop/100 ml ethidium bromide is poured into a cast. A comb placed in one extrem of the gel generates wells where the samples can be loaded. Once the agarose dilution has cooled down, samples are resuspended in 10x DNA loading dye (Peqlab Biotechnologie GmbH). An electric field of 120 V is applied to the gel submerged in 1x TAE buffer until separation has been achieved. Ethidium bromide intercalated in DNA was detected after excitation with ultraviolet light in a Gel Doc 2000 documentation system (BioRad), and fragment size was determined using DNA ladder (Peqlab Biotechnologie GmbH) as comparison.

2.2.2 Reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction allows qualitative detection of gene transcription. In this two-step process, cDNA is first generated from total RNA using the properties of reverse transcriptase. Lastly, the cDNA corresponding to the genes of interest is amplified using specific primers.

Total RNA was extracted using 1 ml TriZOL per 100 mg tissue sample or 10^7 cells. Homogenizer was washed first in a NaOH solution and then in DEPC-H_2O (1 drop diethyl pyrocarbonate per 1 L destilled H_2O, to eliminate RNAses) and finally used for tissue disintegration. Cell suspension was incubated 5 minutes at room temperature and 200 μl chloroform were added per each ml of TriZOL. Samples were vortexed 15 seconds and incubated 3 minutes at room temperature, followed by centrifugation for 15 minutes at 12000 G. Clear supernatant was transferred into fresh tube and 0.5 ml isopropanol was added. After 10 minutes incubation at room temperature,

samples were centrifuged for 30 minutes at 12000 G, followed by a washing step with at least 1 ml ethanol. Samples where then centrifuged for 10 minutes at maximum speed and ethanol was removed. Pellets were allowed to air dry and then solved in 30 to 50 μl DEPC-H_2O. RNA content was measured in a NanoDrop ND-100 spectrometer (Peqlab Biotechnologie GmbH). All centrifugation steps were performed at 4°C and samples were always kept on ice in order to preserve RNA integrity.

cDNA was prepared by reverse transcription using Superscript II reverse transcriptase kit: 1 μg of RNA was incubated with 5 μl random primers in a volume of 12 μl, filled up with DEPC-H_2O. After 10 minutes incubation at 70°C, the mix was placed on ice and 4 μl 5x first strand buffer plus 2 μl DTT and 1 μl dNTPs 10 μM (BioRad) were added. The mix was incubated for 10 minutes at 25°C followed by 2 minutes at 42°C. Superscript reverse transcriptase was added (1 μl per sample) and incubated 50 minutes at 42°C, followed by 15 minutes at 70°C. Samples were used directly for RT-PCR or stored at -20°C.

Finally, specific amplification of the cDNA generated beforehand permits the qualitative determination of gene transcription. For each gene of interest, 1 μl of cDNA was used as template and mixed with 0.5 μl of each oligonucleotide (listed in table 2.1), 2 μl dNTPs (2.5 mM each), 2.5 μl 10x reaction buffer (Amersham), 1 μl $MgCl_2$ 25mM and 0.2 μl Taq polymerase (Amersham) in a final volume of 25 μl. The annealing temperature was calculated by using the mean of each primer melting temperature pair. Elongation time was adjusted following the rule of 60 seconds per 1000 bp of PCR product. Amplified cDNA was analyzed through agarose gel electrophoresis using an appropiate DNA standard as reference.

2.2.3 Targeting construct generation

For the genetic modification of the target gene, a targeting vector was generated that aligns with and replaces the original sequence. Targeting vector construction was performed by standard methods. Genomic DNA covering

Gene - Sense	Sequence
Dectin1 Fwd	ATGAAATATCACTCTCATATAGAGAATCTG
Dectin1 Rev	TTACAGTTCCTTCTCACAGATACTG
Clec2 Fwd	ATGCAGGATGAAGATGGGTATATC
Clec2 Rev	TTAAAGCAGTTGGTCCACTCTTGTC
Clec9a Fwd	ATGCATGCGGAAGAAATATATACCTC
Clec9a Rev	TCATAGTCTCACATCAATCTCCTC
Clec12a Fwd	ATGTCTGAAGAAATTGTTTATGCAAATCTC
Clec12a Rev	CTACCTGCTATCCTCTGGGAG
Clec12b Fwd	ATGTCTGATGAAGTGACCTATGCG
Clec12b Rev	TTAATCCAAATCTTCAATCTTCACCAGG
β-actin Fwd	GCATTGCTGACAGGATGCAG
β-actin Rev	CCTGCTTGCTGATCCACATC
Clec12a-SA Fwd	ATTGCGGCCGCTGCTAACATGTCCTGGACAGG
Clec12a-SA Rev	GCTCTAGAACTGTGGCTAACCTCACAAGC
Clec12a-LA Fwd	CCATCGATAATCCCTCTCATTCTCCTGCC
Clec12a-LA Rev	GAGCTCGAGAAGCATGTGTGACGGCTCATG

Table 2.1: Oligonucleotides used for RT-PCR and targeting vector generation.

the Clec12a locus was amplified from a bacterial artificial chromosome library (clone RPCIB731A2047Q) using the primers listed in table 2.1. A short arm beforehand the first exon of Clec12a locus was generated using the oligonucleotide *Clec12a*-SA Fwd at the 5' end and *Clec12a*-SA Rev for the 3' end. For the long arm, located after the second exon of Clec12a locus, the oligonucleotides *Clec12a*-LA Fwd at the 5' end and *Clec12a*-LA Rev at the 3' end were used. The selected DNA fragments (short and long arm) were assembled into a pBluescript (Stratagene) based targeting vector containing a neomycin-resistance gene in the opposite direction to the open reading frame (kind gift of Dr. Olaf Gross). The short arm was inserted in the vector using NotI and XbaI restriction sites, while the long arm was inserted immediately after the neomycin resistance gene using ClaI and XhoI restriction sites. Homologous recombination replaced the genomic fragment containing the first 2 exons encoding the intracellular and transmembrane domain of Clec12a by the NEO cassette.

2.2.4 Enzymatic linearization of targeting construct

For stem cell electroporation, the targeting construct was linearized by enzymatic digestion to allow homologous recombination. Due to loss through enzymatic digestion and purification steps, at least double amount of DNA is needed of the theoretically required. For this reason, 20 μg of linear DNA were used per electroporation. At least 1 U/μl NotI (New England Biolabs) restriction enzyme was used per 1 μg targeting construct for each enzymatic digestion, in appropriate buffer NEBuffer 3 (New England Biolabs). Incubation was performed overnight at 37°C with smooth shaking (300 rpm). Complete digestion was confirmed through electrophoresis in a 1% agarose (Biozym) gel in TAE buffer loading a dilutions series of the digested DNA.

2.2.5 Phenol and chloroform DNA purification

Many factors affect the success of gene targeting, being purity of the synthetically generated DNA a key component of this process. Purification of DNA was performed using phenol and chloroform. Appropiate volume of linearized DNA was transferred to a 15 ml tube and the same volume of phenol was added. The tube was inverted continuously for 5 minutes, followed by a centrifugation step of 5 minutes at 4000 rpm and at room temperature. The upper phase was collected in a new 15 ml tube, and the same volume of a freshly prepared mixture of 1 part phenol with 1 part chloroform/isoamyloacohol (1:24) was added. Tube was inverted and centrifuged as before. The upper phase was collected again in a new tube and one volume of chloroform was added. Tube was inverted as previously indicated and centrifuged for 5 minutes at 4000 rpm and 4°C. The upper phase was collected in a new tube and 1/10 volume of sodium acetate (3 M) was added and mixed. Two volumes of ice cold ethanol were added and mixture was gently inverted in order to precipitate the DNA. To obtain a complete precipitation, the sample was stored at -20°C for at least 30 minutes. Samples were centrifuged 10 minutes at 4000 rpm and 4°C. Supernatant was discarded and

the DNA pellet was washed in 1 ml 70% ice cold ethanol. The DNA pellet was air dried under sterile conditions and dissolved in sterile PBS to a final concentration of 1 μg/μl.

2.2.6 Preparation of murine embryonic fibroblasts

Embryonic stem cell culture requires a feeder layer of mouse embryonic fibroblasts (MEFs) for optimal growth. For the generation of MEFs embryos were isolated at an age of 13.5 dpc under sterile conditions. Brain and liver were removed, and embryos were meshed through a 100 μm strainer in order to get a cell suspension. Up to 4 embryos were collected in 5 to 8 ml DMEM supplemented with 10% FCS, 1% penicilin & streptomicin, 2 mM L-glutamine and 0,1% β-mercaptoethanol. The cell suspension was centrifuged at 1200 rpm and 5x10^6 cells were plated per 10 cm diameter plate. Cells were splitted before complete confluency was reached.

2.2.7 Embryonic stem cell culture

R1 cells, from the inbred mouse strain 129/Ola, donated by Ronald Naumann from the Max Planck Institute of Molecular Cell Biology and Genetics (Dresden, Germany), were cultured in DMEM supplemented with heat inactivated (by incubating 20 minutes at 65°C) 15% FCS , 1% penicilin & streptomicin, 1% non-essential aminoacids, 1000 U/ml LIF, 6 mM L-glutamine and 0,1 % β-mercaptoethanol. R1 cells were plated over an almost confluent layer of MEFs (see section 2.2.6) pretreated with 1 mg/ml mitomycin C for at least 4 hours and several washing steps with pure DMEM medium. For electroporation, ESCs were harvested and resuspended at a density of 6,4 x 10^6 cells/ml.

2.2.8 Electroporation of stem cells

Chemical, electrical, viral or hybrid methods allow delivery of molecules to the cell interior. Here, transfection of targeting vector was performed using

electroporation. This process involves intense electric impulses that increase the permeability of the plasmatic membrane for a brief period of time. For each electroporation, 20 μl of linearized targeting vector (1 μg/μl) were mixed with 780 μl stem cell suspension (6,4x10^6 cells/ml) in a 0,2 cm gap cuvette (Bio-Rad). A Gene Pulser II Electroporation system (Bio-Rad) was used with the following settings: 340 mV, 250 μF, infinite Ω and 2 mm cuvette gap. Optimal electroporation was achieved when the time constant was between 3,0 and 4,0 msec. The cuvettes were placed at room temperature for 5 minutes immediately after electroporation. After that, they were incubated on ice for 15 minutes to allow cell recovery. Finally 800 μl ESC culture medium were added per cuvette, resuspending cells carefully and plating 800 μl of the electroporated cell suspension per 10 cm diameter plate coated with MMC pretreated MEFs plate. The cells were incubated at 37°C for 24 hours before first medium change.

2.2.9 Selection of targeted stem cells

The targeting vector contains a gene that confers cells with resistance against an antibiotic. This allows selection of stem cells that have acquired the targeting vector, although the integration of the artificially generated nucleic acid might be site unspecific at this point of the selection process. Medium was changed 24 hours after the electroporation. Selection with 200 μg/ml G418 (synthetic neomycin analog) started after 48 hours and was performed every other day. At day 10 after electroporation, the resistent stem cell clones were picked under the stereomicroscope in semi-sterile conditions with a pipette tip in 20 μl volume, placing each clone in a 96 well with 30 μl PBS, adding 25 μl prewarmed trypsin. Each 96 well plate was incubated for 10 minutes at 37°C, followed by blocking with 80 μl ESC medium/well, pipetting up and down to resuspend cells. One third of the cell suspension was distributed into a backup plate and one third into a MMC pretreated MEFs plate. The last third was used for screening of positive clones.

2.2.10 PCR screening of selected clones

After antibiotic selection of targeted stem cells, detection of site-specific recombination of the targeting vector is required. A first PCR screening was performed using pools of 8 clones collected in 500 μl reaction tubes. The tubes were centrifuged 5 minutes at 2500 G and 4°C. Supernatant was discarded and 30 μl H_2O were added for cell lysis, followed by DNA denaturation for 15 minutes at 95°C. Samples were cooled down, centrifuged and 1 μl proteinase K (20 mg/ml) was added per tube. Incubation consisted in a first step of 90 minutes at 55°C, followed by a second step of 10 minutes at 95°C.

For the PCR reaction, 7.5 μl DNA template were used in PCR reaction tubes with 7 μl dNTPs 2.5 mM, 5 μl 10x PCR buffer, 0.75 μl oligonucleotide (forward and reverse, 10 mM), and 0.75 mM DNA enzyme mix from the Expand Long Template PCR system (Roche Diagnostics GmbH). The mix was filled up with destilled H_2O up to a volume of 50 μl. The program consisted of one cycle of 2 minutes at 93°C, 10 cycles of 10 seconds at 93°C for denaturation, 30 seconds at 57°C for annealing and 90 seconds at 72°C for elongation, followed by 1 cycle of 5 minutes at 72°C and final hold at 4°C. Amplified DNA was subjected to agarose gel electrophoresis for analysis. Lastly, the whole process described in this section was repeated with each individual clone from each positive pool.

2.2.11 Southern blot screening

Southern blotting permits the identification of specific DNA sequences combining electrophoresis with probe hybridization techniques. Individual clones from positive pools were screened through Southern blot. Stem cells of positive pools were cultured in dishes without MEFs until confluency was reached. Cells were harvested and resuspended in 4 ml TNE lysis buffer (3.7 ml TNE buffer with 60 μl proteinase K 20 mg/ml, 150 μl pronase E 20 mg/ml and 150 μl 20% SDS) per sample and incubated overnight at 37°C. TNE buffer

consists of 150 mM NaCl, 10 mM Tris pH 7.5, 2 mM EDTA and 1% SDS. DNA was purified using the protocol described in section 2.2.3.

For each clone to be screened, 20 μg of genomic DNA were digested with 300 U of the restriction enzyme XmnI, 7.5 μl appropriate buffer, 0.75 μl BSA, filled with destilled H_2O up to a volume of 75 μl. The digestion mix was incubated overnight at 37°C and moderate shaking. The digested DNA was loaded in a 1% TAE agarose gel and run overnight at 30 V. The day after, the gel was washed first with destilled H_2O followed by a 20 minutes incubation in 0.25 M HCl solution. The gel was newly washed with destilled H_2O and incubated twice in denaturation solution I (1.5 M NaCl, 0.5 M NaOH), 20 minutes each time. Gel was washed again in destilled H_2O, followed by two incubation steps in neutralization solution II (1 M Tris base, 1.5 M NaCl; pH adjusted to 7.4). The gel was finally washed with H_2O. The transfer of DNA into a nitrocellulose membrane was performed overnight, the set up can be found elsewhere (Southern, 2006). The membrane was briefly crosslinked by exposure to UV light, then washed rapidly in destilled H_2O and stored at -20°C or used directly for prehybridisation.

In order to avoid signal noise from unspecific binding, the membrane was prehybridised with Church buffer (1% BSA, 1 mM EDTA, 0.5 M NaPO4 pH 7.2, 7% SDS) for at least 1 hour at 65°C. Labeling of the probe with P^{32} was performed using the Amersham Ready-To-Go DNA labelling beads kit (-dCTP) from GE Healthcare following manufacturer instructions. Purification of labelled probe was performed using illustra Microspin S-200 HR columns (GE Healthcare). The purified radioactive probe was denaturalized 5 minutes at 95°C and then placed for 2 minutes on ice. The prehybridized membrane was then incubated with the probe in fresh Church buffer overnight at 65°C in a rotating oven.

After incubation with the labelled probe, the membrane was washed 3 times for 15 minutes with 2x SSC buffer containing 0.1% SDS, and once for 15 minutes with 0.2x SCC buffer and 0.1% SDS. The membrane was sealed

and placed in a casette with a calcium tungstate screen. Radioactivity was measured and analyzed using ImageQuant software (GE Healthcare).

2.2.12 Generation of chimeric mice

Collection of blastocysts, ES injection in blastocysts, surgical transfer of injected blastocysts into pseudopregnant recipient females as well as first mating of chimeras was performed by the team of Dr. Ronald Naumann, Transgenic Core Facility, Max Planck Institute of Molecular Cell Biology and Genetics in Dresden, Germany.

