TECHNISCHE UNIVERSITÄT MÜNCHEN

TUM School of Medicine and Health

Investigations on the C-type lectin receptor Clec12a and its role in the activation by the adjuvant alum

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Vollständiger Abdruck der von der TUM School of Medicine and Health

der Technischen Universität München zur Erlangung einer Doktorin der Medizinischen Wissenschaft genehmigten Dissertation.

Vorsitz: apl. Prof. Dr. Klaus-Peter Janssen

Prüfende der Dissertation:

1. Prof. Dr. Jürgen Ruland
2. Priv.-Doz. Veit Buchholz

Die Dissertation wurde am 09.11.2023 bei der Technischen Universität München eingereicht und durch die TUM School of Medicine and Health am 13.03.2024 angenommen.

Zusammenfassung

Die Zellen des angeborenen Immunsystems besitzen eine Vielzahl von keimbahnkodierten Rezeptoren, um Pathogene oder andere Gefahren zu erkennen. Eine kontrollierte Aktivierung dieser Mechanismen ist notwendig, um die Gewebshomeostase aufrechtzuerhalten. Zudem nutzen Adjuvanzien diese Rezeptoren, um Impfantworten zu verstärken. Ein bisher nur unzureichend erforschter inhibitorischer Immunrezeptor ist der C-Typ Lectin Rezeptor Clec12a. In dieser Arbeit wird die Rolle von Clec12a bei der durch das Adjuvanz Alum induzierten Immunantwort untersucht.

Abstract

The cells of the innate immune system contain a variety of germ line encoded receptors that enable them to recognize pathogens or other dangers to the body. Controlled activation of these defense mechanisms is essential to maintain tissue homeostasis. Furthermore, adjuvants use these receptors to enhance immune responses to vaccination. In this context, a hitherto insufficiently studied receptor is the inhibitory C-type lectin receptor Clec12a is. This dissertation investigates the role of Clec12a in the immune response inducted by the adjuvant alum.

Table of contents

Table of abbreviations

AML	acute myeloid leukaemia
APC	antigen presenting cell
ATP	adenosine triphosphate
BCR	B-cell antigen receptor
BM	bone marrow
BSA	bovine serum albumin
CBA	cytometric bead array
CD	cluster of differentiation
CLL-1	c-type lectin-like molecule-1
CLR	c-type lectin receptor
CTLD	c-type lectin like domain
DAMP	damage-associated molecular pattern
DC	dendritic cell
DCAL2	dendritic-cell-associated C-type lectin 2
eCBA	enhanced cytometric bead array
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCM	flow cytometry
FCS	fetal calf serum
Flt3L	*fms*-like tyrosine kinase 3 ligand
GFP	green fluorescence protein
GM-CSF	granulocyte–macrophage colony stimulating factor
GM-DC	GM-CSF derived DCs
Gy	gray
h	human
HMGB1	high-mobility group box 1 protein
Ig	immunoglobulin
IFN	interferon
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
LPS	lipopolysaccharides
m	murine
MFI	mean fluorescence intensity
MHC	major Histocompability Complex
MICL	myeloid inhibitory C-type lectin
MINCLE	macrophage inducible Ca^{2+}-dependent lectin receptor
MP	macrophages

MSU	monosodium urate
NADPH	nicotinamide adenine dinucleotide phosphate
NF	nuclear factor
NFAT	nuclear factor of activated T-cells
NLR	NOD-like receptor
NLRP3	NOD-like receptor pyrin-containing protein 3
NOD	nucleotide-binding oligomerization
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffer saline
PRR	pathogen recognition receptor
RBC	red blood cells
RIG	retinoic acid-inducible gene
RLR	RIG-I-like receptor
ROS	reactive oxygen species
SEM	standard error of the mean
SD	standard deviation
SHIP	Src-homology region 2-containing inositol 5’ phosphatase
SHP	Src-homology region 2-containing phosphatase
Src	sarcoma
Syk	spleen tyrosine kinase
TCR	T-cell receptor
T_h-cells	T-helper-cells
TNF	tumour-necrosis factor
TLR	toll-like receptor
T_{reg}-cells	T-regulatory-cells
UA	uric acid

1 Introduction

The immune system not only provides protection against pathogens and other environmental factors, but also plays crucial roles in wound healing and tissue homeostasis. These diverse functions are maintained by multiple complimentary mechanisms provided by the innate and adaptive immune systems. Innate immunity relies on generic, rather than antigen-specific mechanisms to provide a rapid first-line defence against pathogens and tissue damage. These are complimented by the adaptive immune response that generates an array of antigen-specific responses directed against the invading pathogen that persist to provide a long-lasting memory response that protects the host against re-infection; the response vaccines are designed to emulate.

The innate and adaptive immune systems do not exist independently of each other but interact to provide robust protection against the threats posed by viruses, bacteria, parasites and cancers. Inflammation plays a key role in this crosstalk between innate and adaptive immunity. In medicine, inflammation is characterised by five cardinal symptoms: redness, warmth, swelling, pain, and loss of function. These symptoms are a consequence of the body's response to tissue damage and/or an invading pathogen; a complex, multifactorial response that involves soluble molecules such as complement components, proteases and cytokines, immune cells and epithelial cells, endothelial cells and fibroblasts within the affected tissue itself. Properly controlled, inflammation represents a protective response that not only contributes to pathogen elimination, tissue repair and restoration of homeostasis, but also stimulates development of adaptive immunity (Chovatiya & Medzhitov, 2014).This latter "adjuvant" effect is exploited in vaccine development to activate the immune system and drive development of a long lasting immunity to the target of interest.

1.1 The innate immune system

The first immune system barrier encountered by a disruptor of homeostasis is the innate immune system, which consists of various leucocyte-derived cells that can phagocytise or opsonise invaders and that express chemokines, cytokines, vasoactive amines, or proteolytic cascade products.

1.1.1 Myeloid derived leucocytes

Leucocytes can be roughly divided into myeloid-derived cells and lymphocytes. Myeloid-derived leucocytes, such as neutrophils, eosinophils, basophils, macrophages, or myeloid dendritic cells are typically considered components of the innate immune system. These cells express a broad array of pattern recognition receptors (PRRs) that recognise pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) derived from

invading microorganisms and stressed, injured or dying cells, respectively, PRR signalling initiates a cascade of molecular and cellular responses that result in immune activation and inflammation. This inflammatory response is generally protective but exaggerated and insufficient responses can lead to inflammatory diseases or immunodeficiency. Gain-of-function mutations in PRR genes can exacerbate inflammation and lead to inflammatory diseases such as familial Mediterranean fever or cryopyrin-associated periodic syndrome that are caused by mutations in the inflammasome sensors pyrin and NLRP3, respectively (Krainer et al., 2020). Mutations that blunt innate immune responses are also pathogenic as demonstrated by chronic granulomatous disease. This is an immunodeficiency syndrome caused by mutations that make nicotinamide adenine dinucleotide phosphate (NADPH) oxidase dysfunctional resulting in impaired production of reactive oxygen species (ROS) and failure to suppress bacterial infection (Roos et al., 1996). Additionally, since their critical role in homeostasis and disease, components of the innate immune response can represent therapeutic targets for some diseases. For example, TNF inhibitors gain in importance in the therapy of rheumatoid arthritis (Radner & Aletaha, 2015).

Neutrophils are an important cell type and are the most abundant leucocytes found in blood. Bone marrow produces an estimated 10^{11} neutrophils daily and they are the most abundant leukocyte population in blood, representing approximately 50-70% of the total leucocyte population; a value that increases significantly in diseases (Kolaczkowska & Kubes, 2013). In *in vitro* cultures, the half-life of a neutrophil has been estimated to be approximately 12 hours (Kobayashi et al., 2003; Sabroe et al., 2004). Also the exact half-life of circulating neutrophils remains unclear but a majority of studies indicate it is hours rather than days (Murphy & Weaver, 2018; Tak et al., 2013). The primary task of circulating neutrophils is to provide a defence against invading microbes (Nauseef & Borregaard, 2014). This is achieved by recruiting neutrophils to sites of inflammation or infection, by chemokines or cytokines, through a process called chemotaxis. Once recruited, neutrophils can be activated by a variety of stimuli to trigger different cascades. Their effector functions include the phagocytosis of pathogens, the degranulation of antimicrobial molecules, formation of neutrophil extracellular traps (NETs) as well as production of cytokines, chemokines and the destruction of pathogens in phagosomes, through the formation of reactive oxygen species (ROS). These highly potent effector mechanisms provide a rapid first line defence against intruders such as bacterial and fungal infections but lack target specificity making unwanted collateral damage to host tissues mostly unavoidable (Nathan, 2006; van Rees et al., 2016).

Tissue damage caused by neutrophil-dependent mechanisms can be extensive and contribute to the pathogenesis of many chronic inflammatory diseases including rheumatoid arthritis (Fresneda Alarcon et al., 2021). However neutrophils have fulfilled their function, for example, the infection has been removed, they regularly undergo apoptosis and inflammation resolves (Wright et al., 2010). In addition to these direct effects, neutrophils themselves can also release an array of

cytokines and chemokines that amplify inflammatory reactions also by recruiting and activating other immune cells. These include dendritic cells whose activation and mobilisation at sites of inflammation stimulates the adaptive immune system to develop antigen specific immune response. Neutrophils can therefore play a key role in the pathogenesis of sterile, viral and bacterial infections, autoimmune diseases, arteriosclerosis and many other diseases (Jaillon et al., 2013; Mocsai, 2013).

1.1.2 Antigen-presenting cells

Antigen-presenting cells (APC)s can present antigenic peptides bound to Major Histocompability Complex (MHC) proteins to other cellular components of the immune system. When a cell becomes infected or alternatively an APC phagocytoses a pathogen, pathogen-derived peptides are processed and bound to MHC molecules before translocation to the cell surface. MHC class I proteins are loaded with peptides derived from the intracellular or cytoplasmic milieu which can then be recognised by T-cell receptors on cluster of differentiation (CD)8^+ T-cells, which are and MHC I restricted. Virtually all cells are MHC class I^+ making this a generalised mechanism by which the immune system recognise cells infected with viruses or other intracellular pathogens. In contrast, endogenous expression of MHC II proteins is restricted to "professional" APC which present peptides derived from phagocytosed material to naïve $CD4^+$ T-cells, which are MHC II restricted. (Banchereau & Steinman, 1998). The most important three professional APC types are dendritic cells (DC)s, macrophages (MP)s and B-cells; cross talk between the innate immune system and these cells being essential for the effective induction of adaptive immunity (Akira, 2011; Iwasaki & Medzhitov, 2015).

1.2 The Adaptive Immune System

To induce a more specific immune response to pathogens and a "memory" function in case of a reinfection, a second type of immune response is necessary: adaptive immunity. Immature DCs express, as do neutrophils and MPs, surface PRRs that recognise conserved pathogen-derived molecules such as bacterial lipopolysaccharides (LPS). Recognition of these molecules activates and mobilises DCs which migrate into peripheral lymphatic tissue to mature and present antigenic peptides to T-cells. Full activation of antigen-specific T-lymphocytes by DCs not only requires interactions between MHC/peptide complexes at the DC surface and an antigen-specific TcR, but also a variety of co-stimulatory molecules, (Murphy & Weaver, 2018). Only then can the host develop an effective antigen-specific T-cell response and immune memory, insufficient engagement of co-stimulatory molecules resulting in T-cell anergy or death. However, these antigen-specific responses may sometimes target self-antigens leading to autoimmunity.