Targeted R1 (129/OlaHsd genomic background) embryonic stem cells were injected in blastocysts and transferred into C57BL/6JOlaHsd foster mothers. Resulting chimeric animals from two different targeted ES clones (IH12 and IA10) were mated with C57BL/6JOlaHsd mice and germline transmission of the mutant allele was verified by PCR and Southern blot analysis of tail DNA from F1 offspring with agouti coat color. Heterozygotic mice were backcrossed at least 6 times with C57BL/6JOlaHsd, thereof at least one generation with C57BL/6JOlaHsd males to ensure transmission of wild type Y chromosome.

2.2.13 Dissection of embryos

Dissection of embryos was performed as elsewhere described (Shea and Geijsen, 2007). Female mice were mated and vaginal plug was checked every morning and designated as day 0,5 when positive *post coitum*. Pregnant mice were euthanized and the peritoneum was exposed doing an incision in the abdomen. The uterine horns were removed and placed in ice cold PBS. Residual maternal musucular tissue was removed and each embryo was separated by cutting between implantation sites. Embryos were analzyed using a stereomicroscope MZ 75 (Leica) and photographed with a camera PowerShot A95 (Canon).

Gene - Sense	Sequence
Clec12a-Fwd	CTGTATGCCCTTAATACACCTCCTGC
Clec12a-Rev wt	CCATGAACAATGAGGAGAGAAGCC
Clec12a-Rev ko	GGTGGGATTAGATAAATGCCTGC

Table 2.2: Oligonucleotides used for animal genotyping.

2.2.14 Animal genotyping

For genomic DNA extraction, a small piece of mouse tail was cut under sterile conditions and digested with Wizard SV Genomic DNA Purification System (Promega) following producer indications. For PCR amplification of DNA, 3 μl DNA sample were incubated with 2.5 μl 10x PCR buffer No. 1 (Roche), 3.5 μl dNTPs (2.5 mM each), 0.2 μl DNA enzyme mix (Roche), and 0.8 μl of each oligonucleotide (table 2.2) in a final volume of 25 μl completed with destilled H_2O.

Probes were incubated in a MyCycler thermal cycler (Biorad). The program consisted of one cycle of 2 minutes at 95°C, 40 cycles of 15 seconds at 95°C for denaturation, 30 seconds at 57°C for annealing and 90 seconds at 72°C for elongation, followed by 1 cycle of 5 minutes at 72°C and final hold at 4°C.

2.2.15 Isolation of primary cells and cell purification

Immune system cellular effectors can be found in multiple tissues, but they are mainly located in the immune organs: bone marrow, spleen, thymus and lymph nodes. In order to investigate the development of these under basal conditions, hematopoietic organs were surgically removed under sterile conditions. Single cell suspensions were harvested from each tissue and red blood cells were removed resuspending pellets in RBC lysis buffer (eBioscience) for 5 minutes at room temperature. The lysis was blocked with same amount of medium containing 5% FCS. Cell suspensions were centrifuged, resuspended in appropriate medium and cell concentration was finally adjusted to 10^6 cells/ml, unless otherwise indicated.

For B lymphocyte stimulation experiments (section 2.2.21), cells were isolated from thymus, spleen or lymph nodes under sterile conditions and red blood cells were depleted as described before. The cell suspensions were centrifuged 5 minutes at 4°C and supernatant was discarded. Cell pellets were washed in 10 ml/organ MACS buffer and centrifuged as before. After supernatant was discarded once more, pellets were resuspended at a concentration of 40 μl/10^7 cells in MACS buffer. The same amount of cells were incubated 15 minutes at 4°C in 10 μl of anti-$CD4^+$ T cell biotin-antibody cocktail (for T cells) or $B220^+$ B cell biotin-antibody (for B cells), inverting tubes carefully every 5 minutes. Afterwards, supplementary 30 μl MACS buffer and 20 μl anti-biotin MicroBeads were added and incubated for 20 minutes at 4°C inverting every 5 minutes. Further 10 ml MACS buffer were added and the cell suspension was centrifuged for 5 minutes at 4°C and 350 G. Supernatant was discarded and cell pellets were resuspended in 500 μl MACS buffer. Cell suspensions were placed on ice while preparing magnetic columns. LS columns (Miltenyi Biotec) were placed in magnet stand and equilibrated with 3 ml MACS buffer. Cell suspensions were transferred to the columns and flow-through was collected in 15 ml tubes. The columns were washed 3 times with 3 ml MACS buffer and cell suspensions collected were placed on ice for further analysis.

2.2.16 Bone marrow stem cell isolation

Cellular effectors of the immune system derive from pluripotent hematopoietic stem cells found in the bone marrow. Isolation of those and their culture in the presence of specific growth factors enables differentiation into several cell lineages for *in vitro* studies.

Femora and tibiae of mice were removed and rinsed with RPMI using a syringe and a 22-gauge needle. Collected bone marrow was passed through a 100 μm strainer and washed with fresh RPMI medium. For erythrocyte lysis, cells were incubated for 5 minutes with RBC lysis buffer (eBioscience) and blocked with RPMI containing 5% FCS. Afterwards, the cells

were washed centrifuging 5 minutes at 300 G and then resuspended in appropriate medium.

2.2.17 Macrophage differentiation

Bone marrow stem cells of one mouse were plated for 24 hours in DMEM supplemented with 5% FCS, 1% penicilin & streptomycin, 2 mM L-glutamine and 0,1% β-mercaptoethanol. Non-adherent cells were counted the next day and plated at a density of 5 x 10^5 cells/ml in a volume of supplemented DMEM with 10% LCCM corresponding to the format needed for stimulation. On day 4, same volume of fresh medium was added to the cells. On day 7, a sample of cells was collected and analyzed by flow cytometry in order to characterize the differentiation grade using anti-CD11c and anti-F4/80 antibodies.

2.2.18 Dendritic cell differentiation

Bone marrow stem cells were plated at a density of 1 x 10^6 cells/ml in RPMI supplemented with 10% FCS, 1% penicilin & streptomycin, 2 mM L-glutamine, 0,1% β-mercaptoethanol and 20 ng/ml GM-CSF. Ten ml of cell suspension were plated in a 10 cm Petri dish, and 10 ml fresh medium were added on day 4 after culture. On day 7, a sample of the cell suspension was collected and analyzed by flow cytometry in order to characterize the differentiation grade. For this purpose anti-CD11c and anti-MHCII antibodies were used.

2.2.19 *In vitro* stimulation of antigen presenting cells

Upon encounter with PAMPs or DAMPs, the answer of immune cells to these stimuli can be measured by the amount of cytokines they release. In order to investigate the response of APCs to TLR and Dectin-1 ligands *in vitro*, bone marrow derived APCs were generated and stimulated as follows.

Bone marrow derived macrophages were primed with 10μl IFN-γ the day before stimulation and incubated overnight at 37°C. The next day the medium was replaced by fresh DMEM and stimuli were added as follows: Pam3CSK4 0.01 μl/ml, LPS 50 ng/ml, CpG 10 nM, Zymosan 50 μl/ml, Curdlan 100 μl/ml, poly I:C 10 ng/ml, ssRNA40 1 ng/ml and R848 10 ng/ml. Cells were stimulated for the indicated times at 37°C. Supernatants were collected and used for cytokine production analysis. Bone marrow derived dendritic cells were stimulated for the indicated times at 37°C without previous priming.

2.2.20 Cytokine production analysis

The amount of secreted cytokines by stimulated dendritic cells or macrophages was measured by enzyme-linked immunosorbent assay (eBioscience, Becton Dickinson). Nunc MaxiSorp 96 well flat-bottom plates (eBioscience) were coated with capture antibody overnight at 4°C. The day after, plates were blocked with PBS buffer containing 10% FBS for 1 hour at room temperature. The plates were washed at least 3 times with PBS buffer containing 0.05% Tween-20 (pH 7.4). A duplex series of standard dilutions were used as reference, including blank samples. The samples from stimulated dendritic cells or macrophages were pipetted also in duplex form. The plates were incubated at least 2 hours at room temperature or alternatively overnight at 4°C. Plates were washed as before for at least 5 times. Finally, plates were incubated with the detecting antibody diluted in PBS buffer with 10% FBS for up to 2 hours at room temperature. Plates were washed as before for at least 7 times and the substrate solution was added. The incubation took place in the dark for 10 to 30 minutes, depending on the cytokine to detect. The reaction was stopped with 2 N H_2SO_4, and the optical density of the substrate in each well was measured. The results for samples of interest were extrapolated from the formula given by the results of the standard curve.

Alternatively, the beads-based array Mouse Th1/Th2 10plex Ready-to-Use FlowCytomix Multiplex Kit (Bender MedSystems GmbH) was used

according to the manufacturer instructions. The standard dilutions and samples were plated. A mixture of beads was added to all wells together with a biotin-conjugated mixture and plates were incubated protected from light for at least 2 hours at room temperature on a microplate shaker. The plates were washed twice with PBS buffer containing 1% BSA. After washing 50 μl streptavidin-PE solution were added to each well together with 100 μl PBS and 1% BSA. The plates were incubated in the dark for at least 1 hour at room temperature on a microplate shaker. The plates were washed twice as indicated before and finally standards and samples were collected for flow cytometry measurement.

2.2.21 Proliferation assay

Clonal expansion is the cell multiplication characteristic of T and B cells upon encounter with an antigen. Here two approaches were applied to verify the results.

For the DNA quantification approach, purified B cells were centrifuged 5 minutes at 4°C and 350 G, supernatant was discarded and pellets were resuspended in appropriate volume of RPMI medium with 5% FCS, 1% penicilin-streptomycin, 1% L-glutamin and 0,1% β-mercaptoethanol. Cells were placed in 96-well cell culture plates and stimulated with PMA (50 nM), LPS (1 mg/ml) or anti-CD40 (10 μg/ml) for 24 and 48 hours at 37°C. Non-purified samples and non-stained cells were used for parameter settings set-up and as negative control respectively. The CyQUANT Cell Proliferation Assay Kit (Invitrogen) was used for proliferation measurements. The CyQUANT dye was pre-mixed with cell-lysis buffer and added to each sample well at the desired time point. The microplates were incubated for 1 hour at 37°C, and measurement of fluorescence was done with a reader and filters for 480 nm excitation and 520 nm emission. A reference standard curve (created with the same purified cells) was used to convert sample fluorescence values into cell numbers. Measured values were normalized relative to stimulation

start. This dye intercalates with DNA, and using spectrometry techniques it is possible to relatively quantify the amount of DNA content in a sample.

2.2.22 *In vivo* immunizations

Active immunizations consist in the exposure of an organism to an immunogen. They permit the study of the response of the immune system to molecules of different nature. For our model, NP-ovalbumin was used as T-cell dependent immunogen, while TNP-ficoll served as T-cell independent immunogen.

Animals of matching sex and age were selected and were immunized subcutaneously or intraperitoneally with either 200 μg NP-OVA (Biosearch Technologies) and TiterMax (CytRx Corporation) or CpG as adjuvant, or with TNP-Ficoll (Biosearch Technologies). A water-in-oil emulsion was prepared with equal volumes of NP-OVA and TiterMax using a double hub needle and mixing both phases repeatedly times for several minutes.

2.2.23 Immunoglobulin sera levels determination

The levels of immunoglobulins detected in the blood indicate the maturation state of antibody-producing cells. They also reveal an activation of the immune system when elevated. At the contrary, levels below normal ranges can suggest an immature or defective cellular immune response.

For measuring antibody levels, retro-orbital blood samples were collected from anesthesized mice immediately before immunization and once every week for 21 days. Sera were obtained by centrifuging blood samples 30 minutes at 4°C and used directly for analysis or stored at -80°C. Basal sera levels of antibodies were determined by ELISA using the Mouse Isotyping kit (Rockland Inc.) as described in section 2.2.20. Sera levels of antibodies after *in vivo* immunization were performed also by ELISA. Here the plates were coated with NP-OVA or TNP-Ficoll instead.

2.2.24 Protein level determination via Western blotting

The protein content of cellular lysates was determined using the Bradford dye reagent. Bradford dye concentrate (Bio-Rad) was diluted 1:10 in double distilled H_2O. In a 96 well microplate, 1 μl of protein lysate was mixed with 99 μl of diluted Bradford reagent per well, using duplets for each sample. To generate the standard curve, a dilution series of BSA was prepared in the same buffer as the lysates. The absorbance was measured in a spectrophotometer set to 595 nm. Measured values corrected with a blank sample were used for mathematical determination of protein concentration.

Cells were collected after different stimulations and washed twice with cold PBS. Pellets were lysed with lysis buffer. Equal amounts of protein were first mixed with Laemmli buffer and boiled for 5 minutes at 95°C. The samples were separated by electrophoresis in an SDS gel and then transferred to nitrocellulose membranes. These membranes were blocked with skim milk and then incubated with each primary antibody overnight at 4°C under rotation. The blots were then washed and incubated with horseradish peroxidase-labeled secondary antibody. Pierce enhanced chemoluminescence Western Blotting Substrate (Thermo Scientific) was used to visualize binding, and enzyme activity was detected exposing membranes to CL-XPosure film (Thermo Scientific).

2.2.25 Flow cytometry analysis

Flow cytometry allows cell immunophenotyping by distinguishing cell size, granularity, and cell surface marker expression. For this, samples labeled with conjugated-antibodies flow in a fluid stream through a beam of light and laser beams of different wave length. The cell scatters these lights, which is then recognized by different detectors positioned behind filters that allow light within an specific range of wave length to pass through.

For flow cytometry analysis, harvested single cell suspensions were washed with FACS buffer (5% FCS in PBS) and first blocked with anti-

CD16/CD32 antibody to avoid background staining by other detection antibodies via their Fc portions to the Fc receptors. After 5 to 10 minutes incubation at 4°C, samples were washed again with cold FACS buffer and incubated with a mixture of the antibodies of interest for 20 minutes at 4°C. The clinical flow cytometers used were FACS Calibur or FACS Canto II (Becton Dickinson).

2.2.26 Sterile inflammation *in vivo* models

In vivo assays of sterile inflammation were performed with Dr. Konstantin Neumann after his identification of the ligand for Clec12a. Briefly, in order to simulate an sterile inflammation process *in vivo*, freeze-thawed necrotic kidney cells from syngeneic mice or 150 μl PBS were injected into the peritoneum of 8- to 12-weel-old wild type and Clec12a-deficient mice. After 14 hours, the mice were euthanized and the peritoneal cellular content was counted and analyzed by flow cytometry.

In a more physiological approach, mice were sublethally X-ray-irradiated with 1 Gy as elsewhere described (Iyoda et al., 2005) in order to induce selective cell death of double positive thymocytes. After 14 hours, the mice were euthanized and the thymus were removed for cell count and analysis by flow cytometry or alternatively for RNA extraction.

2.2.27 Statistical analysis

Experiments were performed at least three times and graphics show mean values with standard deviation, unless otherwise indicated. Statistical significance, considered as $p<0.05$ (indicated by a single asterisk, or double asteriks when $p<0.01$), was studied using the unpaired two-tailed Student's t test.

2.2.28 Software

Vector and primer design was performed using VectorNTI (Invitrogen) and Gene Construction Kit (Textco BioSoftware, Inc.). Sequence analysis and

alignments were performed with VectorNTI, BLAST (U.S. National Library of Medicine) or ClustalW (European Molecular Biology Laboratory) software. Flow cytometry data were acquired using CellQuest (Becton Dickinson) and analyzed with FlowJo (Tree Star Inc.) software. Bead-based array results were evaluated using FlowCytomix Pro 3.0 software (Bender MedSystems GmbH). Statistics were performed using Office Excel (Microsoft) or GraphPad Prism 5 (GraphPad Inc.).