1.2.1 B lymphocytes

The B-cell arm of the adaptive immune response evolved to provide a humoral antigen-specific defence against pathogens in which antibodies specifically bind to microbes facilitating their recognition and destruction by the immune system, primarily by activating complement and enhancing phagocytosis by macrophages. B-cells carry a randomly rearranged unique receptor for foreign antigens, called B-cell antigen receptor (BCR). It consists of one immunoglobulin molecule and CD79, a heterodimer both containing an immunoreceptor tyrosine-based activation motif (ITAM)s in their cytoplasmic tail (Seda & Mraz, 2015). The characteristics of ITAMs will be explained in chapter 1.3.1.

Antigen-specific B-cell activation requires either BCR crosslinking by a polymeric antigen, a T-cell-independent response, or in the case of monomeric antigens help provided by T-helper-cells, which recognize the same antigen, a T-cell-dependent response. BCR cross linking by polymeric antigens results in ITAM phosphorylation, downstream activation of transcription factors such as nuclear factor (NF)-κB resulting in B-cell proliferation (Ruland & Mak, 2003) and ultimately, differentiation into antibody secreting plasma cells.

When confronted by a non-polymeric antigen B-cells depend on co-activation by T-cells to drive their activation and subsequently, Ig affinity maturation and class switching. This depends on the ability of B-cells to act as professional APCs that express MHC II and the co-stimulatory receptor complex B7 (CD80 and CD86) on their surface; B7 being a ligand for CD28 expressed on the surface of on naïve T-cells {Figure 1.1}. These cognate interactions result in upregulation of CD40 on B-cells which in turn binds to CD40L on the T-cells triggering B-cell proliferation, immunoglobulin affinity maturation and class switching (Murphy & Weaver, 2018). Ultimately, activated B-cells can differentiate into antibody producing cells (plasma cells), some of which are long lived to provide lasting, pathogen-specific defence.

Antibodies consist of two functionally and structurally distinct domains: an antigen binding site and an effector domain that enables them to activate the complement cascade as well as immune cells via their Fc receptors. The amino acid composition of the region that recognises antigens differs substantially between antibody clones and is therefore called the variable region (V-region). Simply explained, the antibody shape results in a Y where the V-region forms the top. In this region the tip areas are especially variable: the hypervariable regions. Through secondary protein structures this generates a very specific antigen-binding site. To increase the affinity for the antigen, mutations are introduced via a process called somatic hypermutation. This somatic hypermutation and random recombination of gene segments results in an immense variability of different antigen binding sites (Teng & Papavasiliou, 2007). The region responsible for effector mechanisms does not vary in structure and is therefore the constant region (C-region), consisting of an Fc-fragment, which forms the base of the Y (Murphy & Weaver, 2018).

Antibodies in mammals can appear in five different isotypes: immunoglobulin

(Ig)A, IgD, IgE, IgG, IgM. The last letter stands for the heavy chain the antibody contains, like α-chain in IgA. IgM is the first immunoglobulin secreted upon antigen recognition, which has the lowest affinity because it did not undergo somatic hypermutation. Together with IgD IgM is expressed on naïve B-cells (Seifert & Kuppers, 2016). However, with the additional help of T-cells, a B-cell can undergo class switch recombination to a more specialized immunoglobulin. This class switch only occurs in the C-region and thus results in a different effector function. IgA is mainly secreted by plasma cells in the mucosa in the mouth, intestinal system and respiratory tract and contributes to immune defence at these barriers; the dominant role of IgE is triggering mast cells activation and histamine release whilst IgG, the smallest immunoglobulin can penetrate tissue easier and is the only one able to cross the placenta to contribute to fetal immunity (Murphy & Weaver, 2018). IgG consists of different subclasses, IgG1, IgG2a, IgG2b, IgG3 and IgG4. These subclasses differ in their affinity to bin to Fc-receptors and activate complement (Bruhns et al., 2009). So, although IgG1 is the most common subclass, IgG3 has a high affinity to complement C1q leading to stronger complement activation (Kapur et al., 2014). And isolated IgG2 elevation for instance, is seen in irritatable bowel disease (Engelhart et al., 2017). Their different roles, however, are still being investigated.

1.2.2 T lymphocytes

T-cells recognise their antigen in the context of MHC molecules via their T-cell receptor (TCR). A majority of T-cells express TCRs consisting of an α- and a β-polypeptide chain, which are similar in structure to the fab-fragments (C-region) of immunoglobulins. Other T-cell subsets exist that express TCRs composed of a γ- and a δ-chain (Murphy & Weaver, 2018), but in the context of vaccination, the focus of this dissertation, $CD4^+$ and $CD8^+$ T-cells, which are αβ-T-cells, are the most relevant.

Apart the TCR and MHC there are other surface proteins crucial for T-cell activation. These include CD4 and CD8 that define T-cell populations with different effector functions as well as costimulatory molecules without which T-cell activation fails resulting in anergy or apoptosis. In addition, TCRs interact with CD3 which is required to mediate downstream signalling (Murphy & Weaver, 2018). Once a naïve T-cell is activated, it secretes different cytokines, depending on its T-cell type, enhancing different mechanisms of the immune system {Figure 1.1}.

$CD8^+$ T-cells, also called cytotoxic T-cells, recognise antigens presented in the context of MHC I molecules and are particularly responsible for the direct elimination of infected cells by secreting perforins and granzymes. Perforins lead to pores in the membrane while granzymes are proteases which infiltrate the target cell resulting in caspase activation and apoptosis.

$CD4^+$ T-cells on the other hand “help” other immune cells to be activated in a manner appropriate to the challenge the immune system is faced e.g., endogenous, bacteria, fungi, and are therefore called T-helper (T_h)-cells. T_h-cells differentiation is orchestrated by cytokines resulting in numerous T_h subsets defined by expression of

transcription factors and secretion of cytokines that determine their effector function (Figure 1.1). This is a complex and continually expanding area of research, hence the following serves only to provide an incomplete overview of this topic. Briefly, T_h1-cells produce type I interferons (IFN)-γ and thereby induce a cascade of responses that provide defence against intracellular bacteria and protozoa. In contrast T_h2-cells secrete cytokines that drive B-cell proliferation and immunoglobulin class switching. These include IL-4 which for instance mediate class switching to IgE and IgG1 to enhance eosinophil activation. Other subsets induce T_h17-cells which play important roles in anti-fungal immunity by inducing inflammation although this may also induce collateral tissue damage especially at the beginning of T_h differentiation (Bedoya et al., 2013).

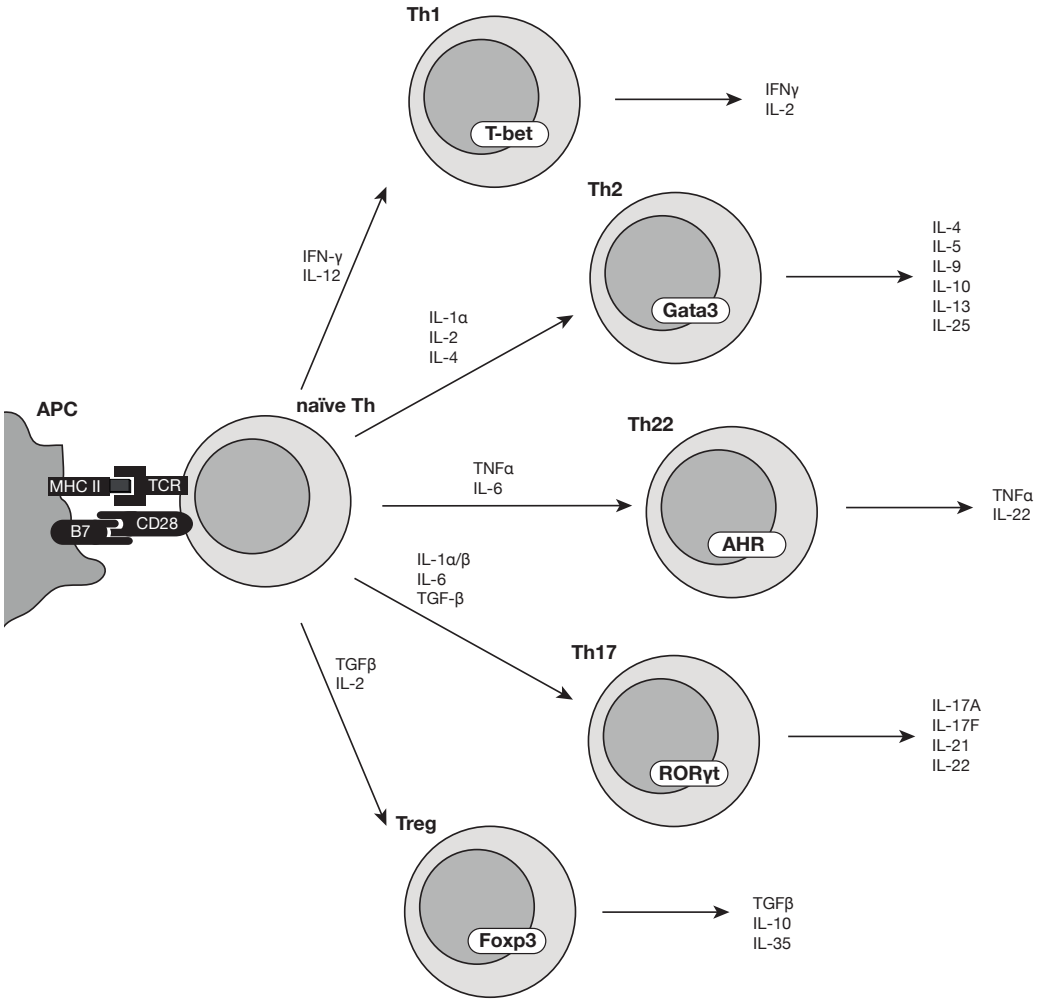

Figure 1.1 Activation and differentiation of T-cells

Dendritic cells present the processed antigen to $CD4^+$ naïve T_h-cells to stimulate these to differentiate into divergent types of T_h-cells. Depending on the cytokines present, the naïve T_h-cell differs into distinct T_h-cell, which in turn produces their special cytokines to activate or inhibit further immune response. Adapted from (Fard et al., 2016; Magee et al., 2012)

This is controlled by a subset T-regulatory (T_{reg})-cells that are anti-inflammatory and regulate differentiation of effector T-cell response. T_{reg}-cells play crucial roles in maintaining homeostasis, limiting collateral tissue damage and development of autoimmunity, for example by producing IL-10 and inhibiting differentiation and activation T_h1- and T_h17-cells. However, T_{reg}-cells might also contribute to pathogen survival if they inhibit inflammatory responses before pathogen removal is complete, whilst ineffective generation of T_{reg}-cell response can result in chronic inflammation (Murphy & Weaver, 2018). In either case, dysfunction of T_{reg}-cells may lead to autoimmune disease, immune deficiency or cancer (Miyara et al., 2011), although more recent studies show promising progress in exploiting T_{reg}-cells as tools to prevent from graft versus host disease (Brunstein et al., 2016).