Image editing was performed with Photoshop CS2 and Illustrator CS2 (Adobe Systems Inc.). The manuscript was generated using TEXnicCenter editor and JabRef v2.5 for bibliography management.

Chapter 3

Results

3.1 Expression profile of C-type lectins

A novel signaling pathway downstream of the C-type lectin Dectin-1 has been previously described which involves the adaptor molecule Card9, a key role player in NF-κB signaling (Gross et al., 2006). The gene coding for Dectin-1 is located in the NK gene cluster. Other genes coding for not well characterized proteins lie within the same cluster, very proximate to the Dectin-1 *locus*. A search in the Ensembl browser identified these genes as other related C-type lectins, including Clec2, Clec9a, Clec12a and Clec12b. In order to investigate the transcription levels of these genes in the immune system, a RT-PCR was performed using mRNA from tissues or purified cell types from C57Bl6/J animals.

To ensure equivalent loads of the samples, the housekeeping gene β-actin was used as a control. The RT-PCR analysis shows a wide transcription range for most C-type lectins. Dectin-1 transcription has been detected in thymus, spleen, lymph nodes and bone marrow, and also in liver and slightly in brain. Dectin-1 has also been detected in B cells, neutrophils, dendritic cells and macrophages, but not in T cells nor natural killer cells. Clec2, Clec9 and Clec12a share a very similar expression, with detection in thymus spleen, bone marrow and liver, while only Clec2 is expressed in lymph nodes.

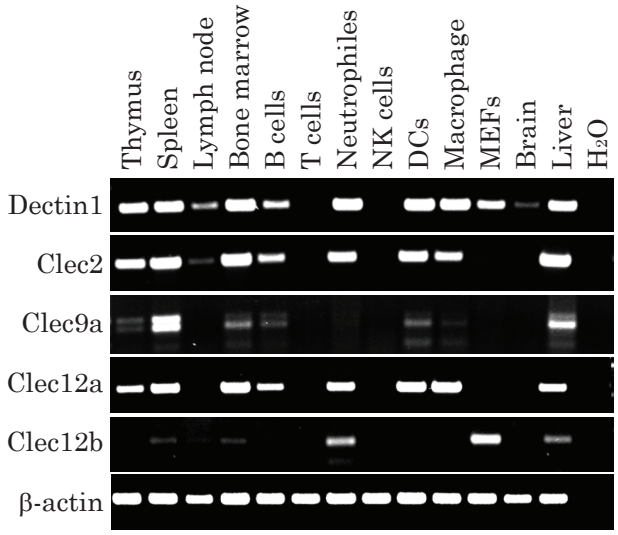

Figure 3.1: RT-PCR screening of C-type lectins. mRNA was obtained from tissues or purified cell types in order to generate cDNA. RT-PCR was performed with specific primers for each C-type lectin located close to Dectin-1 in the NK gene cluster.

Furthermore, these genes are transcribed in B cells, neutrophils, dendritic cells and macrophages. Clec12b shows a different pattern of expression when compared to the other C-type lectins. Very slight expression of Clec12b was detected in spleen and bone marrow, and stronger in neutrophil granulocytes and murine embryonic fibroblasts.

3.2 Gene targeting of Clec12a

In order to generate a Clec12a-deficient mouse model, a targeting vector was used with a short arm carrying the identical sequence before the 5' end of the *Clec12a* gene, and a 5 Kb long arm with the sequence from the 3' end of *Clec12a* second exon and including exons 4 and 5, with a neomycin casette in inverted transcription direction between short and long arm (figure 3.2-A). Through homologous recombination, the two first exons of the *Clec12a* gene are replaced by the neomycin resistance gene, which disrupts the reading frame therefore impeding transcription.

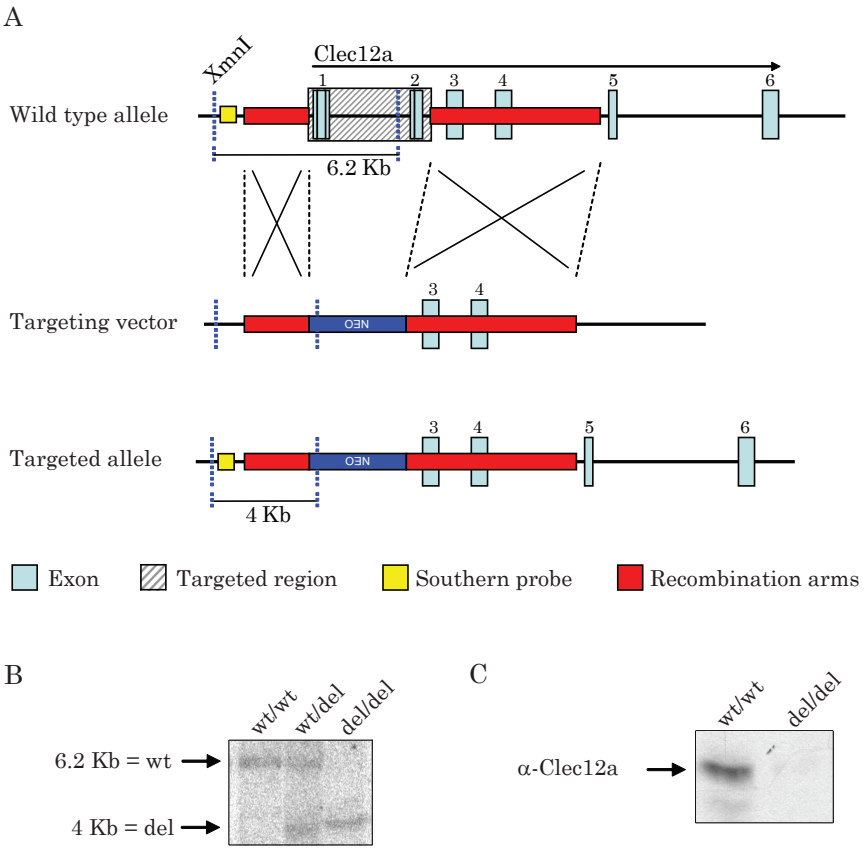

Figure 3.2: Generation of a Clec12a-deficient mouse model. A) Targeting strategy for generating Clec12a-deficient mice. Dark blue dotted line represents XmnI restriction sites. B) Confirmation of site specific recombination was confirmed by Southern blot using XmnI digested genomic DNA from bone marrow. C) Protein expression was confirmed by Western blot using lysates from bone marrow and an antibody specific against the extracellular domain of Clec12a.

The replacement by the NEO casette deletes the wild type XmnI restriction site, placing an inherent XmnI restriction site at a different position in the genome. This permitted identification of specific recombination using a probe at the 5' end of the gene. For this, genomic DNA from targeted embryonic stem cell clones was extracted, digested with the XmnI restriction enzyme and then probed by Southern blot, were the detection of different lengths indicates that appropiate recombination has taken place. Out of 2400 neomycin-resistant clones, 14 turned specific for the desired recombination, resulting in a recombination rate of 0,58%. Germline transmission was obtained from two independent clones, for which specific targeting was assessed again via Southern blot using genomic DNA extracted from the bone marrow of wild type and Clec12a-deficient animals (figure 3.2-B).

In order to confirm that the rest of the gene could not be expressed, Western blot was performed to ensure that Clec12a protein production was abolished. For this purpose, lysates from bone marrow cells were used and probed with an antibody specific against the extracellular domain of the Clec12a protein, coded by exons 4, 5 and 6 of the gene. Figure 3.2-C shows that Clec12a was not detected in the Clec12a-deficient mice. Interestingly, in the Western blot is it possible to see two bands for Clec12a in the wild type sample, which suspectedly correspond to the alternative splicing during *Clec12a* gene expression.

3.3 Clec12a might play a role during embryonic development

The targeting of *Clec12a* takes place in one allele, thereby generating heterozygotic mice. Interbreedings of these animals generated less than 13% homozygotic mice for the deletion, as shown in figure 3.3-A. Following mendelian ratios, approximately 25% homozygotic mice should be born, therefore we investigated whether there were any recognizable defects during development in Clec12a-deficient embryos. Dissection of 12.5 dpc embryos revealed a neu-

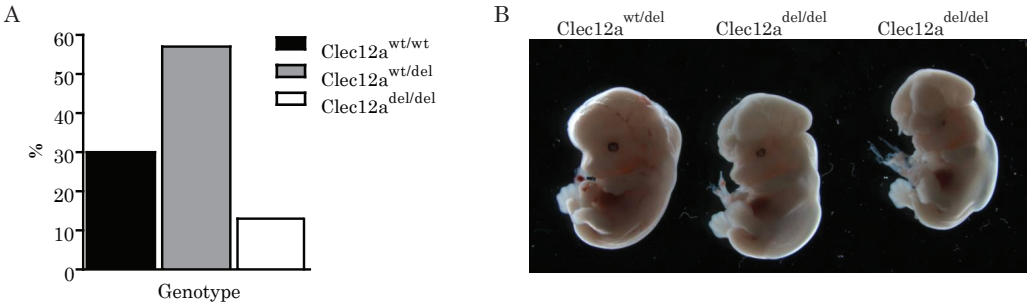

Figure 3.3: Clec12a might play a role during embryonic development. A) Heterozygotic crossings of animals generated from two different targeted stem cell clones give raise to 13% homozygotic mice (clone IH12 n=266; clone IA10 n=226). B) Three different embryos were dissected at an age of 12.5 dpc and PCR genotyped using a piece of the tail.

ral tube closure defect in some of the homozygotic specimens, as shown in figure 3.3-B. However, about 13% homozygotic embryos could be obtained from two independent embryonic stem cell clones succesfully targeted (IA10 and IH12), which were apparently healthy and did not show obvious phenotypic abnormalities.

3.4 Normal cellular development of the immune system

Approximately two thirds of the Clec12a deficient embryos develope normally into healthy newborns. Those animals do not present major abnormalities and have a normal lifespan compared to their littermates: both wild type and Clec12a-deficient mice showed no signs of disease along 24 months of observation. Due to the expression of Clec12a in immune cell subsets detected via RT-PCR it was first investigated whether Clec12a could be involved in immune system development. First of all, mice were dissected and no gross abnormality was encountered in organ development. Regarding the lymphoid system, thymus, lymph nodes and spleen were collected and total cell count was evaluated.

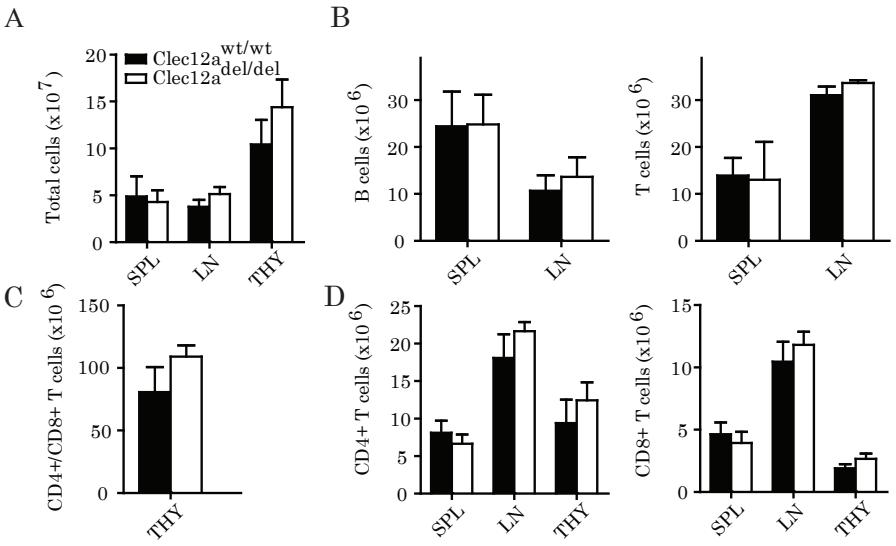

Figure 3.4: Normal cellularity in lymphoid organs. A) Organs from 8-12 week old wild type (n=3) or Clec12a-deficient mice (n=3) were collected and cellularity was evaluated by total cell counting. B) Cell type from lymphoid organs was studied by flow cytometry using B220, CD3 and TCRβ antibodies; percentages of positive surface marker expression were used to calculate absolute cell number. C) CD4 and CD8 antibodies were used to quantify the double positive T cells in the thymus, and D) CD4 and CD8 T cell subsets in other lymhpoid organs.

Proliferative diseases of the hematological system can initially manifest themselves through altered cellular counts. There were no significant differences between wild type and Clec12a-deficient mice in the total amount of cells in the thymus, the lymph nodes nor in the spleen (figure 3.4-A). Also B and T cell compartment showed no difference in absolute numbers between wild type and Clec12a-deficient lymph nodes and spleens (figure 3.4-B), nor in their relative distribution. T cells develop in the thymus into CD4 or CD8 T cells along several selection steps. First, progenitors turn into double CD4 and CD8 expressing cells, to later on express only one of these two surface proteins. Double positive cells in the thymus (figure 3.4-C) as well as CD4 and CD8 compartments in thymus, lymph nodes and spleen in Clec12a deficient animals were similar to wild type specimens.

3.5 Normal distribution, differentiation and activation status of T cell subsets

The effector cells in the adaptive immune system are the lymphoid B and T cell lineages. Flow cytometry confirmed the normal distribution of B and T cells in lymph nodes and spleen of Clec12a-deficient mice, using the surface markers B220 and TCRβ for B and T lymphocytes respectively (figure 3.5). Also a similar distribution of the CD4 and CD8 T cell subsets in thymus, lymph nodes and spleen among wild type and Clec12a-deficient animals was found (figure 3.6-A), with some minimal but not significant differences (i.e. lymph nodes).

However, knowing that Clec12a might act as a negative immune modulator, the activation status of each subset was investigated. For this, gated lymphocytes were analyzed for expression of known T cell activation markers. Upon activation, T cells show an apparent downregulation of CD4 or CD8 (depending on the subset) and TCRβ surface molecules. The first is redistributed through increased cellular size (detected by the forward scatter), while the latter is physiologically internalized to prevent further stimulation.

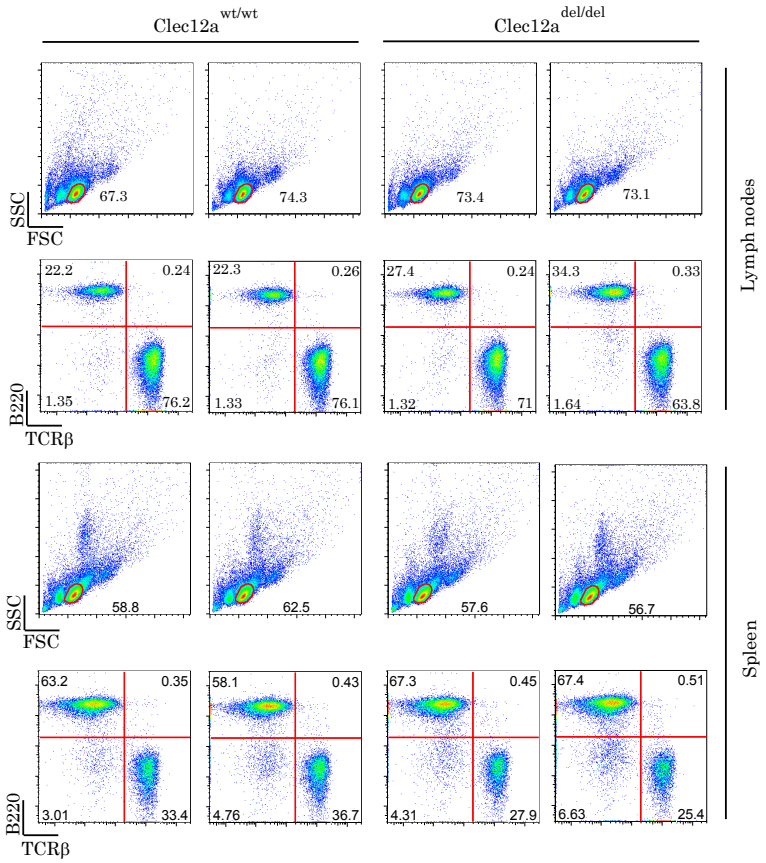

Figure 3.5: Normal distribution of B and T cell subsets. Single cell suspension of lymph nodes and spleen was incubated with fluorochrome-labelled antibodies against TCRβ and B220. Each column represents one 8-12 week old animal (wild type n=2, Clec12a-deficient n=2). For the indicated organ, upper panels show forward against side scatter for live/dead cell discrimination. Numbers indicate percentage of events within the gate. Lower panels show cells expressing TCRβ against B220 surface marker; numbers for each quadrant indicate percentage of total events within the viable cell gate. This analysis is representative of >3 experiments.

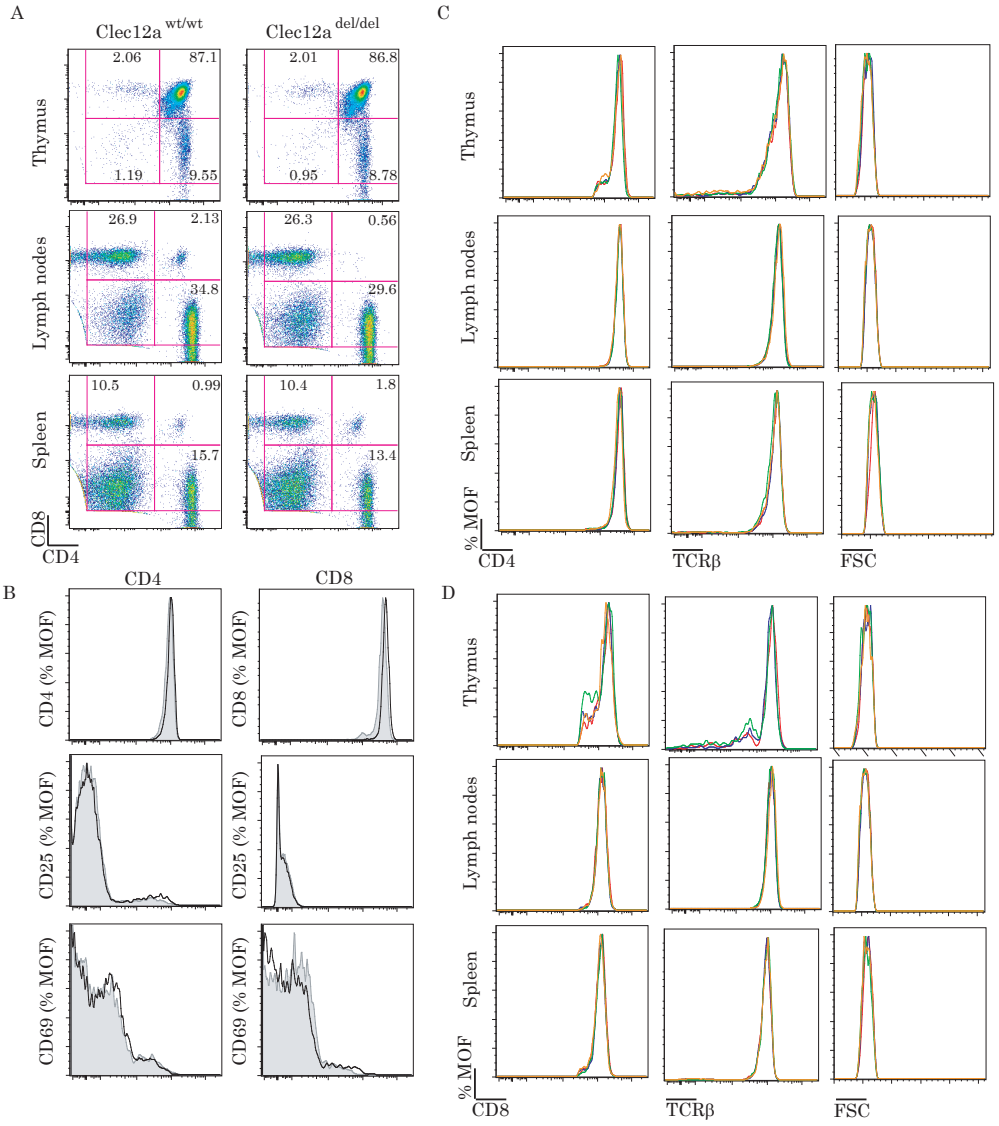

Figure 3.6: Differentiation into CD4 and CD8 T cell subsets. A) Single cell suspensions from lymphoid organs were analyzed for CD4 and CD8 expression. Each column represents one animal. B) The histograms show expression profile of the CD4 subset in the left column and CD8 in the right column, and the activation markers CD25 and CD69 (wild type as grey area, Clec12a-deficient as a black line). TCRβ expression and cellular size are ploted in histograms for the CD4 (C) and CD8 (D) subset, including expression of CD4 and CD8 surface marker respectively (wild type n=2 as red and blue lines; Clec12a-deficient mice n=2 as green and orange lines). This analysis is representative of 3 experiments.

Therefore shifts of these markers to the left in the flow cytometry pannels would indicate cell activation. In contrast, CD25 and especially CD69 are proteins expressed during early phases of T cell activation. Shifts to the right would indicate cell activation. None of these changes were observed among wild type or Clec12a-deficient animals in thymus, lymph nodes nor in the spleen (figures 3.6-B to D).

3.6 Normal B cell maturation, differentiation, activation status and proliferation

As shown in figure 3.1, Clec12a was detected in murine B lymphocytes. Therefore we asked whether lack of Clec12a could play a role in B cell differentiation and maturation. As shown before, total counts of B cells were similar among wild type and Clec12a-deficient mice. Since IgM is specific for immature B cells and IgD is only expressed once maturation has been initiated, we used these two surface markers to analyse B cell maturation. Although Clec12a-deficient B lymphocytes showed lower IgD expression levels, which could indicate a more immature phenotype, generally this difference was not statistically significant (figure 3.7).

B lymphocytes differentiate also into distinct subsets with their own characteristics. Regarding their localization (among other features), marginal zone and follicular B cells can be distinguished by the expression of CD21 and CD23 surface markers respectively. Regarding these two subsets, no quantitative differences were found among wild type and Clec12a-deficient mice, as shown in figure 3.8-A.

Upon activation, B lymphocytes undergo several cytological changes that can be detected. For instance, activated B cells increase in size, what can be measured by the forward scatter of a flow cytometer. Also the expression of surface cellular markers changes. The expression of CD62L diminishes, promoting the detachment of the cells and its consequent migration through the body to sites of infection or inflammation. In contrast the expression of

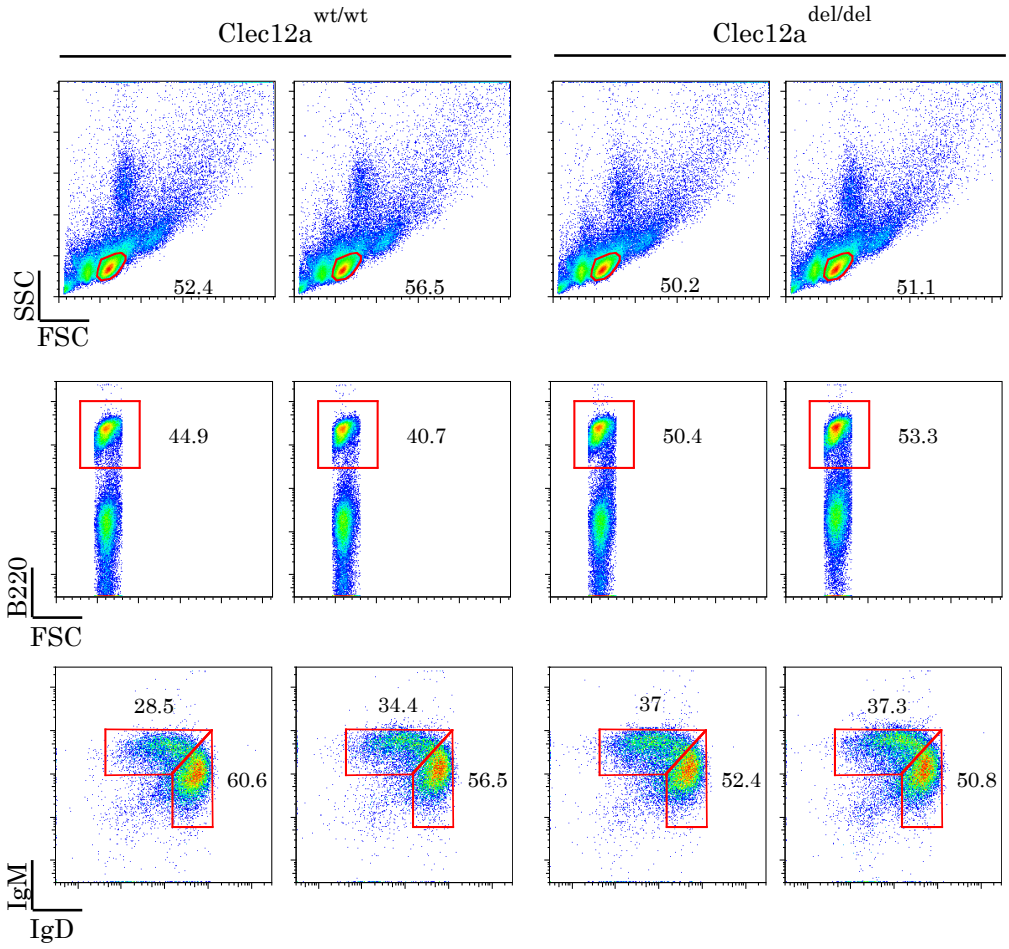

Figure 3.7: Normal B cell maturation and activation status. Groups of 2 or 3 animals of same sex and age were analysed: spleens from 8 to 12 weeks old mice were analyzed by flow cytometry. Viable B lymphocytes were gated using forward and side scatters (higher panels). $B220^+$ cells were gated (medium panels) and maturation status was evaluated via expression of IgM and IgD (lower panels). This analysis is representative of >3 experiments.

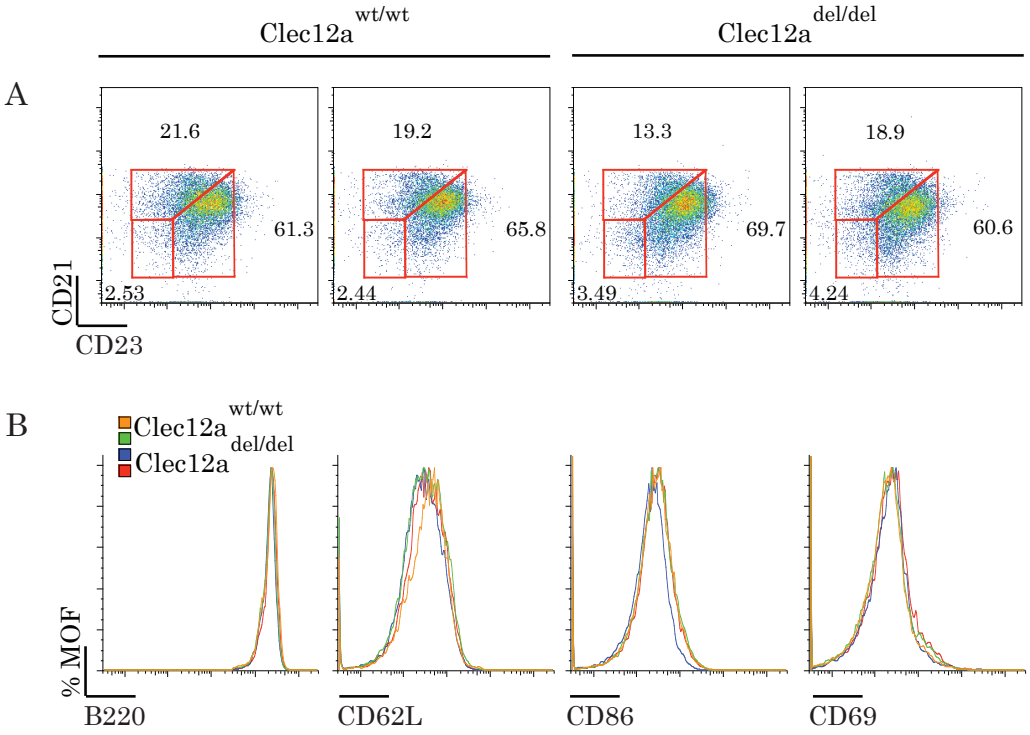

Figure 3.8: Distribution of follicular and marginal zone B cells and activation status. A) B220 expressing cells in the spleen were gated and CD21 and CD23 plotted in order to distinguish follicular from marginal zone B cells. Each panel represents one animal, the experiment is representative of >3 analysis. B) B220 expressing cells were plotted in an histogram (% maximum of fluorescence) against B220 surface marker, CD62L, CD86 and CD69 activation markers (wt = red and blue lines; ko = green and orange lines).

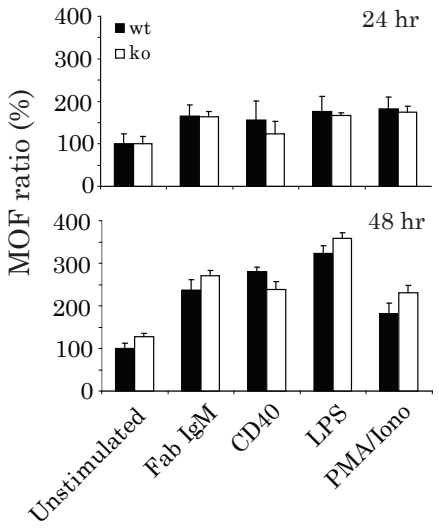

Figure 3.9: B cell proliferation. Clonal expansion was quantified via CyQuant Proliferation assay. Purified B cells were incubated with Fab IgM (10 mg/ml), antibody CD40 specific (10 mg/ml), LPS (10 mg/ml) and PMA/Ionomycin (10 nM) for 24 (upper panel) or 48 hours (lower panel). CyQuant dye and repressor were added and incubated for 1 hour at 37°C. Fluorescence was measured and proportional mean of fluorescence (MOF) values compared to unstimulated cells are plotted. Black bars represent wild type animals, empty bars represent Clec12a-deficient mice. This analysis is representative of 2 experiments.

co-activator molecules such as CD86 is enhanced. Analysis of these markers didn't reveal any difference in the basal activation status among B cells from wild type or Clec12a-deficient mouse (figure 3.8-B).

Clonal expansion is a key component of the humoral immune response and is needed for a high and rapid production of sufficient antibody levels. B cell proliferation can be measured using different approaches. Here measurement of intracellular DNA content was used to determinate cellular proliferation. Using a dye that binds DNA, this technique confirmed that the lack of Clec12a did not influence the proliferation of B cells upon stimulation with LPS, CD40-L, Fab IgM or PMA-ionomycin (a combination that activates the protein kinase C and mobilizes Ca^{+2} through the plasmatic membrane, stimulating intracellular signal transduction), as shown in figure 3.9).