1.3 Pattern Recognition Receptors

Germline encoded PRRs are proteins that play a key role in activating innate immune responses following infection or tissue damage. PRRs are able to recognise microorganisms. They do so, by recognizing conserved molecular motifs derived from pathogens – pathogen-associated molecular patterns (PAMPs) – or damaged cells – damage associated molecular patterns (DAMPs). For example, some PRRs such as Toll-like receptor (TLR) 4 recognise lipopolysaccharides (LPS) from gram-negative bacteria (Hoshino et al., 1999) as PAMPs. Importantly, PRRs not only sense exogenous, but also endogenous molecules released from damaged cells: DAMPs.

Alternatively, disrupting cellular integrity results in release of certain cellular constituents including potassium ions, adenosine triphosphate, uric acid, high-mobility group box 1 protein (HMGB1) and several S100 calcium-binding proteins (Bianchi, 2007; Hornung et al., 2008) all of which are DAMPs that through activation of PRRs result in an acute inflammation, which is called sterile inflammation. These different causes of inflammation are likely to have different characteristics. Obtaining deeper insight into how they result in either a physiological or a continuing pathological outcome would be helpful for understanding the mechanism for later use in treatment development.

Several classes of PRRs have been characterized including transmembrane Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic proteins such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide-binding oligomerisation domain-containing protein (NOD)-like receptors (NLRs) and absent in melanoma-2 (AIM2)-like receptors (ALRs) (Li & Wu, 2021). These receptors are mainly expressed by innate immune cells, while some are also expressed by lymphocytes or even non-immune cells.

These PRRs recognise a wide variety of PAMPs and therefore play a crucial role in triggering early host defence against many different pathogens (Beutler & Rietschel, 2003). TLRs are transmembrane receptors found on the cell surface and in endolysosomal compartments and therefore detect extracellular and phagocytosed

PAMPs. The best-known member of this group is TLR4, which recognises LPS, as mentioned above.

In contrast NLRs are cytoplasmic PRRs that can detect both PAMPs and DAMPs, although their complete recognition profile of NLRs is still under investigation. An important representative of the NLRs is the NOD-like receptor pyrin-containing protein 3 (NLRP3), which is activated by very diverse triggers including crystals that damage lysosomes after phagocytosis.

RLRs are cytoplasmic PRRs for RNA virions, such as Herpesviridae or othomyxoviridae, located in the cytoplasm or the cell nucleus (Liu et al., 2018). Their activation of the inflammatory cascade leads to an amplified induction of interferon (IFN) leading to a “antiviral environment” in the afflicted cell (Lee et al., 2019).

1.3.1 C-type lectin receptors

The proteins of the C-type lectin group all contain an independently folding, modular carbohydrate-recognising domain (CRD), which in most cases binds sugars in a Ca^{2+}-dependent manner. However, a proportion of C-type lectins lack Ca^{2+} binding sites and are referred to as C-type lectin-like domain (CTLD) proteins (Zelensky & Gready, 2005). Depending on their cytoplasmic tail, CTLD can be divided into different groups, most of which include receptors with immunity-regulating capacities, containing immunoreceptor tyrosine-based activation motifs (ITAMs) or ITAM-like motifs {Figure 1.2}. These motifs regulate induction of a variety of key cellular responses, including phagocytosis and degranulation, cell migration, adhesion, proliferation and differentiation, and gene induction (Fodor et al., 2006; Kaczmarek et al., 2013; Zitvogel et al., 2010). Following receptor engagement, the endoplasmic ITAM containing tyrosine becomes phosphorylated by Src kinases.

However, the immune system contains both activating and inhibitory pathways, and some C-type lectin receptors (CLRs) contain immunoreceptor tyrosine-based inhibition motifs (ITIMs) {Figure 1.2}. Their phosphorylation leads to phosphatase activation and thus to an inhibition of an ITAM induced pathway.

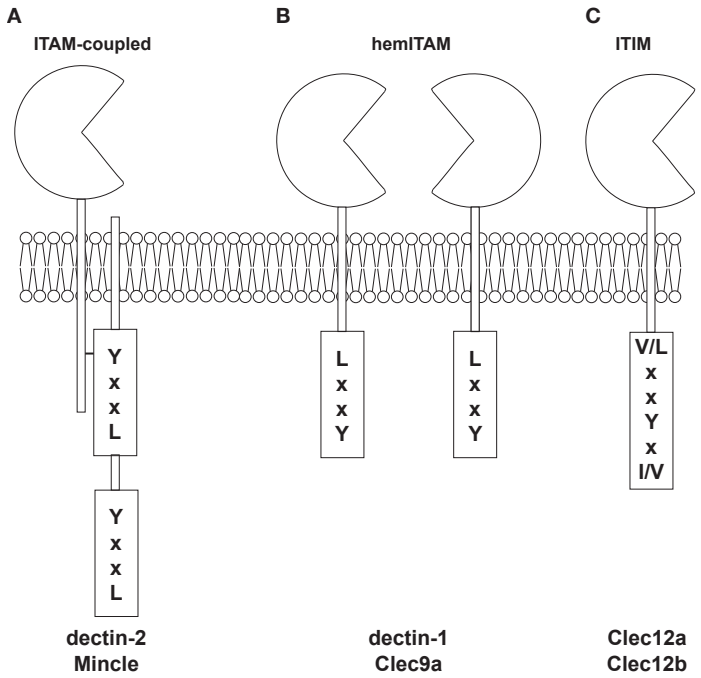

Figure 1.2 Intracellular signalling motifs of myeloid CLRs

The binding motifs contain a four-amino acid sequence whereas Y stand for tyrosine, L for leucine, I for isoleucine V, for valine and x can stand for any amino acid

A CLRs like dectin-2 associate with intracellular ITAM-bearing motifs like the FcRγ chain containing a tandem repeat of YxxL/I and thereby activate Syk

B hemITAMs only possess one amino acid sequence and therefore needs two receptors to completely activate Syk

C Inhibitory CLRs containing an ITIM can recruit SHP-1 and/or SHP-2

This figure was adapted from (Sancho & Reis e Sousa, 2012)

Similar to other PRRs, CLRs can sense both exogenous and endogenous molecules and can influence the immune system in an activating or inhibitory manner. For example, dectin-1, also known as Clec7a, recognises β-glucans, residing in the cell wall of fungi (Blanco-Menendez et al., 2015). Clec9a, also known as dendritic cell NK lectin group receptor-1 (DNGR-1) recognizes the actin filaments of damaged or dead cells (Ahrens et al., 2012; Zhang et al., 2012). Furthermore, Clec4e, also known as macrophage inducible Ca^{2+}-dependent lectin receptor (Mincle) senses spliceosome associated protein (SAP)-130 in necrotic cells (Yamasaki et al., 2008). In contrast, Clec12a, an ITIM bearing CLR, recognizes monosodium urate crystals (MSU) released by cells undergoing apoptosis (Neumann et al., 2014).

The signalling pathways of activating CLRs has been well studied: Once an ITAM or hemITAM containing CLR has bound to its activating ligand, the tyrosines of the ITAM or hemITAM are phosphorylated by Src kinases. Two associated phosphorylated tyrosines subsequently lead to the recruitment and activation of the Spleen tyrosine kinase (Syk) (Mocsai et al., 2010).

Syk also mediates the signalling in B lymphocytes downstream of the ITAM-

containing BCR and downstream of various other immune receptors like Fc-receptors (Mocsai et al., 2010). In myeloid cells, Syk activation leads to downstream activation of protein kinase C delta type (PKCδ), which in turn activates caspase recruitment domain-containing protein 9 (CARD9) (Strasser et al., 2012). The CARD9/B-cell lymphoma 10 (BCL10)/Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) pathway activates NF-κB {Figure 1.3}, which induces inflammatory genes and also activates the NLRP3 inflammasome, which induces IL-1β maturation following infection by Candida albicans (Gross et al., 2009), as well as IL-18 and pyroptosis, an inflammatory type of cell death (Keyel, 2014). These cytokines induce inflammation and the stimulation of a T_h17 immune response (Martinon et al.2009). Furthermore, Martinon et al. demonstrated that MSU in gout also activates the NLRP3 inflammasome via Syk, resulting in IL-1β production (Martinon et al., 2006).

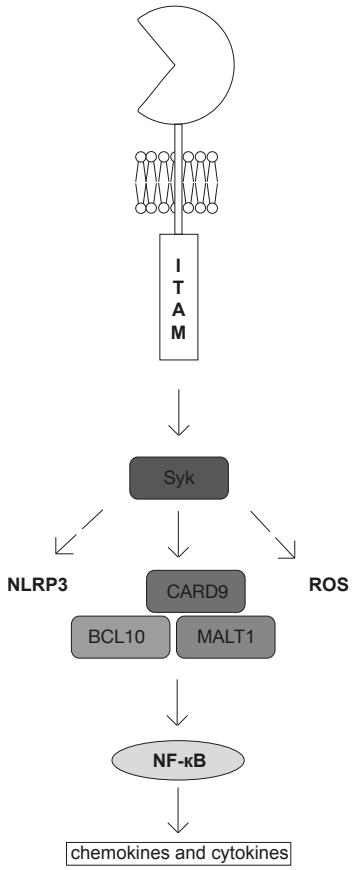

Figure 1.3 Syk pathway

Syk is recruited and activated by ITAM containing CLRs. Syk activation leads to the production of pro inflammatory cytokines, the production of ROS and the activation of the NLRP3 inflammasome

Independent of the pathway described above, Syk activation in myeloid cells leads to NADPH oxidase activation and ROS generation, the so-called respiratory burst (Sancho & Reis e Sousa, 2012). Other receptors may collaborate with CLRs, as

dectin-1 appears to require the assistance of Complement receptor 3 (CD11b) for phagocytosis (van Bruggen et al., 2009) and ROS production (Li et al., 2011), which can also signal via Syk (Mocsai et al., 2006).

1.4 Clec12a

One insufficiently characterised ITIM-containing CLR is Clec12a, which is also known as myeloid inhibitory C-type lectin (MICL), dendritic cell (DC)-associated C-type lectin 2 (DCAL2) or C-type lectin-like molecule-1 (CLL-1) and encoded within the dectin-1-cluster (Sobanov et al., 2001). Clec12a is a type II transmembrane receptor, whose cytoplasmic tail signals through one ITIM. It becomes phosphorylated after receptor binding, resulting in the phosphatase recruitment of Src-homology region 2-containing phosphate (SHP)-1 and SHP-2. Clec12a phosphorylation has been shown to exert an inhibitory effect on Syk signalling (Han et al., 2004; Marshall et al., 2004). Some ITIM-containing receptors recruit the inhibitory Src-homology region 2-containing inositol 5' phosphate (SHIP). However, Pyz et al. did not detect any recruitment of SHIP after Clec12a activation (Pyz et al., 2008). SHP-1, like other inhibitory signalling molecules, acts downstream of a variety of immune inhibitory receptors that counterbalance activating immunoreceptors (van Rees et al., 2016).

Clec12a is found on eosinophils and basophils, on bone marrow-derived DCs and MPs, and on B lymphocytes but does not appear to be expressed on peripheral blood natural killer cells or mature $CD4^+$ cells (Lahoud et al., 2009; Marshall et al., 2006; Pyz et al., 2008). Moreover, murine (m)Clec12a has been found to be highly expressed on peripheral blood monocytes, neutrophils, and plasmacytoid DCs (Marshall et al., 2004). In general, mClec12a appears to have a broader cellular distribution than hClec12a. Furthermore, hClec12a is expressed on primary acute myeloid leukaemia blasts and may represent a promising diagnostic and therapeutic target (Bakker et al., 2004). Additionally, hClec12a is downregulated in reaction to inflammation, which could indicate that Clec12a modulates cellular activity in the absence of inflammatory stimuli (Marshall et al., 2006; Pyz et al., 2008). Furthermore, shortly before the start of my dissertation, in the laboratory or Jürgen Ruland, Clec12a was sown to bind to dead cells and inhibits inflammation in response to certain necrotic cells, indicating Clec12a may inhibit PRRs activated by dead cells (Neumann et al., 2014). Notably, two activating CLRs had also been shown to also recognize dead cells: Mincle and Clec9a (Sancho et al., 2009; Yamasaki et al., 2008). The CTLD of human Clec12a (hClec12a) shares 49% identity with the CTLD of murine Clec12a (mClec12a).

1.5 Potential ligands to Clec12a

Recent research demonstrated Clec12a is activated by certain necrotic cells. Neumann et al. demonstrated that Clec12a directly recognises monosodium urate crystals (MSU), which may form following release of uric acid (UA) from dying cells. Binding of MSU to Clec12a inhibits ROS production and inflammation (neutrophil influx) (Neumann et al., 2014).

1.5.1 MSU

MSU is a crystallized form of UA that forms in the presence of physiological levels of sodium and at physiological pH. UA is the final product from the purine degradation. In gout, MSU forms deposits in joints and other parts of the body leading to local inflammation. It is evident that a high concentration of UA in blood, induced for example by a purine rich diet, is the main risk factor for precipitation of MSU. MSU induces sterile inflammation by the activation of the NLRP3 inflammasome and further activates immune cells including DCs and MPs (Martinon et al., 2006).

Back in 1849 Alfred Garrod linked high levels of UA to gouty arthritis in human beings. Other mammals are protected from gout due to the enzyme uricase, which converts UA to allantoin, which is much more soluble than UA. Later on it was determined that high levels of UA also have a correlation to cardiovascular pathologies and hypertension (Feig et al., 2008; Rock et al., 2013).

Additionally, Shi et al. identified MSU as an endogenous DAMP of dying cells that can induce adaptive immune responses against foreign antigens (Yan Shi et al., 2003). Dead cells release UA, which is also important during treatment malignancies with cytotoxic medications, as high levels of UA contribute to the development of tumour lysis syndrome (Gupta & Moore, 2018).

If activating transmembrane receptors for MSU crystals exist, is still unclear. MSU crystals can activate Syk in dendritic cells by direct lipid membrane binding independent of transmembrane proteins (Ng et al., 2008) and in neutrophils by a mechanism involving CD16 and CD11b (Barabe et al., 1998). CD11b seeming to be crucial for phagocytosis in these cells. Phagocytosis of MSU in turn leads to activation of the NLRP3 inflammasome (Martinon et al., 2006).

While the induction of inflammation by MSU crystals as evidenced in gout is well established, its role in activating the adaptive immune responses is less clear. MSU crystals induce upregulation of co-stimulatory molecules by dendritic cells and co-injection of MSU or UA with a foreign antigen induces $CD8^+$ T-cell responses (Kanevets et al., 2009; Y. Shi et al., 2003). The increased $CD8^+$ T-cell response has also been shown to enhance anti-tumor immunity in certain preclinical models (Y. Wang et al., 2015). Similar to the widely used adjuvant alum, MSU crystals also induce a strong antigen-specific T_h2-type immune response characterized by production of IgE or IgG1 (Kool et al., 2011). One report even suggests that alum requires uric acid production for its adjuvanticity (Kool et al., 2008). Despite these adjuvant effects of

MSU, autoimmunity does not seem to develop in patients in gout, suggesting the adjuvant effect is not strong enough to induce adaptive responses to self-proteins (Dalbeth et al., 2021).

1.5.2 Alum, other crystals and vaccination

MSU is not the only crystal activating the immune system. There are many crystals that can harm the body by inducing inflammation (Mulay & Anders, 2016). One crystal, for instance, is Silica, which is a natural quartz, the main constituent of sand. Permanently inhaled silica leads to an inflammatory lung disease. Still, inflammation induced by crystals can also be exploited in medical treatments as in vaccination. Many antigens are insufficiently immunogenic to induce a long lasting, high titre antibody response. This problem can be overcome by adding so called adjuvants to the vaccine formulation. These enhance the immune response to the antigen and thus induce a far stronger antibody production.

Alum, a mixture of aluminium salts, principally aluminium potassium sulphate and aluminium hydroxide, has been used for a long time as an immunopotentiator in vaccines (Glenny, 1930). However, although alum is known to activate PRRs, the mechanism by which it enhances the immune response is not completely clear. Nonetheless, alum is still the most regularly used adjuvant in clinical applications (Shah et al., 2017). Alum, similar to MSU crystals, activates the complement system, dendritic cells, the NLRP3 inflammasome, and induces inflammatory cell death, which leads to the release of DAMPs.

However, the role of any one of these activities in respect to its activity as an adjuvant promoting antibody production remains unclear (Kool et al., 2012). For example, two studies found the NLRP3 inflammasome is required for alum-induced antibody production, whilst other studies found normal antibody production in response to alum (Eisenbarth et al., 2008; Franchi & Nunez, 2008; Kool et al., 2008; Li et al., 2008). Intriguingly, alum injected intraperitoneally increases the local level of UA suggesting this may play a key role in its role as an adjuvant, a concept supported by the observation its adjuvant effects are reduced in the presence of uricase (Kool et al., 2008).

Atomic force microscopy demonstrated that similar to MSU crystals, alum binds to plasma membrane lipids of DCs. This induces Syk activation, which seems to be required for the adjuvant effect of alum (Flach et al., 2011). Recently, the same group showed that an intracellular protein containing an ITAM sequences senses the deformation of the plasma membrane by solid structures like alum (Mu et al., 2018). This is in line with findings that alum enhances uptake and presentation of alum-associated antigens (Ghimire et al., 2012). Recent findings indicate that strong binding of antigens to alum particles is required for the adjuvanticity, suggesting formation of alum-antigen particles enhance transport, uptake and presentation of antigens (Moyer et al., 2020).

Together, these papers indicate that there are several mechanisms by which alum and MSU crystals may alter adaptive immune responses. If Clec12a exclusively binds to MSU crystals or also alum, and if it regulates any adaptive immune responses is unknown.

1.6 Investigative approach and objectives

This dissertation focuses on the inhibitory PRR, Clec12a. Before the start of this dissertation, the laboratory of Jürgen Ruland generated Clec12a-deficient mice, and identified necrotic cells and MSU crystals as Clec12a ligands. Preliminary *in vivo* models had also already established Clec12a inhibited MSU crystal-induced and necrotic cell-induced influx of neutrophils at sites of inflammation *in vivo*.

The first objective of this dissertation was to perform experiments using Clec12a-deficient mice to extend these initial findings to another model of cell death induced sterile inflammation. Experimental cell death can be induced extracorporeally by many different methods for example by a freeze-thaw cycling. Within the body exposure to gamma irradiation or X-ray radiation induces cell death (Fujita et al., 1997). Since double-positive thymocytes (positive for CD4 and CD8) are particularly susceptible to gamma radiation, they die soon after exposure to low-dose irradiation compared to other cell types. This model was chosen because this inflammation partly depends on the activating CLR Mincle (Yamasaki et al., 2008), which may be sensitive to inhibition by the inhibitory Clec12a. Another method to induce death of thymocytes *in vivo* is intraperitoneal injection of dexamethasone (Fearnhead et al., 1994).

The second objective was to determine if Clec12a also recognizes other immunostimulatory crystals than MSU, in particular alum. This was investigated *in vitro* by the direct binding to Clec12a-recombinant fusion proteins, green fluorescence protein (GFP)-induction of Clec12a-CD3ζ hybridoma, control of upregulation of costimulatory receptors and Syk induced ROS production following MSU or alum stimulation of neutrophils, dendritic cells and macrophages.

The third objective was to investigate if Clec12a regulates adaptive immune responses to MSU crystals or alum using *in vivo* antibody production and T cell activation in response to ovalbumin as read outs.

2 Mice and Materials

2.1 Mice

CL57BL/6J mice were purchased from the Jackson Laboratory. Clec12a$^{-/-}$ mice were bred as previously described (Neumann et al., 2014). Knockout mice and wild-type mice were cross-bred over at least eight generations. Only mice of the same genetic background were used. Sex matched mice were used at the age of 8-12 weeks for all experiments. All mice were bred and housed under specific pathogen-free conditions, according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines in „Zentrum für präklinische Forschung“. The Regierung von Oberbayern approved all animal experiments.

2.2 Chemicals and reagents

All media and supplements were purchased from Gibco if not stated otherwise.
Medium used:
RPMI 5%: RPMI-1640 medium supplemented with 5% fetal calf serum (FCS) not heat inactivated, 1% penicillin plus streptomycin, 1% L-glutamine and 0.1% 2-mercaptoethanol.
RPMI 10%, DMEM 5%, DMEM 10% are supplemented respectively.

A detailed description of additional chemicals and reagents and kit information is provided in the respective methods section.

2.3 Cell stimulation

Name	Company
Imject Alum	Thermo Scientific, stock 40mg/ml
Aluminiumhydroxide Gel	Sigma-Aldrich
MSU	InvivoGen or Sigma-Aldrich (crystalized from UA) (Schiltz et al., 2002)
Silica crystals (1.5 mm)	Alfa Asar
Zymosan	Sigma-Aldrich
Curdlan	Wako
LPS from E. coli 0111:B4	InvivoGen

2.4 Flow cytometry

Antibodies listed below were conjugated to one of the fluorochromes: fluorescin isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin cyanin 5 (PE-Cy5),

phycoerythrin cyanin 7 (PE-Cy7), peridinin-chlorophyll proteins (PerCP), allophycocyanin (APC), allophycocyanin cyanin 7 (APC-Cy7) or eFluor450 (PacBlue). Only the Fc-blocking antibody CD16/32 was unconjugated.