3.7 Normal basal levels of immunoglobulins and humoral responses

Due to the suspected inhibitory function of Clec12a, we wanted to test whether lack of our protein of interest could lead to an above-average ac-

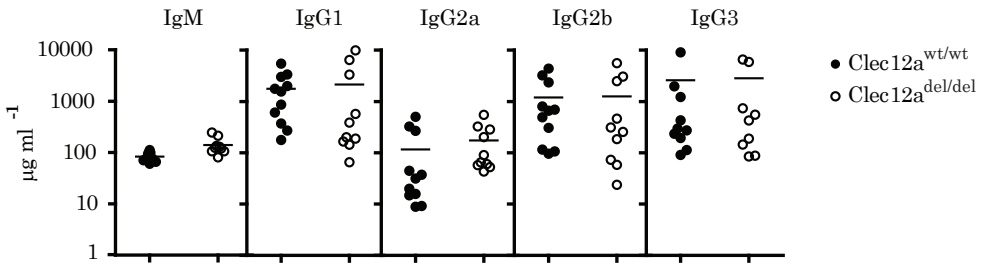

Figure 3.10: Basal serum levels of immunoglobulins. Basal immunoglobulin isotype levels in serum were determined by ELISA in 8-12 week old wild type (filled circles, n=11) and Clec12a-deficient (open circles, n=10) mice. These results are representative of 3 experiments.

tivation status of B cells. In order to confirm this hypothesis, basal levels of different immunoglobulins in the serum of wild type and Clec12a-deficient animals were messured (figure 3.10). Enzyme-linked immunosorbent assays against IgM and subtypes of IgG showed no quantitave differences in immunoglobulin content in the sera of wild type and Clec12a-deficient animals. Although basal immunoglobulins levels appeared to be normal, we asked whether Clec12a could be involved in the development of proper humoral responses. To investigate this, *in vivo* immunizations with NP-ovalbumin as T cell dependent and with TNP-Ficoll as independent antigens were performed. No significant differences were observed among both groups (figure 3.11).

3.8 Normal innate immune cell differentiation

Extracellular signals are crucial for cell differentiation. *In vitro* differentiation of bone marrow-derived macrophages and dendritic cells can be achieved using specific molecules in the culture medium. The presence of LCCM drives the differentiation of bone marrow stem cells into a homogeneous population of macrophages, while GM-CSF drives pluripotential stem cells towards the myeloid dendritic cell lineage with a purity of almost 70%.

The differentiation of macrophages and dendritic cells can be detected via relatively high expression levels of MHCII surface molecule using flow

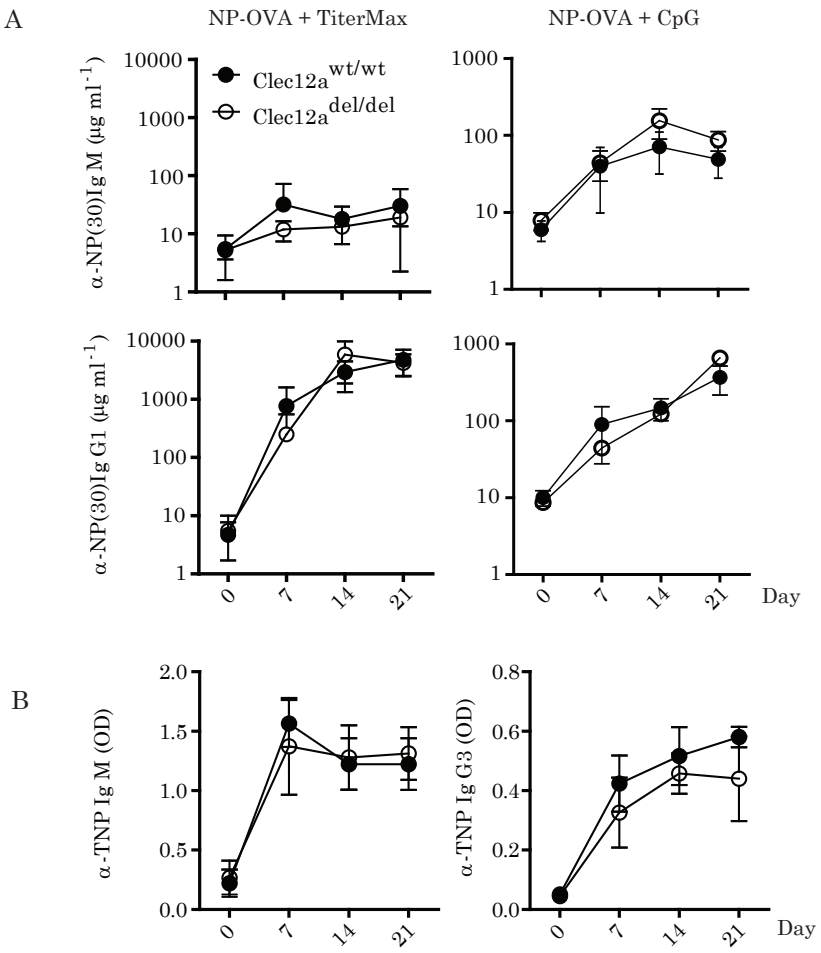

Figure 3.11: ***In vivo*** **immunizations**. A) Normal humoral response and isotype switching in Clec12a-deficient mice is shown. Mice were immunized with the T cell dependent antigen NP-ovalbumin and the adjuvants CpG (wt n=3, ko n=3) or TiterMax (wt n=7, ko n=8), and specific anti-NP immunoglobulin levels in serum were determined by ELISA in 8-12 week old mice. B) Normal humoral response and isotype switching in Clec12a-deficient mice when immunized with the T cell independent antigen TNP-Ficoll (wt n=3, ko n=3). These results are representative of 3 experiments.

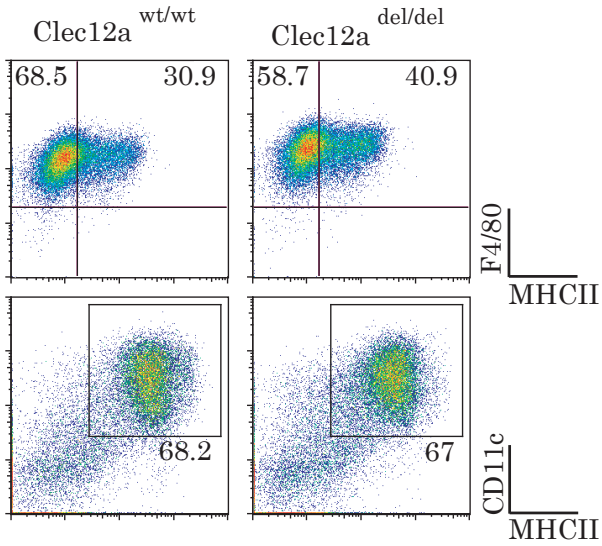

Figure 3.12: Differentiation into antigen presenting cells. Bone marrow stem cells were cultured with LCCM (upper panel) or GM-CSF (lower panel) for 7 days and surface marker expression was analyzed via flow cytometry with antibodies against F4/80 and MHCII for macrophages and with CD11c and MHCII for dendritic cells. These results are representative of >3 experiments.

cytometry. While macrophages especifically express the marker F4/80, dendritic cells can be identified by the presence of CD11c in the cellular surface. Culture of bone marrow-derived stem cells in the presence of these growth factors generated similar levels of differentiation independently of the origin of the stem cells (figure 3.12).

3.9 Cytokine production of APCs upon PRR stimulation

C-type lectins can function as *bona fide* pathogen recognition receptors in a similar way to Toll-like receptors. They could also modulate TLR activation, since it has been shown that cross-talk among both families occurs in some cases. Therefore we tested whether Clec12a could be involved in the detection of or in the response modulation to any ligand known for CLRs or TLRs. For

this purpose, *in vitro* bone marrow derived macrophages and dendritic cells were stimulated with a palette of TLRs and CLRs ligands. Cytokine release is a good tool to quantitavely measure how efficiently an antigen triggers the innate immune response in APCs. From eleven different cytokines tested (IFNγ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-17, TNFα and GM-CSF), no significant differences were found among both animal groups (figure 3.13).

Since Clec12a is supposed to be an inhibitory receptor, it could be hypothesized that its function is to counteract with activating receptors such as TLRs or CLRs. These receptors signal through different signaling cascades involving MAP kinases or the NFκB signaling pathway, therefore we investigated the phosphorilation status of several kinases upon stimulation with PAMPs. No differences have been observed at different time points, and delayed kinetics could also be excluded (figure 3.14).

3.10 Function of Clec12a in dead cell sensing

While this work was ongoing, Dr. Konstantin Neumann (postdoctoral researcher at Prof. Ruland's group) used biochemical and *in vitro* cellular assays to identify potential ligands for Clec12a. He could demonstrate that both murine and human Clec12a protein bind to dead cells from different organs, with highest levels of binding found for kidney tissue (data not shown).

Non-physiological cell death is known to be a rich source of DAMPs which can trigger a local sterile inflammatory response. The influx of neutrophils (the first cell type to be recruited to the inflammatory site) to the injury site serves as an indicator of this initial immune response.

According to these data, the role of Clec12a in the context of sterile inflammation was investigated *in vivo*. For this purpose dead cells or PBS were injected in the peritoneum of wild type and Clec12a-deficient mice. Clec12a-deficient mice exhibited a greater recruitment of neutrophils into the peritoneum when injected with dead cells in comparison to the wild type

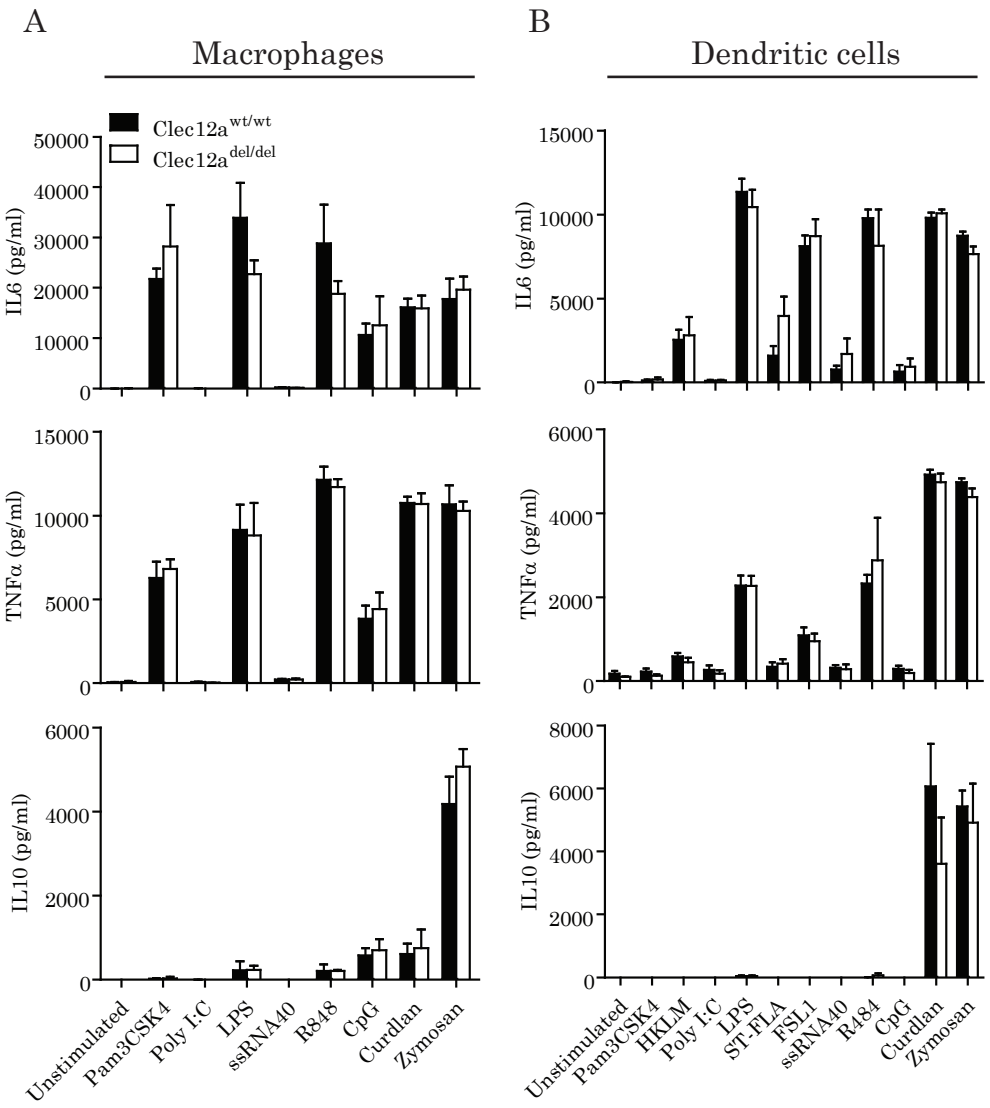

Figure 3.13: Stimulation of APCs with TLR and Dectin stimuli. A) Bone marrow derived macrophages were primed for at least 4 hours with IFNg and then stimulated with a TLR and Dectin stimuli palette for 24 hours. Supernatants were collected and cytokines were measured by ELISA and bead-based array (wt=filled bars, ko=empty bars). B) Bone marrow derived dendritic cells were stimulated with the same stimuli palette and cytokine concentration was assessed by ELISA and bead-based array (wt=filled bars, ko=empty bars). Results are representative of at least 3 experiments and standard deviations show mean values of triplets.

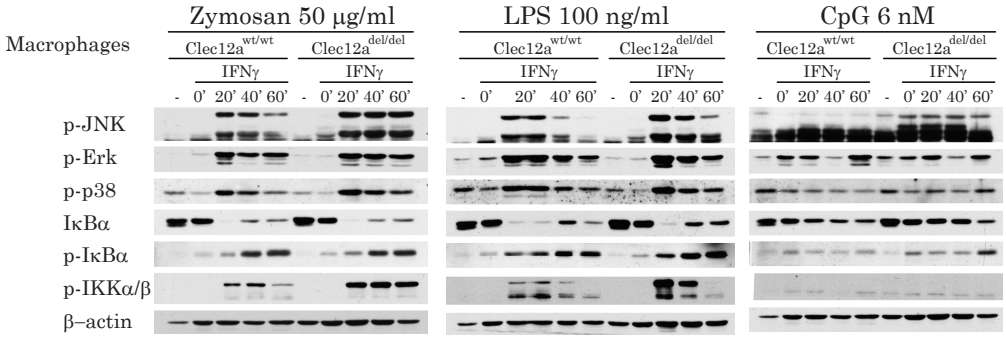

Figure 3.14: MAPK cascade activation after TLR stimulation of APCs. Bone marrow derived macrophages were generated by 7 days culture with LCCM. Differentation was ensured via flow cytometry and cells were primed (-, without prime) with IFNγ for at least 4 hours and then stimulated with TLR or Dectin stimuli for the indicated times. Total cells lysates were used for protein extraction and probed for the indicated proteins (p=phosphorylated).

animals (figure 3.15-A). To simulate dead cell-induced sterile inflammation *in vivo*, animals were X-ray-irradiated to induce the selective killing of $CD4^+$-$CD8^+$ thymocytes. Also here Clec12a-deficient animals showed an exacerbated recruitment of neutrophils into the thymus (figure 3.15-B). Finally, the expression of the neutrophil-recruiting chemoattractants CXCL1, CXCL10 and TNFα was compared among irradiated wild type and Clec12a-deficient mice. Also here the expression was increased in the group of Clec12a-deficient animals (figure 3.15-C).

All these data demonstrate that Clec12a is an important negative regulator of sterile inflammatory responses to cell death *in vivo*.