Name	**Label**	**Company**
7-AAD Viability Staining Solution	PerCP	eBioscience
Fixable Viability Dye eFluor 506	APC-7	eBioscience
α-human Clec12a (50C1)	PE	Southern Biotech
α-mouse CD3 (145-2C11)	APC-Cy7	eBioscience
α-mouse CD3 (145-2C11)	FITC	eBioscience
α-mouse CD4 (GK1.5)	PacBlue	eBioscience
α-mouse CD4 (GK1.5)	PE-Cy5	eBioscience
α-mouse CD8a (5H10)	APC	AbD Serotec
α-mouse CD11b (M1/70)	PE-Cy5	eBioscience
α-mouse CD19 (1D3)	PE-Cy7	eBioscience
α-mouse CD25 (PC61.5)	PE	eBioscience
α-mouse CD86 (GL1)	PE	eBioscience
α-mouse F4/80 (BM8)	PE-Cy7	eBioscience
α-mouse Gata3 (TWAJ)	PE	eBioscience
α-mouse Gr-1 (RB6-8C5)	APC	eBioscience
α-mouse Gr-1 (RB6-8C5)	PacBlue	eBioscience
α-mouse IFNγ (XMG1.2)	FITC	eBioscience
α-mouse IL-4 (11B11)	APC	eBioscience
α-mouse IL-22 (1H8PWSR)	PE-Cy5.5	eBioscience
α-mouse Ly6c (AL-21)	FITC	BD Biosciences
α-mouse Ly6c (HK1.4)	APC	eBioscience
α-mouse MHC II (M5/114.15.2)	FITC	eBioscience
α-mouse MHC II (M5/114.15.2)	APC-Cy7	eBioscience
α-mouse Tbet (4B10)	PE-Cy7	eBioscience
α-mouse TCRβ (H57-597)	FITC	eBioscience
α-mouse TCR γ/∂ (eBioGL3)	PE	eBioscience
α-mouse TNFα (MP6-XT22)	PE	BD/Pharmingen

3 Methods

3.1 Reporter Cells: generation and stimulation

Reporter cells are designed to show ligand binding to a receptor. All reporter cells were previously generated by Konstantin Neumann.

In short: the extracellular and transmembrane domain of the requested receptor on the surface of a t-cell hybridoma A5 is fused with CD3ζ. Here: cDNA encoding the transmembrane and extracellular domains of mouse Clec7a (NCBI: NP_064392.2 aa 44–244), mouse Clec9a (NCBI: NP_001192292.1 aa 31–264), mouse Clec12a (NCBI: NP_808354.1 aa 41– 267) and human Clec12a (NCBI: NP_612210 aa 40–265). Cells were counted and put in a 24 well plate with $5x10^5$ cells/ml in 500µl. Following, cells were stimulated with different concentrations of alum, MSU and Curdlan. Ligation to the receptor leads by nuclear factor of activated T-cells (NFAT) activation to GFP expression (Andersen et al., 2001; Bot et al., 1996; Neumann et al., 2014). This was analysed by EVOS FL, life technologies.

3.2 Recombinant CLR extracellular domains

Recombinant CLR extracellular domains are designed to show ligand binding to a receptor. All CLR fusion proteins were previously generated by Konstantin Neumann.

Briefly, cDNA encoding the transmembrane and extracellular domains of mouse Clec7a (NCBI: NP_064392.2 aa 44–244), mouse Clec9a (NCBI: NP_001192292.1 aa 31–264), mouse Clec12a (NCBI: NP_808354.1 aa 41– 267) and human Clec12a (NCBI: NP_612210 aa 40–265) were fused either to the C terminus of IgG1-Fc in the expression vector pFuse-hIgG1-Fc2 (Invivo Gen) or to a Strep-tag II (IBA Life Sciences) Isoleucine Zipper cassette (Watzl, 2006) with the protein sequence IHHHHHHGLSAWSHPQFEKGGGSGGGSGGGSWSHPQFEKGRMKQIEDK IEEILSKIYHIENEIARIKKLIGERGIS. Constructs were amplified in 293T cells (Neumann et al., 2014). Fusion proteins were stained with anti-human Fc-PE and measured in FACS Canto II.

3.3 Bone marrow isolation

Bone marrow (BM) was taken from all long bones in RPMI 5% and put through a 100µm mesh. Cells were centrifuged for 5 minutes at 350xg. Supernatant was discarded. Red blood cells (RBC) in the BM were lysed by adding 4ml of RBC lysis buffer (HISS Diagnostics) for maximum 5 minutes. Cells were washed in RPMI 5% and taken up in 10ml RPMI with 5% FCS.

3.4 In vitro culturing of bone marrow derived dendritic cells (BMDCs) and macrophages (BMDMs)

Cells were isolated as described above, counted and spread to gain dendritic cells (DC) on a Petri plate with $4x10^6$ cells in 10ml RPMI 10% plus 20ng/ml recombinant mouse granulocyte–macrophage colony stimulating factor (GM-CSF) (Immunotools). For macrophages (MP), cells were spread either $8x10^6$ cells on a 10cm plate in 10ml or $2x10^7$ cells on a 15cm plate in 20ml DMEM 10% plus 10% LCCM (obtained from supernatant of L929 cells). Cells were incubated at 37°C. Next day non-adherent MP were taken, counted again and spread on a new plate as described above. Dendritic cells were left untouched. On d=3 5ml of corresponding medium was added. On d=5 corresponding medium was renewed. On d=7 cells were harvested. Adherent cells were detached by plate incubation with 5ml of PBS-5mMEDTA for 15 minutes at 37°C, stopped and washed from the plate with 4ml RPMI 5% or DMEM 10% respectively.

3.5 In vitro culturing of Flt3L derived dendritic cells

Bone marrow cells were counted and resuspended at $2x10^6$ cells/ml containing 200ng/ml human recombinant Flt3L (eBioscience). Cells were plated at 5ml/well on 6 well plates and cultured for 9 days without disturbing (Xu et al., 2007). Adherent cells were detached by plate incubation with 5ml of PBS-5mMEDTA for 15 minutes at 37°C, stopped and washed from the plate with 4ml RPMI 5%.

3.6 In vitro activation of bone marrow cells and dendritic cells

Bone marrow cells (the majority of which are neutrophils) or dendritic cells from wild-type and mutant mice were counted and taken up in in RPMI 5% in a final concentration after adding stimuli of $2x10^6$ cells/ml. Cells were stimulated in different concentrations of alum, MSU, Curdlan or Zymosan for different periods. For upregulation of surface markers cells were analysed in flow cytometry.

3.7 Measurement of ROS production

A 98-wells-flat-well-plate (Thermo Fischer scientific) was blocked with 300µl DMEM plus 10% FCS and stored in the fridge one day before the experiment. Cells used were washed in Optimem with 5% FCS. Cells were counted and laid out in 100µl with $2x10^5$ cells per well. Cells were stimulated with 50µl in total. Stimulatory crystals were mixed with Optimem plus 5% FCS containing 150µM Luminol (Sigma-Aldrich). If used, inhibitors as R406, PP2 or PP3 (all Calbiochem) were added 15 minutes prior stimulation in a concentration 16x the stimuli and incubated at 37°C. ROS production was measured by chemilumineszenz every 2 minutes in a Luminometer (Berthold

Technologies, Mithras LB 940) at 37°C for 60 minutes.

3.8 Model of death thymocytes due to total body irradiation

Total body irradiation leads to double-positive thymocytes killing, which results in neutrophil influx into the thymus (Uchimura et al., 2000). The protocol was adapted from (Yamasaki et al., 2008):

Mice were total-body-irradiated with 1 Gy. After 14 hours mice were sacrificed. Thymus was removed and meshed trough a 100µm and a 70µm mesh with 1,75ml DMEM. Cells were centrifuged at 400xg for 5 minutes. Supernatant was taken for cytokine measurement. Cells were lysed with 2ml RBC lysis buffer for 5 minutes, stopped with RPMI 5%, counted and stained for flow cytometry analyses.

3.9 Model of death thymocytes due to dexamethasone injection

Injecting dexamethasone can also induce death of T cells (Xing et al., 2015). Mice were injected i.p. with 100µg Dexamethasone. Protocol was continued as described above for irradiation.

3.10 FACS analysis for cell activation after stimulation

Flow cytometry is a measurement procedure to analyse single cells. Every cell, previously fluorescently labelled, bypasses a laser that is coupled to a set of detectors, which measure the light scatter and fluorescence of each. By that size and structure of the surface of cells can be determined. Surface markers can be labelled utilizing fluorescently labelled antibodies to quantify these. After cell fixation and permeabilisation, even intracellular cell proteins can be determined by flow cytometry. Here, flow cytometry was used to ascertain and quantify immune cells after cell death in vivo or to determine expression or upregulation of surface proteins due to stimulation.

After respective cell preparation and counting, ideally $2x10^6$ cells were washed and resuspended in FACS buffer (PBS, 3% FCS) and incubated with 200µl of 1:200 diluted CD16/32 to block free Fc-receptors and the requested fluorochrome-conjugated antibodies for 20 minutes at 4°C. Antibodies were diluted either 1:200 or 1:400 in FACS buffer. 2ml FACS buffer was added to each sample tube and centrifuged at 400xg for 5 minutes. Supernatant was discarded. Cells were vortexed and resuspended in 600µl FACS buffed for analysis. In case of an amine-reactive live/dead (L/D) cell stain, this stain was the first step: Cells were washed once with FCS free PBS and stained with Amin diluted 1:1000 for 30 minutes on ice. After washing with FACS buffer it was continued as above.

FACS data were acquired on a BD FACS Canto II (BD Biosciences) and analysed with FlowJo 10 software (Tree Star).

3.11 Cytokine measurement via cytometric bead assay using flow cytometry

This method quantifies cytokines by bound fluorescent beads analysed by flow cytometry (FCM). The T_h1/T_h2 CBA-kit (eBioscience) was used. Briefly, frozen supernatant was thawed carefully on ice and was used undiluted. Standard was prepared 1:20 in Assay Buffer provided and serial diluted 1:3 for standard 1 to 7. Standard 8 was blank. On 96-well v-plate s25µl of standard was applied to the first column. Samples were added with 25µl per well. 25µl Bead Mix and 50µl Biotin-Conjugate Mix were added to all wells. Plate was incubated for 2 hours on a shaker at 500rpm at room temperature. Beads were washed with 150µl of Assay Buffer on all wells and centrifuged at 3000xg for 5 minutes. Supernatant was discarded, wash repeated. Subsequently, 150µl Streptavidin-PE-Mix (eBioscience) was added to all wells and incubated for 1 hour on a shaker at 500rpm at room temperature. Plate was washed once. Beads were resuspended in 100µl Assay buffed for analysis.

Cytokine data were acquired on a BD FACS Canto II (BD Biosciences) and analysed with FlowCytomixPro software.

3.12 Cytokine measurement via enhanced CBA

This method can detect even very low levels of cytokines. Cytokine levels were analysed using an enhanced cytometric bead array (eCBA) (BD Biosciences). Briefly, frozen supernatant or serum was thawed carefully on ice. Serum was diluted 1:4 in Assay Diluent, supernatant stayed undiluted. Standard was used in a concentration of 200000 fg/ml and 1:3 serial diluted. All samples were spread on a 96 v-well plate. Capture beads were vortexed for at least 15 seconds, diluted in Capture Bead Diluent and added to all samples. Following 2 hours of incubation at room temperature, Detection Reagent (Part A) was directly added to all wells and incubated for 2 hours. Plate was centrifuged for 5 minutes at 500xg and washed two times with wash buffer provided. Enhanced Sensitivity Detection Reagent (Part B) was added to all wells and incubated for 1 hour. Afterwards plate was washed twice. Samples were resuspended in wash buffer and analysed.

Cytokine data were acquired on a BD FACS Canto II (BD Biosciences) and analysed with FlowJo 10 software (Tree Star).

3.13 Immunisation of mice and blood sampling

The intention of an immunisation is to induce production of antibodies against the injected antigen. The solution of the antigen is mixed with an adjuvant to activate the immune response and therefore enhance the antibody production. A frequently used model antigen is ovalbumin (OVA).

Mice were injected with 20µg OVA (Invivo Gen), diluted in NaCl 0.9% (Fresenius Kabi), plus a certain amount of the adjuvant (alum or MSU) as described in results intraperitoneally (i.p.) on day 0. On day 28 an immunisation boost of 20µg OVA only was injected i.p. On day 7,14,21,35 and 42 blood samples were taken from the cheek. Serum was frozen in 2ml micro tubes from BD Bioscience at -20°C.

3.14 OVA-specific immunoglobulin measurement via Enzyme-Linked Immunosorbent Assay

The Enzyme-Linked Immunosorbent Assay (ELISA)-method quantifies immunoglobulins in serum by labelling antibody-antigen interaction with alkaline phosphatase.

Detection of serum immunoglobulins (except for IgE) of Clec12a$^{-/-}$ and wild-type mice was performed using the Mouse Immunoglobulin Panel for ELISA manufactured by Southern Biotech, using a protocol established by Konstanze Pechloff.

On day 1 a 98-flat-well maxisorb plate (Thermo scientific) was coated with 50µl on the first row with goat anti mouse IgH/L capture antibody diluted 1:500. The remaining wells were coated with OVA c=20µg/ml. The plate was stored covered in the fridge over-night. On day 2 liquid was discarded thoroughly. All wells were blocked with 400µl of blocking buffer for 1 hour at room temperature. Afterwards plates were washed three times in washing buffer. The frozen serum to be measured was thawed carefully on ice. All sera were diluted in blocking buffer. The concerning dilutions are displayed in results. The first spot of row A was covered with 100µl standard: purified mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA or total Ig respectively c=2µg/ml, row A 2-8 received 100µl of samples. Row B-H was lain out with 66,7µl blocking buffer. Dilution series was started 1:3 (33,3µl) for row B-H respectively. Plate was incubated at room temperature for one hour rocking. Liquid was discarded thoroughly and subsequently washed 3 three times. 50µl AP-labelled goat anti mouse screening antibody was diluted 1:250, added to all wells and incubated at room temperature for one hour rocking. Following the incubation, the plate was washed five times. 100µl substrate buffer (with freshly added AP substrate from Sigma Aldrich) was added on the wells and directly measured in a microplate reader (Tecan Group), using the Magellan software to detect the serum immunoglobulin levels. Time course measurements (5, 10, 15 and 20 minutes) were performed at a measurement wavelength of 450nm. The reference wavelength for background noise was 570nm.

The serum concentrations of immunoglobulins were determined by standard-curve calculations.

Reagents:

blocking buffer	1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)
washing buffer	1x PBS (Biochrom) solved in distilled water, 0,05% Tween)

3.15 IgE measurement

IgE was measured with anti-OVA IgE (mouse) EIA Kit (Cayman Chemical). Briefly, plates from Cayman Chemical were pre-coated with anti-mouse IgE and blocked. Serum was diluted 1:25 and 1:50 together with standard applied to the plated. Following 2 hours of incubation at room temperature rocking, plate was washed four times with wash buffer. OVA conjugate was added on the wells and incubated for 1 hour, and washed four times afterwards. Streptavidin-HRP was added for 30 minutes on the wells. Following incubation, the plate was washed four times and TMB was added. After approximately 15 minutes standard 1 reaches 0.5 of the optical density. This was monitored on 650nm on the plate reader. Stop Solution was added and results were measured at a wavelength of 450nm in a microplate reader (Tecan Group). The serum concentrations of immunoglobulins were determined by standard-curve calculations.

Reagents:

Wash buffer	5ml vial Wash Buffer concentrate, 2l water, 1ml Polysorbate 20
Assay buffer	10ml Immunoassay Buffer Concentrate, 90ml water
Ovalbumin-Biotin Conjugate	lyophilized Ovalbumin-Biotin Conjugate, 12ml Assay buffer
Streptavidin-HRP	1,2ml Streptavidin-HRP, 10,8ml Assay buffer

3.16 Statistical analysis

All results were analysed for statistical significance with GraphPad Prism 4 software using unpaired two-tailed student t-test or Mann–Whitney U test.

* ≈ $p < 0.05$, ** ≈ $p < 0.01$

4 Results

4.1 The role of Clec12a in homeostasis

Clec12a$^{-/-}$ mice were previously generated in the laboratory of Jürgen Ruland (Neumann et al., 2014). Clec12a$^{-/-}$ mice are apparently healthy, showing neither any obvious signs of immune or inflammatory disease under steady state conditions, nor differences in the numbers of myeloid or lymphoid cells compared to wild-type mice (Neumann et al., 2014). In line with these observations, Clec12a$^{-/-}$ mice and wild-type mice have similar blood cytokine levels under homeostatic conditions {Figure 4.1}. It was also previously demonstrated Clec12a binds to dead cells including freeze thawed or mechanically disrupted splenocytes, thymocytes, kidney and liver cells. This recognition demonstrated to inhibit the activation of neutrophils and the production of some chemokine or cytokines such as CXCL1 or tumour necrosis factor (TNF) (Neumann et al., 2014). In general, dead or dying cells express or release molecules such as uric acid, which subsequently forms MSU crystals under physiological conditions, to activate the immune system, triggering a sterile inflammatory response and removal of the dead cells. The above study therefore identifies Clec12a as a modulator of the response of the innate immune system to tissue damage.

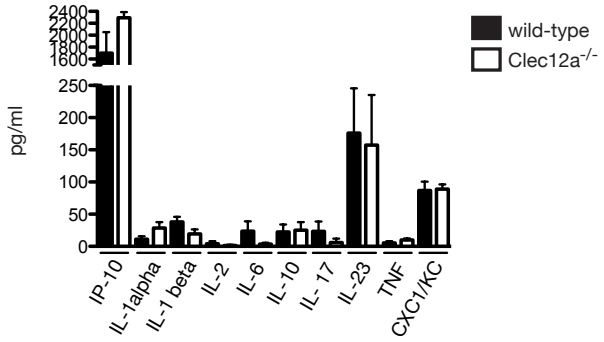

Figure 4.1 Clec12a $^{-/-}$ mice have normal levels of cytokines and chemokines in blood

Serum was taken from untreated mice. Levels of cytokines in wild-type and Clec12a$^{-/-}$ mice were analysed by a cytometric bead array. Data are means + SD of eight mice per group

4.2 The role of Clec12a in the reaction to dead cells

4.2.1 Clec12a becomes activated in the presence of dead cells and regulates inflammation

To investigate the physiological significance of Clec12a, thymocytes apoptosis was induced by low dose irradiation in Clec12a$^{-/-}$ mice and wild-type mice (Yamasaki et al., 2008). Consistently, after low-dose irradiation, neutrophil influx was observed in the thymus of all mice, but was increased significantly in Clec12a$^{-/-}$ mice compared to wild-type mice {Figure 4.2A}.

Similar data were obtained when this experiment was repeated using intraperitoneal injection of dexamethasone used to induce thymocyte cell death. Once again, neutrophil recruitment into the thymus was significantly increased in Clec12a$^{-/-}$ mice compared to wild-type mice {Figure 4.2B}.

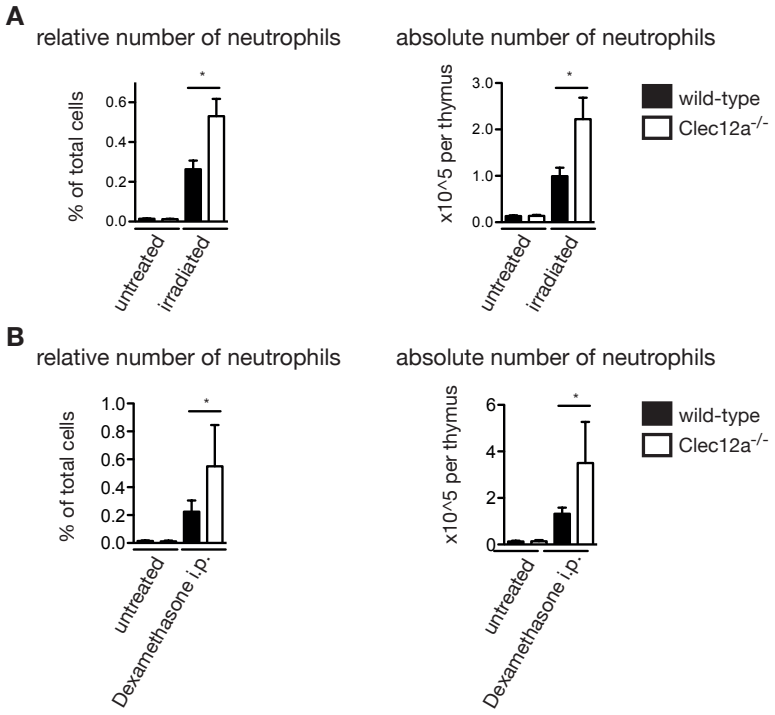

Figure 4.2 Clec12a regulates sterile inflammation in response to cell death in vivo

A wild-type and Clec12a$^{-/-}$ mice were left untreated or were total body-irradiated with 1 gray (Gy). Fourteen hours later, the fraction and total cell number of CD11b^{+}Gr-1^{hi} neutrophils in the thymus was analysed by flow cytometry. Data are means + SD of 12 mice per group for irradiated and 5 mice per group for untreated.

B wild-type and Clec12a$^{-/-}$ mice were left untreated or were injected with 100µg of dexamethasone. Fourteen hours later, the fraction and total cell number of CD11b^{+}Gr-1^{hi} neutrophils in the thymus was analysed by flow cytometry. Data are means + SD of 5 mice per group for dexamethasone injected and 5 mice per group for untreated.

4.2.2 Clec12a modulates cytokine production, due to sterile inflammation, in response to cell death

Celc12a binds specifically to monosodium urate (MSU) (Neumann et al., 2014), a crystal formed following release of uric acid by dying cells (Yan Shi et al., 2003). As earlier described, MSU signals via Syk (Mocsai et al., 2010). Furthermore, MSU has also been shown to activate the NLRP3 inflammasome, which in turn results in IL-1β production (Martinon et al., 2006). Together, these results suggest that Clec12a might inhibit the inflammasome, resulting in reduced IL-1β release. *In vitro*, however Clec12a deletion did not show any effect on MSU-induced IL-1β production (Neumann et al., 2014).

In contrast dexamethasone injection and low dose irradiation both resulted in significantly elevated levels of IL-1α in the thymus of Clec12a-deficient mice compared to wild-type controls {Figure 4.3A and C}. Intriguingly, TNF, which acts as a neutrophil-recruiting chemoattractant, and IL-1β were only elevated in Clec12a-deficient mice by dexamethasone induced cell death {Figure 4.3 B} but not low dose irradiation {Figure 4.3D}.