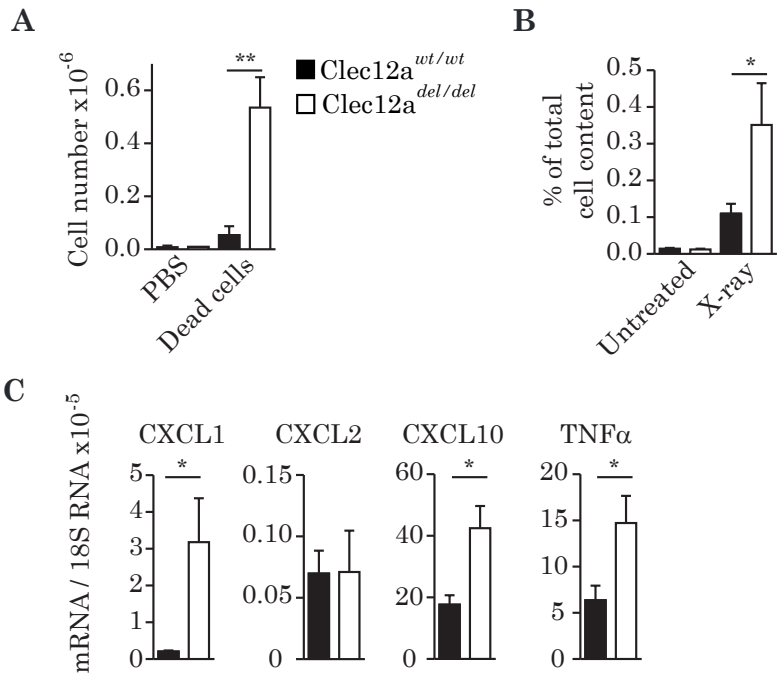

Figure 3.15: Function of Clec12a in dead cell sensing. A) Wild type (n=5) and Clec12a-deficient mice (n=5) were injected intraperitoneally with PBS or freeze-thawed kidney cells from syngeneic mice. After 14 hours, CD11b^{+}Gr-1^{high} neutrophils recruited into the peritoneum were measured by flow cytometry. B) Both wild type and Clec12a-deficient mice (n=6 per group) were irradiated with 1 Gy of X-rays. After 14 hours, CD11b^{+}Gr-1^{high} neutrophils recruited in the thymus were analyzed by flow cytometry. C) Thymus RNA from irradiated mice from the previous experiment was isolated and quantified by q-PCR. Levels of the indicated cytokines were normalized with 18S RNA. For all diagrams, black bars represent wild type animals and empty bars indicate Clec12a-deficient mice. The results are representative of two independent experiments.

Chapter 4

Discussion

4.1 Role of Clec12a in embryonic development

The first observation made after germ line transmission of the Clec12a targeted gene was the reduced number of viable Clec12a-deficient animals. In contrast to the 25% expected homozygotic offspring, less than 13% Clec12a-deficient mice were born. This fact might indicate that the function of Clec12a is at least up to some extent required for developmental processes. Interestingly, some of the Clec12a-deficient embryos showed exencephaly, a lethal type of neural tube closure defect during embryonic development.

The neural tube is a structure present during early embryonic developmental stages that gives places to the brain and the spinal cord. It originates from the neural groove, a depression in the neural plate, and it starts folding and closing centrally in both cranial and caudal directions, leaving the extremes open which are called the anterior and posterior neuropores. These orifices also close after certain time, but defects in these processes lead to neural tube closure defects that derive in malformations of the central nervous system, very often with lethal consequences for the embryos. Up to date, more than two hundred mutant mouse models with neural tube defects at different penetrance grades have been identified (Harris and Juriloff, 2007, 2010). However, the exact mechanisms why specific genes are indis-

pensable during embryogenesis remain mostly unknown, as this is the case of Clec12a. Also whether compensations mechanisms by other proteins could explain why some embryos survive despite the lack of Clec12a needs to be clarified. Further studies in this direction are required to understand the role of Clec12a at early embryonic stages.

Interestingly, another C-type lectin appears to be indispensable for prenatal development. Clec-2-deficient mice are lethal due to abnormal development of lymphoid vessels at embryonic stages (Suzuki-Inoue et al., 2010). The natural ligands of Clec-2 are the snake venom rhodocytin and the endogenous podoplanin (Suzuki-Inoue et al., 2006), a molecule expressed in glomerular podocytes, lymphatic endothelial cells, fibroblastic reticular cells or in several cancer cell types, just to cite some examples (Astarita et al., 2012). Recently, it has been shown how the interaction between the podoplanin expressed in fibroblastic reticular cells (a stromal cell type responsible for the maintenance of the extracellular matrix scaffold in lymph nodes) and its receptor Clec-2 expressed in platelets is required for vascular integrity (Herzog et al., 2013). During inflammatory responses, a flux of lymphocytes from the blood vessels towards the T zone of the lymph nodes occurs to be activated by antigens. This migration takes place through the high endothelial venules, which are specialized postcapillary vessels. The interaction of podoplanin and Clec-2 in this interface is responsible for the release of sphingosine-1-phosphate by platelets, which in turn enhances the expression of VE-cadherin, a protein involved in intercellular junctions and which defect is known to generate interstitial edema and hemorrhage (Herzog et al., 2013). Apparently, these interactions and cellular responses are required to preserve vascular integrity during the increased efflux of lymphocytes through the vessels. Using chimeric mouse models, it has been shown that the interaction of podoplanin with Clec-2 is also critical for the maintenance of vascular integrity at sites of inflammation in an immune complex-induced inflammation model in the skin and also in an LPS-induced inflammation model in the lung (Boulaftali et al., 2013). In these models, mice transfused with Clec-2-deficient platelets

presented hemorrhage at the sites of inflammation. All this data indicate a non-immune role for Clec2 that appears to be essential already during development.

On the other hand, other CLR-deficient mouse models haven't shown survival defects during embryogenesis nor major abnormalities after birth. For example, Dectin-1 deficient animals were viable, and as in the case of Clec12a, they didn't show any gross abnormality or altered peripheral leukocyte counts (Taylor et al., 2007). Clec9a-deficient mice developed normally and showed no alterations in the immune cell populations, however no data has been published about the genotype rates obtained after crossing heterozygotic animals (Sancho et al., 2009). Also mice deficient in Clec4a2, an ITIM-bearing receptor of the Dectin-2 cluster, are born healthy at the expected mendelian ratios (Fujikado et al., 2008).

4.2 Role of Clec12a in the immune system

4.2.1 Clec12a in immune cell development

The lack of Clec12a did not affect the development and maturation of immune cell populations. Exhaustive analysis of the different cell populations in primary lymphoid organs, in the spleen and the lymph nodes did not show significant differences in the cellular number nor in surface marker profiles. Also *in vitro* differentiation of bone marrow-derived macrophages and dendritic cells was not altered by the absence of Clec12a.

Interestingly, the C-type lectin Dcir (Clec4a2), which is also an ITIM-bearing receptor, has been shown to be involved in the homeostasis of dendritic cell and $CD4^+$-T cell development. Mice deficient in Clec4a2 exhibited increased numbers of dendritic cells in the lymph nodes and of activated $CD4^+$-$CD62L^{low}$ T cells in aged mice, while the CD8 T cell subset remained unaffected. Also levels of autoantibodies were significantly elevated in Clec4a2-deficient mice (Fujikado et al., 2008). These data indicate that Clec4a2 is a key player in the maintenance of the developmental home-

ostasis of different immune cell types and its lack derives in the late-onset development of autoimmune diseases.

4.2.2 Clec12a is dispensable for TLR modulation after selective TLR stimulation

It is known that different TLRs can cooperate to enhance an immune response. For instance, synergistic stimulation of TLR4 and TLR7 leads to a significant increased production of IL-1β and IL12-p70 (Napolitani et al., 2005). Parallel activation of TLR2 and TLR4 in mouse macrophages leads to higher levels of secreted IL-6 and TNFα than by independent stimulation (Bagchi et al., 2007). There are also several examples of TLR/NLR synergy. For instance the co-activation of NOD1 and TLR2 in human monocytes which induces TNFα, while activation of NOD1 alone produced minimal levels of this cytokine (van Heel et al., 2005). Also the C-type lectin Dectin-1 has been shown to synergize with TLRs for inflammatory gene regulation, but not for phagocytosis or ROS production (Gantner et al., 2003; Underhill, 2007). Since microbes might express different PAMPs, it is clear that only one microbe can be detected by several PRRs. In the last years it has become evident that a cross-talk among PRRs exists. However the mechanisms how these cooperations occur are not fully understood. Enhanced gene transcription due additive NFκB activation, different NFκB isoforms induced, activation of different MAPKs signaling cascades or alteration in activation kinetics might explain this cross-talk (Underhill, 2007).

On the other side, several PRRs can counteract their actions to regulate the immune response, and C-type lectins have an important role in this aspect. For instance Dectin-1 collaborates with TLR2 to induce enhanced IL-6, IL-10 and IL-23 cytokine production in murine DCs, while it down-regulates IL-12 relative to the levels produced after solely activation of TLR2 (Dennehy et al., 2009). Clec4c, a C-type lectin that couples with ITAM-bearing adaptor molecules, inhibits up-regulation of the co-stimulatory molecules CD86 and CD40 after TLR9 activation in plasmacytoid DCs (Jähn et al., 2010).

Interestingly, Dectin-1 and Clec4c are related to ITAMs and although a positive action is expected, they can also down-regulate specific pathways to shape the immune response.

All these data have shown that co-activation of PRRs leads to a cross-talk among the receptors responsible for immune response trimming. The results presented in this work however indicate that Clec12a does not recognize known TLR or Dectin-1 agonists nor modulates cytokine production after stimulation of those receptors with their respective ligands. However it is not possible to exclude that Clec12a could detect a different PAMP than the ones tested. On the other hand, it is well known that TLRs can detect also DAMPs (Chen and Nuñez, 2010). It is possible that Clec12a could cross-talk with other PRRs and modulate for instance TLR or CLR signaling *in vivo* upon detection of DAMPs released by necrotic cells, in the context of sterile inflammation or also in an infectious setting, since tissue damage and cell death are inherently linked to pathogenic invasion.

Furthermore, Clec12a could be involved in different cellular processes than inflammatory cytokine production upon stimulation with TLR or Dectin-1 ligands, i.e. APC maturation, migration or microbial killing.

4.2.3 Clec12a as a negative regulator of cell death sensing

Based in the biochemical and *in vitro* cellular data that suggest dead cells to harbour the ligand of Clec12a, it could have been demonstrated that Clec12a functions as an inhibitory innate immune receptor for cellular death. The lack of Clec12a induced an exacerbated influx of neutrophils to the site of inflammation. The levels of the cytokine TNFα and the chemokines CXCL1 and CXCL10, but not CXCL2, were increased in the thymus of irradiated Clec12a-deficient animals. While this work was being finalized, Dr. Konstantin Neumann subsequently identified monosodium urate crystals as one specific ligand for Clec12a (data not shown). These data indicate that

Clec12a counteracts the activity of other stimulatory receptors which also sense molecules released by dead cells *in vivo* (figure 4.1).

In the recent years, different C-type lectins have been involved in the recognition of DAMPs associated to cellular death. This is the case of Clec9a, an ITAM-bearing C-type lectin that senses F-actin, a cytoskeletal protein that is released upon loss of cell membrane integrity (Ahrens et al., 2012; Zhang et al., 2012). The C-type lectin Mincle senses SAP-130, a self ribonucleoprotein found in necrotic bodies (Yamasaki et al., 2008). Interestingly, this interaction also leads to the production of CXCL2 and TNFα which induces neutrophil recruitment to the site of damage. The fact that Clec12a deficiency provokes an increased TNFα production and neutrophil recruitment suggests that Clec12a could actually work as the counterpartner of Mincle. However, whether these two receptors interact specifically in the sensing of cell death remains to be further investigated.

4.3 Clinical implications for Clec12a

4.3.1 Clec12a in autoimmune diseases

Lack of Clec12a did not affect humoral immune responses in mice. However, it would be interesting to investigate whether Clec12a is relevant for the development of secondary immune responses by memory B cells performing re-immunizations. Due to the inhibitory function expected for Clec12a, we also hypothesized that Clec12a-deficient mice would develop an autoimmune disease. However, no signs of disease were observed in mice along 24 months of observation under non-challenged homeostatic conditions and specific pathogen free housing. Levels of autoantibodies in Clec12a-deficient animals were under physiological limits and not significantly different than those of wild type animals (data not shown in this work).

Expression of Clec12a has been linked to rheumatoid arthritis in humans, an autoimmune disease that affects predominantly the joints and is characterized by the presence of autoantibodies against the Fc portion of IgG

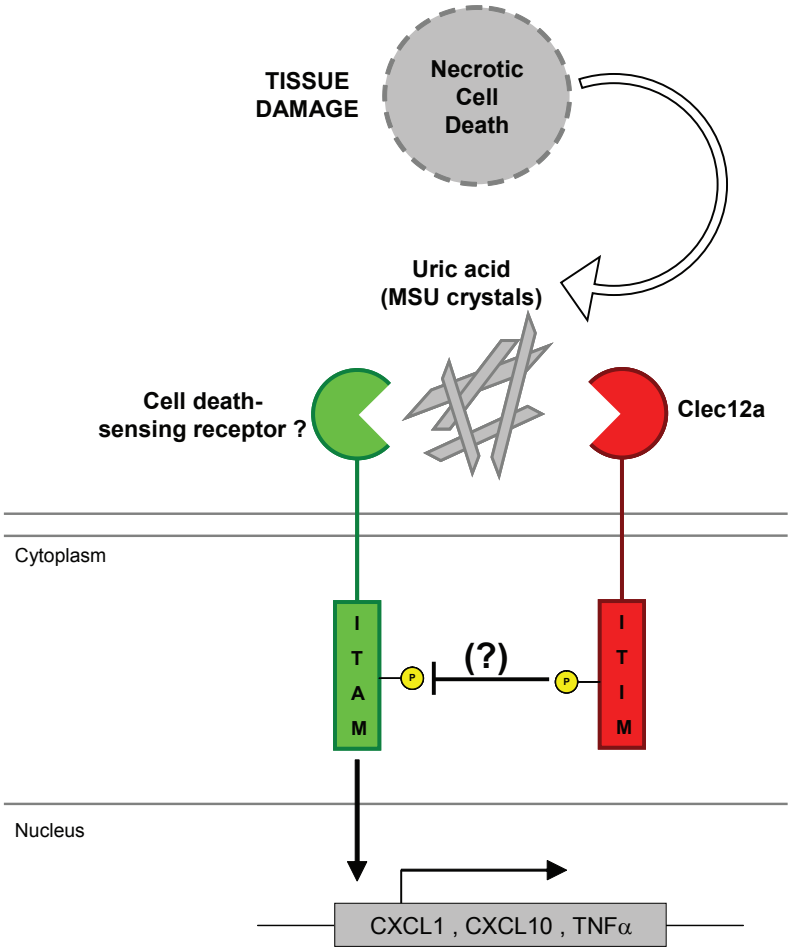

Figure 4.1: Clec12a counteracts inflammatory responses to sterile cell death *in vivo*. Upon binding to a ligand released due to cell death, Clec12a counteracts the activation of other stimulatory receptors by downregulating the expression of chemokines and cytokines, therefore limitating the recruitment of neutrophils to the site of inflammation.

and antibodies against citrullinated peptides. Using microarray technology with samples of synovial tissue fibroblasts from patients with rheumatoid arthritis, it has been shown that increased expression of Clec12a correlated with levels of rheumatoid factor concentrations in serum (Galligan et al., 2007). The mechanistical relationship between the disease and the enhanced transcription of Clec12a remains however to be further investigated.

Uric acid is a chemical compound normally found in soluble form in the cellular cytoplasm and also at determined levels in the blood. It is a final metabolite resulting from the physiological metabolism of purine nucleotides. However, it can be released to the cytoplasm after cellular necrosis for instance due to tissue damage. Hyperuricemia (high concentrations of uric acid in the blood) is known to be the cause of gout, a type of acute inflammatory arthritis. In this medical condition, excess of uric acid crystallizes in form of monosodium urate crystals which precipitate in tissues like joints, kidney, etc. Elevated uric acid levels have been linked to gout rather than to rheumatoid arthritis. However, the fact that autoantibodies can lead to tissue damage and therefore cell death suggest that Clec12a could be involved in the inflammatory component of this autoimmune disease.

Interestingly, the ITIM-bearing C-type lectin Clec4a2 has been related to two mouse models of autoimmune diseases. Clec4a2-deficient mice showed no major defects at younger ages, but developed spontaneous joint abnormalities and produced autoantibodies at later stages resembling rheumatoid arthritis (Fujikado et al., 2008).