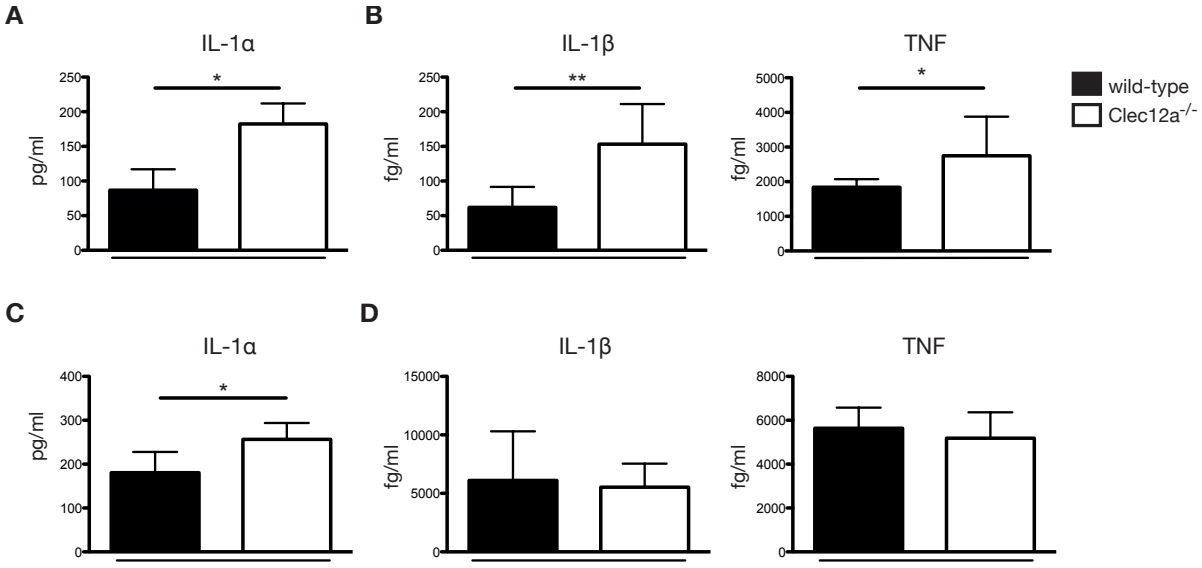

Figure 4.3 Cytokine regulation in the thymus after induction of cell death

A Supernatant of meshed thymus of dexamethasone injected mice was analysed with cytometric bead arrays for concentration of indicated cytokines in wild-type or Clec12a$^{-/-}$ mice

B Supernatant of meshed thymus of dexamethasone injected mice was analysed with enhanced cytometric bead arrays for concentration of indicated cytokines in wild-type or Clec12a$^{-/-}$ mice

C Supernatant of meshed thymus of irradiated mice was analysed with FCM for concentration of indicated cytokines in wild-type or Clec12a$^{-/-}$ mice

D Supernatant of meshed thymus of irradiated mice was analysed with enhanced cytometric bead arrays for concentration of indicated cytokines in wild-type or Clec12a$^{-/-}$ mice

Data were analysed with FlowCytomix. Data are means + SD of three mice per group and are representative of one or two independent experiments.

4.3 Clec12a specifically acts as a receptor for alum

4.3.1 Clec12a-CD3ζ reporter cells are activated by MSU and alum

As next step it was important to test, if Clec12a also recognizes other immunomodulatory crystals. For this, previously constructed reporter cells were used in which the T-cell hybridoma A5 (Bot et al., 1996) expresses a nuclear factor of activated T-cells (NFAT)-controlled green fluorescence protein {Figure 4.4}.

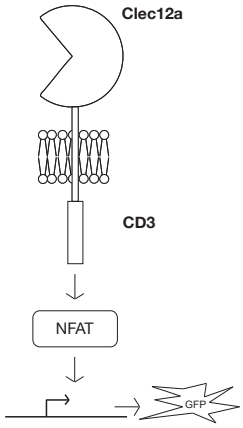

Figure 4.4 Clec12a-CD3ζ hybridoma induce GFP upon MSU or alum ligation

The transmembrane receptor Clec12a was fused on the intracellular domain of CD3. The ligation of MSU or alum results in NFAT activation, which in turn activates the IL-2 promoter transcribing the GFP reporter gene. Figure adapted from (Neumann et al., 2014)

The cells express CLR fusion-proteins (mClec7a, mClec9a, mClec12a, or hClec12a), consisting of their extracellular and transmembrane domains and the cytoplasmic signalling domain of CD3ζ. Ligation of these CLR-CD3ζ fusion proteins results in NFAT activation, which subsequently results in GFP expression {Figure 4.4} (Andersen et al., 2001). These reporter cell lines were then used to compare the ability of MSU and alum to induce GFP expression, using as a positive control Curdlan, which binds to Clec7a and induces high levels of GFP expression in Clec7a-CD3ζ reporter cells {Figure 4.5}. Clec9a-CD3ζ reporter cells showed high background fluorescence for all different conditions. As anticipated from previous studies human and murine Clec12a-CD3ζ reporter cells upregulated expression of GFP after MSU stimulation {Figure 4.5}.

However, the miouse Clec12a-CD3ζ reporter cell line also upregulated GFP expression when incubated with alum (Imject alum, Thermo Fisher Scientific). Neither MSU nor alum induced GFP expression in Clec7a-CD3ζ reporter cells indicating these were Clec12a dependent responses. Furthermore, the response induced by alum appears higher than that induced by MSU in mouse Clec12a-CD3ζ reporter cells,

although this was not observed human Clec12a-CD3ζ reporter cells {Figure 4.5}

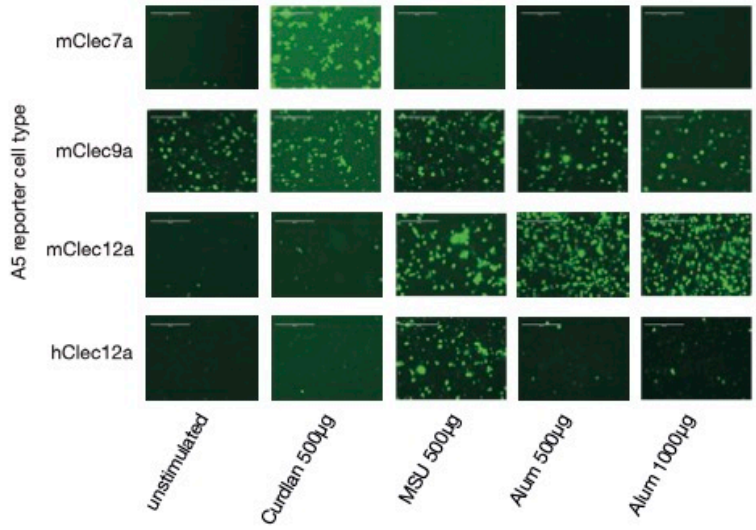

Figure 4.5 reporter cells recognise MSU and alum as ligands to Clec12a

2,5x10^5 A5 reporter cells were incubated with Curdlan 500µg, MSU 500µg, alum 500mg, alum 1000µg in a total volume of 500µl for 24 hours. GFP fluorescence was analysed by fluorescence microscopy. Data are representative of two independent experiments

4.3.2 Clec12a interaction with MSU or alum can be blocked by a monoclonal antibody

How proteins could specifically bind to a crystal surface remains unclear. To exclude the possibility Clec12a binds non-specifically to these crystal surfaces, the ability to block this interaction was tested using a monoclonal antibody against the extracellular domain of Clec12a.

CLR-Fc-fusion proteins were previously constructed (Neumann et al., 2014), fusing either the human or murine Clec12a extracellular domain with a Fc-component of immunoglobulin G (IgG) {Figure 4.6}. Incubating these fusion proteins with MSU or alum in the presence or absence of the monoclonal antibody or an isotype control revealed the antibody reduced binding of both the ligands of Clec12a {Figure 4.7A}. Fusion proteins containing Clec7 and Clec9a did not bind to MSU or alum, again indicating this interaction is Clec12a specific.

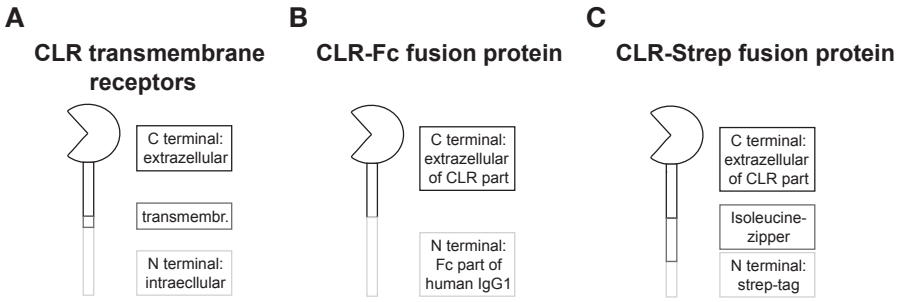

Figure 4.6 CLR fusion proteins

A schematic CLR

B and **C** schematic CLR fusion proteins

These observations not only identify alum as an additional ligand for Clec12a, but based on its co-recognition of MSU also indicates Clec12a does not specifically recognise the uric acid in MSU crystals, but rather a crystal surface structure common to both ligands.

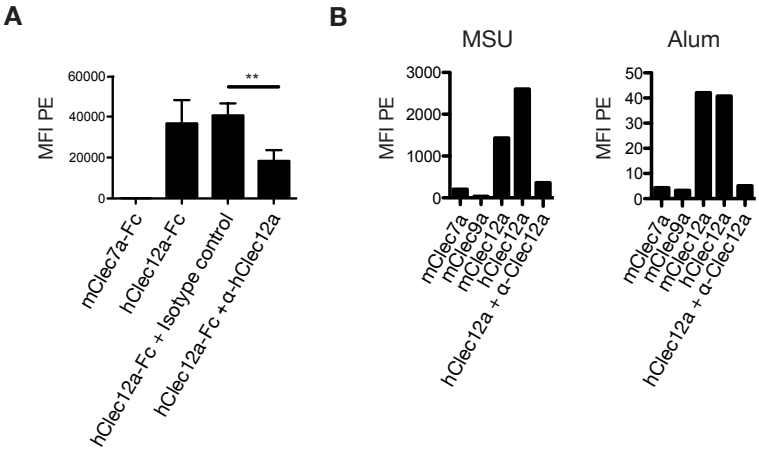

Figure 4.7 MSU and alum bind to Clec12a fusion proteins

A CLR-Fc-fusion proteins were incubated with 20µg/ml monoclonal blocking antibodies to human Clec12a or an isotype control for 20 minutes at room temperature. Following, 100µg MSU was added and incubated for 2 hours at 4°C. Crystals were washed and stained with anti-IgG-PE and analysed in a flow cytometer.

B CLR-Strep-fusion proteins were incubated with 20µg/ml monoclonal blocking antibodies to human Clec12a for 20 minutes at room temperature. Following, 100µg MSU and 80µg alum were added and incubated for 24 hours at 4°C. Crystals were washed and stained with anti-IgG-PE and analysed in a flow cytometer.

Data were analysed with FlowJo. Data are means + SD of three and two independent experiments.