It is long known that the formation of autoantibodies and immune complexes can be the cause or the result of tissue damage. Both can be found in autoimmune diseases like systemic lupus erythematosus, rheumatoid arthritis, progressive systemic sclerosis, etc. Although Clec12a deficiency did not generate spontaneous autoimmune diseases in mice, it would be interesting to investigate whether Clec12a has, as a negative regulator of sterile inflammation, a protective effect in animal models with these clinical settings.

4.3.2 Clec12a in malignant diseases

The average life span of Clec12a-deficient animals was not significantly different from wild type animals, and lack of Clec12a could not be related to early formation of tumors or development of hematological proliferative diseases at a long term.

Interestingly, expression of human Clec12a has been associated to several tumor cell lines of myeloid origin and its expression has been found in blasts of patients with acute myeloid leukemia (Bakker et al., 2004). Furthermore, Clec12a expression in hematopoietic stem cells appears to be a useful marker specific for residual disease in patients with acute myeloid leukemia (Larsen et al., 2012; van Rhenen et al., 2007). Whether Clec12a is involved in the pathogenesis of AML has not been investigated yet.

Another C-type lectin, Clec2, has been shown to bind the endogenous ligand podoplanin, which is expressed by healthy kidney cells, endothelial lymphatic cells and also on the surface of different tumor cells. The interaction of podoplanin with Clec2 expressed in platelets leads to clot formation, while its interaction with Clec2 expressed in myeloid dendritic cells triggers motility processes, leading DCs from peripheral sites towards the T cell zone in the lymph nodes. Apparently, the combination of migratory phenomena and lymphangiogenesis resulting from Clec2 and podoplanin interactions appears to be involved in the invasiveness and metastasic properties of certain tumorigenic cells (Martín-Villar et al., 2010; Suzuki-Inoue et al., 2007).

4.3.3 Clec12a in immunotherapy

Independently of the effect of Clec12a in intracellular signaling cascades, the endocytic ability of this receptor remains to be further investigated. Several independent groups have identified Clec12a as a target molecule to deliver antigenic particles to dendritic cells. Using labelled monoclonal antibodies, it has been demonstrated that Clec12a can internalize antibodies targeted to the extracellular domain of the protein (Bakker et al., 2004; Chen et al.,

2006; Lahoud et al., 2009). Antigen bound to an antibody directed against Clec12a induced potent humoral responses (Lahoud et al., 2009). Indeed the neighbouring C-type lectin Clec9a has been also succesfully used for antigen delivery to enhace vaccination, resulting in effective cross-presentation by dendritic cells to T cells and the generation of cytolitic T cell responses (Caminschi et al., 2008; Sancho et al., 2008). These results present C-type lectins expressed in dendritic cells, such as Clec12a, as a promising therapeutic tool with several applications including vaccine development and tumor therapy.

However, although targeting receptors with antibodies represents a widely accepted way to investigate the function of cell surface molecules, the approaches mentioned before do not necessarily represent the homeostatic conditions in the body and therefore they do not fully represent the physiological role of Clec12a *in vivo*.

4.4 Conclusion and future perspectives

C-type lectins are emerging as non-TLR receptors in the innate immunity. However, their ligands, their mechanisms of action and signaling pathways require further study.

Summarizing the results presented in this work, we conclude that Clec12a might play a crucial role already during murine embryonic development. Regarding the immune system development, deficiency of Clec12a did not influence the gross differentiation of myeloid nor lymphoid lineages. Regarding the innate immune system, Clec12a is dispensable for the innate immune response to selective TLR or Dectin-1 stimulation, although it might modulate the immune response after TLR or Dectin-1 stimulation *in vivo* under certain conditions.

Interestingly, it could be genetically demonstrated that Clec12a acts as a negative regulator of sterile inflammatory processes upon cell death. On the other hand, Mincle sensing of dead cells is independent of the canonical

residues of the carbohydrate recognition domain typical for C-type lectins (Yamasaki et al., 2008). Taking all these data together, further investigations are required to understand how the interaction of innate immune receptors with DAMPs released upon cell death takes place.

Furthermore, cellular death can be the result of different agents: mechanical or thermal trauma, irradiation, chemical or metabolic toxicity, ischemia, infective agents, etc. The possibility that Clec12a plays a role in the homeostasis of the innate immune response upon cell death due to all these agents remains to be studied in detail. Moreover, the inhibitory activity of Clec12a upon sterile inflammation and its antigen delivery capacity excel Clec12a as a potential tool for clinical indications, like vaccine improvement or immunotherapy. While Clec12a expression levels have been linked to autoantibody levels in serum of patients with rheumatoid arthritis, the question whether different single nucleotide polymorphisms for Clec12a exist and whether these correlate with clinical outcomes like rheumatoid arthritis or other autoimmune diseases remains unanswered.

In conclusion, the characterization of the C-type lectin Clec12a has provided new insights into the field of immune homeostasis and promises positive perspectives in a better understanding of innate immunity to danger-associated molecular patterns and sterile inflammatory processes.

Bibliography

Ahrens, S., Zelenay, S., Sancho, D., Han?, P., Kjær, S., Feest, C., Fletcher, G., Durkin, C., Postigo, A., Skehel, M., Batista, F., Thompson, B., Way, M., e Sousa, C. R., and Schulz, O. (2012). F-actin is an evolutionarily conserved damage-associated molecular pattern recognized by DNGR-1, a receptor for dead cells. *Immunity*, 36(4):635–645.

Alexopoulou, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*, 413(6857):732–738.

Astarita, J. L., Acton, S. E., and Turley, S. J. (2012). Podoplanin: emerging functions in development, the immune system, and cancer. *Front Immunol*, 3:283.

Bagchi, A., Herrup, E. A., Warren, H. S., Trigilio, J., Shin, H.-S., Valentine, C., and Hellman, J. (2007). MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. *J Immunol*, 178(2):1164–1171.

Bakker, A. B. H., van den Oudenrijn, S., Bakker, A. Q., Feller, N., van Meijer, M., Bia, J. A., Jongeneelen, M. A. C., Visser, T. J., Bijl, N., Geuijen, C. A. W., Marissen, W. E., Radosevic, K., Throsby, M., Schuurhuis, G. J., Ossenkoppele, G. J., de Kruif, J., Goudsmit, J., and Kruisbeek, A. M. (2004). C-type lectin-like molecule-1: a novel myeloid cell surface marker associated with acute myeloid leukemia. *Cancer Res*, 64(22):8443–8450.

Barrow, A. D. and Trowsdale, J. (2006). You say ITAM and I say ITIM, let's call the whole thing off: the ambiguity of immunoreceptor signalling. *Eur J Immunol*, 36(7):1646–1653.

Batista, F. D. and Harwood, N. E. (2009). The who, how and where of antigen presentation to B cells. *Nat Rev Immunol*, 9(1):15–27.

Belkaid, Y. (2007). Regulatory T cells and infection: a dangerous necessity. *Nat Rev Immunol*, 7(11):875–888.

Boulaftali, Y., Hess, P. R., Getz, T. M., Cholka, A., Stolla, M., Mackman, N., Owens, A. P., Ware, J., Kahn, M. L., and Bergmeier, W. (2013). Platelet ITAM signaling is critical for vascular integrity in inflammation. *J Clin Invest*, 123(2):908–916.

Brown, G. D. and Gordon, S. (2001). Immune recognition. A new receptor for beta-glucans. *Nature*, 413(6851):36–37.

Browne, E. P. (2012). Regulation of B-cell responses by Toll-like receptors. *Immunology*, 136(4):370–379.

Burnet, F. M. (1961). Immunological recognition of self. *Science*, 133(3449):307–311.

Cambi, A., Netea, M. G., Mora-Montes, H. M., Gow, N. A. R., Hato, S. V., Lowman, D. W., Kullberg, B.-J., Torensma, R., Williams, D. L., and Figdor, C. G. (2008). Dendritic cell interaction with Candida albicans critically depends on N-linked mannan. *J Biol Chem*, 283(29):20590–20599.

Cambier, J. C. (1995). Antigen and Fc receptor signaling. the awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). *J Immunol*, 155(7):3281–3285.

Caminschi, I., Proietto, A. I., Ahmet, F., Kitsoulis, S., Teh, J. S., Lo, J. C. Y., Rizzitelli, A., Wu, L., Vremec, D., van Dommelen, S. L. H., Campbell, I. K., Maraskovsky, E., Braley, H., Davey, G. M., Mottram, P., van de

Velde, N., Jensen, K., Lew, A. M., Wright, M. D., Heath, W. R., Shortman, K., and Lahoud, M. H. (2008). The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement. *Blood*, 112(8):3264–3273.

Chen, C.-H., Floyd, H., Olson, N. E., Magaletti, D., Li, C., Draves, K., and Clark, E. A. (2006). Dendritic-cell-associated C-type lectin 2 (DCAL-2) alters dendritic-cell maturation and cytokine production. *Blood*, 107(4):1459–1467.

Chen, G. Y. and Nuñez, G. (2010). Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol*, 10(12):826–837.

Coats, S. R., Pham, T.-T. T., Bainbridge, B. W., Reife, R. A., and Darveau, R. P. (2005). MD-2 mediates the ability of tetra-acylated and penta-acylated lipopolysaccharides to antagonize Escherichia coli lipopolysaccharide at the TLR4 signaling complex. *J Immunol*, 175(7):4490–4498.

Colmenares, M., Puig-Kröger, A., Pello, O. M., Corbí, A. L., and Rivas, L. (2002). Dendritic cell (DC)-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN, CD209), a C-type surface lectin in human DCs, is a receptor for Leishmania amastigotes. *J Biol Chem*, 277(39):36766–36769.

Daëron, M., Jaeger, S., Pasquier, L. D., and Vivier, E. (2008). Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. *Immunol Rev*, 224:11–43.

Dennehy, K. M., Willment, J. A., Williams, D. L., and Brown, G. D. (2009). Reciprocal regulation of IL-23 and IL-12 following co-activation of Dectin-1 and TLR signaling pathways. *Eur J Immunol*, 39(5):1379–1386.

Drickamer, K. (1999). C-type lectin-like domains. *Curr Opin Struct Biol*, 9(5):585–590.

e Sousa, C. R. (2004). Activation of dendritic cells: translating innate into adaptive immunity. *Curr Opin Immunol*, 16(1):21–25.

Engering, A., Geijtenbeek, T. B. H., and van Kooyk, Y. (2002). Immune escape through C-type lectins on dendritic cells. *Trends Immunol*, 23(10):480–485.

Fujikado, N., Saijo, S., Yonezawa, T., Shimamori, K., Ishii, A., Sugai, S., Kotaki, H., Sudo, K., Nose, M., and Iwakura, Y. (2008). Dcir deficiency causes development of autoimmune diseases in mice due to excess expansion of dendritic cells. *Nat Med*, 14(2):176–180.

Galligan, C. L., Baig, E., Bykerk, V., Keystone, E. C., and Fish, E. N. (2007). Distinctive gene expression signatures in rheumatoid arthritis synovial tissue fibroblast cells: correlates with disease activity. *Genes Immun*, 8(6):480–491.

Gallucci, S., Lolkema, M., and Matzinger, P. (1999). Natural adjuvants: endogenous activators of dendritic cells. *Nat Med*, 5(11):1249–1255.

Gantner, B. N., Simmons, R. M., Canavera, S. J., Akira, S., and Underhill, D. M. (2003). Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J Exp Med*, 197(9):1107–1117.

Garrett, W. S., Gordon, J. I., and Glimcher, L. H. (2010). Homeostasis and inflammation in the intestine. *Cell*, 140(6):859–870.

Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., Figdor, C. G., and van Kooyk, Y. (2000). DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell*, 100(5):587–597.

Geijtenbeek, T. B. H. and Gringhuis, S. I. (2009). Signalling through C-type lectin receptors: shaping immune responses. *Nat Rev Immunol*, 9(7):465–479.

Graham, L. M. and Brown, G. D. (2009). The Dectin-2 family of C-type lectins in immunity and homeostasis. *Cytokine*, 48(1-2):148–155.

Gris, D., Ye, Z., Iocca, H. A., Wen, H., Craven, R. R., Gris, P., Huang, M., Schneider, M., Miller, S. D., and Ting, J. P.-Y. (2010). NLRP3 plays a critical role in the development of experimental autoimmune encephalomyelitis by mediating Th1 and Th17 responses. *J Immunol*, 185(2):974–981.

Gross, O., Gewies, A., Finger, K., Schäfer, M., Sparwasser, T., Peschel, C., Förster, I., and Ruland, J. (2006). Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature*, 442(7103):651–656.

Han, Y., Zhang, M., Li, N., Chen, T., Zhang, Y., Wan, T., and Cao, X. (2004). KLRL1, a novel killer cell lectinlike receptor, inhibits natural killer cell cytotoxicity. *Blood*, 104(9):2858–2866.

Harris, M. J. and Juriloff, D. M. (2007). Mouse mutants with neural tube closure defects and their role in understanding human neural tube defects. *Birth Defects Res A Clin Mol Teratol*, 79(3):187–210.

Harris, M. J. and Juriloff, D. M. (2010). An update to the list of mouse mutants with neural tube closure defects and advances toward a complete genetic perspective of neural tube closure. *Birth Defects Res A Clin Mol Teratol*, 88(8):653–669.

Hashimoto, C., Hudson, K. L., and Anderson, K. V. (1988). The Toll gene of drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell*, 52(2):269–279.

Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M., and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*, 410(6832):1099–1103.

Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., and Akira, S. (2002). Small antiviral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol*, 3(2):196–200.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000). A Toll-like receptor recognizes bacterial DNA. *Nature*, 408(6813):740–745.

Herzog, B. H., Fu, J., Wilson, S. J., Hess, P. R., Sen, A., McDaniel, J. M., Pan, Y., Sheng, M., Yago, T., Silasi-Mansat, R., McGee, S., May, F., Nieswandt, B., Morris, A. J., Lupu, F., Coughlin, S. R., McEver, R. P., Chen, H., Kahn, M. L., and Xia, L. (2013). Podoplanin maintains high endothelial venule integrity by interacting with platelet CLEC-2. *Nature*, 502(7469):105–109.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the lps gene product. *J Immunol*, 162(7):3749–3752.

Hu, X., Chen, J., Wang, L., and Ivashkiv, L. B. (2007). Crosstalk among Jak-STAT, Toll-like receptor, and ITAM-dependent pathways in macrophage activation. *J Leukoc Biol*, 82(2):237–243.

Isakov, N. (1997). ITIMs and ITAMs. the Yin and Yang of antigen and Fc receptor-linked signaling machinery. *Immunol Res*, 16(1):85–100.

Iwasaki, A. and Medzhitov, R. (2010). Regulation of adaptive immunity by the innate immune system. *Science*, 327(5963):291–295.

Iyoda, T., Nagata, K., Akashi, M., and Kobayashi, Y. (2005). Neutrophils accelerate macrophage-mediated digestion of apoptotic cells in vivo as well as in vitro. *J Immunol*, 175(6):3475–3483.

Jähn, P. S., Zänker, K. S., Schmitz, J., and Dzionek, A. (2010). BDCA-2 signaling inhibits TLR-9-agonist-induced plasmacytoid dendritic cell activation and antigen presentation. *Cell Immunol*, 265(1):15–22.

Janeway, C. A. (1992). The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today*, 13(1):11–16.

Janeway, C. A. and Medzhitov, R. (2002). Innate immune recognition. *Annu Rev Immunol*, 20:197–216.

Jurk, M., Heil, F., Vollmer, J., Schetter, C., Krieg, A. M., Wagner, H., Lipford, G., and Bauer, S. (2002). Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nat Immunol*, 3(6):499.

Kanneganti, T.-D., Ozören, N., Body-Malapel, M., Amer, A., Park, J.-H., Franchi, L., Whitfield, J., Barchet, W., Colonna, M., Vandenabeele, P., Bertin, J., Coyle, A., Grant, E. P., Akira, S., and Núñez, G. (2006). Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature*, 440(7081):233–236.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K. J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C.-S., e Sousa, C. R., Matsuura, Y., Fujita, T., and Akira, S. (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature*, 441(7089):101–105.

Kono, H. and Rock, K. L. (2008). How dying cells alert the immune system to danger. *Nat Rev Immunol*, 8(4):279–289.

Korn, T., Bettelli, E., Oukka, M., and Kuchroo, V. K. (2009). IL-17 and Th17 cells. *Annu Rev Immunol*, 27:485–517.

Lahoud, M. H., Proietto, A. I., Ahmet, F., Kitsoulis, S., Eidsmo, L., Wu, L., Sathe, P., Pietersz, S., Chang, H.-W., Walker, I. D., Maraskovsky, E., Braley, H., Lew, A. M., Wright, M. D., Heath, W. R., Shortman, K., and Caminschi, I. (2009). The C-type lectin Clec12A present on mouse and human dendritic cells can serve as a target for antigen delivery and enhancement of antibody responses. *J Immunol*, 182(12):7587–7594.

Larsen, H. ., Roug, A. S., Just, T., Brown, G. D., and Hokland, P. (2012). Expression of the hMICL in acute myeloid leukemia-a highly reliable dis-

ease marker at diagnosis and during follow-up. *Cytometry B Clin Cytom*, 82(1):3–8.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., and Hoffmann, J. A. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell*, 86(6):973–983.

Lowell, C. A. (2011). Src-family and Syk kinases in activating and inhibitory pathways in innate immune cells: signaling cross talk. *Cold Spring Harb Perspect Biol*, 3(3).

Marshall, A. S. J., Willment, J. A., Lin, H.-H., Williams, D. L., Gordon, S., and Brown, G. D. (2004). Identification and characterization of a novel human myeloid inhibitory C-type lectin-like receptor (MICL) that is predominantly expressed on granulocytes and monocytes. *J Biol Chem*, 279(15):14792–14802.

Martín-Villar, E., noz, B. F.-M., Parsons, M., Yurrita, M. M., Megías, D., Pérez-Gómez, E., Jones, G. E., and Quintanilla, M. (2010). Podoplanin associates with CD44 to promote directional cell migration. *Mol Biol Cell*, 21(24):4387–4399.

Martinon, F., Mayor, A., and Tschopp, J. (2009). The inflammasomes: guardians of the body. *Annu Rev Immunol*, 27:229–265.

Matzinger, P. (1994). Tolerance, danger, and the extended family. *Annu Rev Immunol*, 12:991–1045.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nat Rev Immunol*, 1(2):135–145.

Medzhitov, R. and Janeway, C. (2000). Innate immune recognition: mechanisms and pathways. *Immunol Rev*, 173:89–97.

Meylan, E., Tschopp, J., and Karin, M. (2006). Intracellular pattern recognition receptors in the host response. *Nature*, 442(7098):39–44.

Murhpy, K. M., Travers, P., and Walport, M. (2008). *Janeway's Immunobiology*. Garland Science, 7th (revised) edition.

Napolitani, G., Rinaldi, A., Bertoni, F., Sallusto, F., and Lanzavecchia, A. (2005). Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol*, 6(8):769–776.

Noguchi, E., Homma, Y., Kang, X., Netea, M. G., and Ma, X. (2009). A Crohn's disease-associated NOD2 mutation suppresses transcription of human IL10 by inhibiting activity of the nuclear ribonucleoprotein hnRNP-A1. *Nat Immunol*, 10(5):471–479.

Oka, K., Sawamura, T., Kikuta, K., Itokawa, S., Kume, N., Kita, T., and Masaki, T. (1998). Lectin-like oxidized low-density lipoprotein receptor 1 mediates phagocytosis of aged/apoptotic cells in endothelial cells. *Proc Natl Acad Sci U S A*, 95(16):9535–9540.

O'Neill, L. A. J., Golenbock, D., and Bowie, A. G. (2013). The history of toll-like receptors - redefining innate immunity. *Nat Rev Immunol*.

Osorio, F. and e Sousa, C. R. (2011). Myeloid c-type lectin receptors in pathogen recognition and host defense. *Immunity*, 34(5):651–664.

Pyz, E., Huysamen, C., Marshall, A. S. J., Gordon, S., Taylor, P. R., and Brown, G. D. (2008). Characterisation of murine MICL (CLEC12A) and evidence for an endogenous ligand. *Eur J Immunol*, 38(4):1157–1163.

Radding, C. M. (1973). Molecular mechanisms in genetic recombination. *Annu Rev Genet*, 7:87–111.

Remer, K. A., Brcic, M., and Jungi, T. W. (2003). Toll-like receptor-4 is involved in eliciting an LPS-induced oxidative burst in neutrophils. *Immunol Lett*, 85(1):75–80.

Robinson, M. J., Sancho, D., Slack, E. C., LeibundGut-Landmann, S., and e Sousa, C. R. (2006). Myeloid C-type lectins in innate immunity. *Nat Immunol*, 7(12):1258–1265.

Rock, K. L., Latz, E., Ontiveros, F., and Kono, H. (2010). The sterile inflammatory response. *Annu Rev Immunol*, 28:321–342.

Ruland, J. (2008). CARD9 signaling in the innate immune response. *Ann N Y Acad Sci*, 1143:35–44.

Ruland, J. and Mak, T. W. (2003). Transducing signals from antigen receptors to nuclear factor kappa B. *Immunol Rev*, 193:93–100.

Sancho, D. and e Sousa, C. R. (2012). Signaling by myeloid C-type lectin receptors in immunity and homeostasis. *Annu Rev Immunol*, 30:491–529.

Sancho, D., Joffre, O. P., Keller, A. M., Rogers, N. C., Martínez, D., Hernanz-Falcón, P., Rosewell, I., and e Sousa, C. R. (2009). Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature*, 458(7240):899–903.

Sancho, D., Mourão-Sá, D., Joffre, O. P., Schulz, O., Rogers, N. C., Pennington, D. J., Carlyle, J. R., and e Sousa, C. R. (2008). Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. *J Clin Invest*, 118(6):2098–2110.

Schwandner, R., Dziarski, R., Wesche, H., Rothe, M., and Kirschning, C. J. (1999). Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem*, 274(25):17406–17409.

Shea, K. and Geijsen, N. (2007). Dissection of 6.5 dpc mouse embryos. *J Vis Exp*, (2):160.

Shi, Z., Cai, Z., Sanchez, A., Zhang, T., Wen, S., Wang, J., Yang, J., Fu, S., and Zhang, D. (2011). A novel Toll-like receptor that recognizes vesicular stomatitis virus. *J Biol Chem*, 286(6):4517–4524.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med*, 189(11):1777–1782.

Southern, E. (2006). Southern blotting. *Nat Protoc*, 1(2):518–525.

Steinman, L. (2007). A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med*, 13(2):139–145.

Stuart, L. M., Takahashi, K., Shi, L., Savill, J., and Ezekowitz, R. A. B. (2005). Mannose-binding lectin-deficient mice display defective apoptotic cell clearance but no autoimmune phenotype. *J Immunol*, 174(6):3220–3226.

Suzuki-Inoue, K., Fuller, G. L. J., García, A., Eble, J. A., Pöhlmann, S., Inoue, O., Gartner, T. K., Hughan, S. C., Pearce, A. C., Laing, G. D., Theakston, R. D. G., Schweighoffer, E., Zitzmann, N., Morita, T., Tybulewicz, V. L. J., Ozaki, Y., and Watson, S. P. (2006). A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. *Blood*, 107(2):542–549.

Suzuki-Inoue, K., Inoue, O., Ding, G., Nishimura, S., Hokamura, K., Eto, K., Kashiwagi, H., Tomiyama, Y., Yatomi, Y., Umemura, K., Shin, Y., Hirashima, M., and Ozaki, Y. (2010). Essential in vivo roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2-deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. *J Biol Chem*, 285(32):24494–24507.

Suzuki-Inoue, K., Kato, Y., Inoue, O., Kaneko, M. K., Mishima, K., Yatomi, Y., Yamazaki, Y., Narimatsu, H., and Ozaki, Y. (2007). Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells. *J Biol Chem*, 282(36):25993–26001.

Takeda, K. and Akira, S. (2005). Toll-like receptors in innate immunity. *Int Immunol*, 17(1):1–14.

Takeda, K., Takeuchi, O., and Akira, S. (2002). Recognition of lipopeptides by Toll-like receptors. *J Endotoxin Res*, 8(6):459–463.

Takeuchi, O. and Akira, S. (2008). MDA5/RIG-I and virus recognition. *Curr Opin Immunol*, 20(1):17–22.

Taylor, P. R., Tsoni, S. V., Willment, J. A., Dennehy, K. M., Rosas, M., Findon, H., Haynes, K., Steele, C., Botto, M., Gordon, S., and Brown, G. D. (2007). Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat Immunol*, 8(1):31–38.

Underhill, D. M. (2007). Collaboration between the innate immune receptors dectin-1, TLRs, and NODs. *Immunol Rev*, 219:75–87.

Underhill, D. M. and Ozinsky, A. (2002). Toll-like receptors: key mediators of microbe detection. *Curr Opin Immunol*, 14(1):103–110.

van Heel, D. A., Ghosh, S., Butler, M., Hunt, K., Foxwell, B. M. J., Mengin-Lecreulx, D., and Playford, R. J. (2005). Synergistic enhancement of toll-like receptor responses by nod1 activation. *Eur J Immunol*, 35(8):2471–2476.

van Kooyk, Y. and Geijtenbeek, T. B. H. (2003). DC-SIGN: escape mechanism for pathogens. *Nat Rev Immunol*, 3(9):697–709.

van Rhenen, A., van Dongen, G. A. M. S., Kelder, A., Rombouts, E. J., Feller, N., Moshaver, B., van Walsum, M. S., Zweegman, S., Ossenkoppele, G. J., and Schuurhuis, G. J. (2007). The novel AML stem cell associated

antigen CLL-1 aids in discrimination between normal and leukemic stem cells. *Blood*, 110(7):2659–2666.

Weis, W. I., Taylor, M. E., and Drickamer, K. (1998). The C-type lectin superfamily in the immune system. *Immunol Rev*, 163:19–34.

Yamasaki, S., Ishikawa, E., Sakuma, M., Hara, H., Ogata, K., and Saito, T. (2008). Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nat Immunol*, 9(10):1179–1188.

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y.-M., Gale, M., Akira, S., Yonehara, S., Kato, A., and Fujita, T. (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol*, 175(5):2851–2858.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol*, 5(7):730–737.

Zelensky, A. N. and Gready, J. E. (2005). The C-type lectin-like domain superfamily. *FEBS J*, 272(24):6179–6217.

Zhang, D., Zhang, G., Hayden, M. S., Greenblatt, M. B., Bussey, C., Flavell, R. A., and Ghosh, S. (2004). A toll-like receptor that prevents infection by uropathogenic bacteria. *Science*, 303(5663):1522–1526.

Zhang, J.-G., Czabotar, P. E., Policheni, A. N., Caminschi, I., Wan, S. S., Kitsoulis, S., Tullett, K. M., Robin, A. Y., Brammananth, R., van Delft, M. F., Lu, J., O'Reilly, L. A., Josefsson, E. C., Kile, B. T., Chin, W. J., Mintern, J. D., Olshina, M. A., Wong, W., Baum, J., Wright, M. D., Huang, D. C. S., Mohandas, N., Coppel, R. L., Colman, P. M., Nicola, N. A., Shortman, K., and Lahoud, M. H. (2012). The dendritic cell recep-

tor Clec9A binds damaged cells via exposed actin filaments. *Immunity*, 36(4):646–657.

List of Abbreviations

ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CLR	C-type lectin receptor
CpG	Cytosine nucleotide next to a guanine nucleotide
CRD	Carbohydrate recognition domain
CTL	Cytolytic T lymphocyte
CTLD	C-type lectin domain
dCTP	Deoxycytidine triphosphate
del	Deleted
dsRNA	Double-stranded ribonucleic acid
DAMP	Danger-associated molecular pattern
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modidified Eagle's medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
dpc	Day post-conception
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting

FCS	Fetal calf serum
FSC	Forward scatter
hClec12a	Human Clec12a
IFN	Interferon
IKK	Inhibitor of kappa B kinase
IL	Interleukin
IRF	Interferon-regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitor motif
ko	Knock out
LA	Long arm
LCCM	L-cell conditioned media
LIF	Leukemia inhibitory factor
LN	Lymph nodes
Malt1	Mucosa associated lymphoid tissue translocation protein 1
mClec12a	Murine Clec12a
MEF	Murine embryonic fibroblast
MOF	Mean of fluorescence
mRNA	Messenger ribonucleic acid
NFκB	Nuclear factor kappa B
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain-like receptor
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffer saline
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PRR	Pathogen recognition receptor
RBC	Red blood cell
RNA	Ribonucleic acid
rpm	Revolutions per minute

RIG	Retinoic acid-inducible gene
RLH	Retinoic acid-inducible gene-like helicases
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SA	Short arm
SDS	Sodium dodecylsulfate
SHIP	Src-homology region 2-containing inositol 5' phosphatase
SHP	Src-homology region 2-containing phosphatase
SNP	Single nucleotide polimorfism
SPL	Spleen
Src	Sarcoma
TAE	Tris-acetate-ethylenediaminetetraacetic acid
Taq	*Thermus aquaticus*
TGFβ	Transforming growth factor beta
TREM	Triggering receptor expressed on myeloid cells
THY	Thymus
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor
U	Unit
UV	Ultraviolet
VDJ	Variable, Diverse, and Joining gene segments
wt	Wild type
Xmn	*Xanthomonas manihotis*

Aminoacids

A	Alanine
C	Cysteine
D	Aspartic acid (aspartate)
E	Glutamic acid (glutamate)

F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
X	Any amino acid
Y	Tyrosine

Units

G	Acceleration of gravity
M	Molar
mM	Millimolar
μF	Microfaraday
μg	Microgram
μl	Microliter
mV	Milivolt
ng	Nanogram
pg	Picogram
°C	Celsius degree

List of Figures

List of Tables

Acknowledgements

This PhD thesis was performed at the 3rd Medical Department (Oncology and Hematology) and the Institute of Molecular Immunology of the Klinikum Rechts der Isar (Technical University of Munich) under the supervision of Univ.-Prof. Dr. Jürgen Ruland (currently Director of the Institute of Clinical Chemistry and Pathobiochemistry): I want to thank him for giving me the opportunity of joining his excellent team and especially this project. The years under his supervision will remain as a remarkable stage in my scientific career.

I want to thank Univ.-Prof. Dr. Ulrike Protzer, Institute of Virology, and Priv.-Doz. Dr. Philipp P. Jost, 3rd Medical Department, for kindly mentoring my doctoral thesis. I deeply appreciate their readiness to participate in this project. Their discussions have significantly enriched my scientific knowledge and my passion for research.

I also want to thank the whole team of Univ.-Prof. Dr.med. Jürgen Ruland: postdocs, doctoral students and technical assistants, who always have been a supporting and encouraging figure for excellent work. Thanks to Dr. Olaf Gross for introducing me to this project and the enthralling field of innate immunity. Thanks also to Dr. Konstantin Neumann, whose collaboration and his efforts to identify the ligand of Clec12a greatly contributed to enhance the success of this challenge. Special mention goes to Nicole Han-

neschläger for her excellent teamwork and technical skills.

Furthermore, I would like to thank my family, to my father and especially my sister Ana, for being my support from the distance, even through the most difficult time of our lives. This work is dedicated to my mother, who unfortunately could not see the completion of this project.

Thanks to Renate and Gerhard: your kind help contributed to the finishing of this work. And last but not least, I would have never been able to accomplish my goals without the support of my husband: Gabor, only you know how much and in which way you have contributed to the success of this project.