4.4 Cellular response due to stimulation of Clec12a with MSU or alum

4.4.1 Regulation of activation markers due to Clec12a stimulation

Clec12a$^{-/-}$ mice exhibit a higher influx of neutrophils in response to dead thymocytes *in vivo,* suggesting Clec12a may modulate neutrophil activation in response to MSU and alum. To test this, upregulation of CD11b or CD11c, which are markers of neutrophil activation, was analysed after stimulation with MSU and alum *in vitro*. No differences in expression of these activation markers ware observed for either ligand in wild-type cells compared with Clec12a$^{-/-}$cells {Figure 4.8A}

Alum and MSU both can upregulate expression of co-stimulatory molecules essential for T-cell activation on DCs (Kool et al., 2008; Y. Shi et al., 2003). Thus, upregulation of CD80 and MHC II in response to MSU and alum stimulation was analysed in wild-type and Clec12a$^{-/-}$ DCs. However, despite using different concentrations and stimulation durations (additional data not shown) neither ligand significantly modulated expression of these surface markers in a Clec12a-dependant manner {Figure 4.8B}.

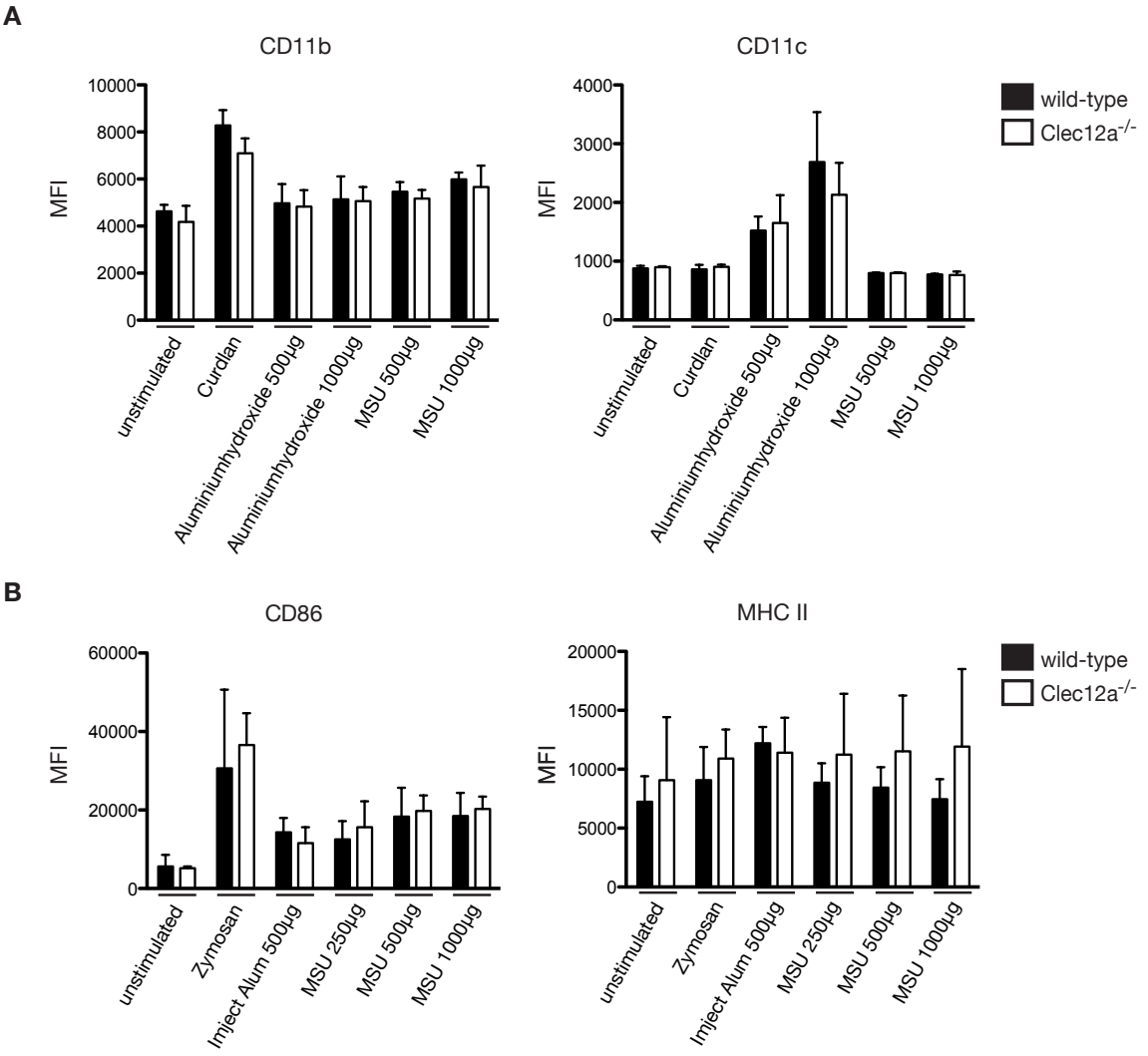

Figure 4.8 *in vitro* stimulation with alum or MSU does not lead to Clec12a regulation

A 1x10^6 purified neutrophils of wild-type or Clec12a$^{-/-}$ mice were stimulated each for 6 hours. Cells were harvested and analysed by flow cytometry. Data are means + SD of three mice per group and two independent experiments.

B 1x10^6 GM-DCs of wild-type or Clec12a$^{-/-}$ mice were stimulated each for 6 hours. Cells were analysed by flow cytometry. Data are means + SD of five mice per group and three independent experiments.

4.4.2 Clec12a diminishes alum-induced ROS production in neutrophils

In response to microbial infection cells of the innate immune system such as neutrophils produce reactive oxygen species (ROS). However, ROS will not only damage invading pathogens, but also has the potential to cause significant tissue damage. Previous studies demonstrate MSU crystals induce neutrophils to produce ROS by NADPH oxidase. (Gaudry et al., 1993). Moreover, some of CLRs have been shown to regulate the immune response via Syk (Mocsai et al., 2010). Clec12a contains an ITIM and it is therefore predicted to inhibit this Syk pathway. Together these observations suggest Clec12a may act as a negative modulator of ROS production in response to MSU or alum stimulation *in vitro*.

To test this hypothesis, bone marrow cells, which mainly consist of neutrophils (Swamydas et al., 2015), were isolated from Clec12a$^{-/-}$ and wild-type mice and incubated with different stimuli. As shown in Figure 4.9A, alum hardly induced any ROS in wild-type bone marrow cells, whereas Clec12a-deficient cells responded to alum stimulation with robust ROS production. This Clec12a-depenent effect on ROS production was stronger for alum than MSU. In contrast, ROS production in response to silica was not regulated by Clec12a; indicating that not all types of crystals inducing ROS are regulated by Clec12a. This result was verified using purified neutrophils {Figure 4.9C} with the beta glucan curdlan as negative control. Pure Clec12a$^{-/-}$ neutrophils showed increased ROS production compared to wild-type neutrophils in response to distinct alum preparation and MSU but not curdlan. MSU induced ROS production seemed to be more elevated compared to the ROS production after stimulation with the alum preparation of aluminiumhydroxide.

To determine if induction of ROS production by MSU was Syk and/or SRC dependent, these proteins were inhibited using R406, a Syk inhibitor (Braselmann et al., 2006), and PP2, an inhibitor of the Src family kinases (Hanke et al., 1996). PP3 is the inactive analogue of PP2 and was used as control {Figure 4.9B}. ROS production in response to MSU was inhibited in wild-type and Clec12a$^{-/-}$ bone marrow cells incubated by both R406 or PP2, indicating both Src and Syk kinases are required for ROS production under the conditions tested.

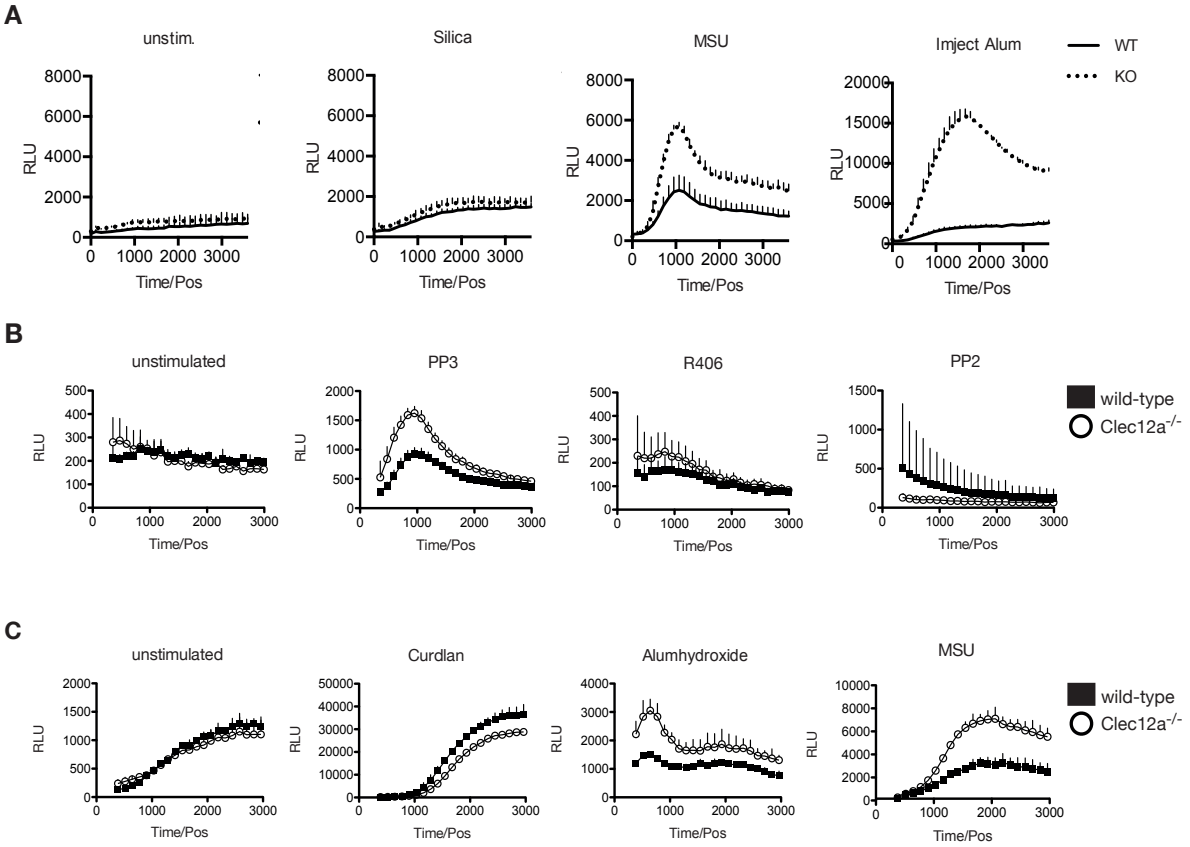

Figure 4.9 Clec12a signals via Syk

A Bone marrow cells of wild-type or Clec12a$^{-/-}$ mice were incubated with medium, 200µg Silica (as negative control), 500µg MSU or 500µg Imject Alum in the Presence of 50µM Luminol. Emitted light was recorded in 2 minutes intervals for 50 minutes. Data are means + SEM of three mice and two independent experiments.

B Bone marrow cells of wild-type or Clec12a$^{-/-}$ mice were prior incubation with MSU 1000µg, incubated with different inhibitors. PP3 (as negative control), R406 (Syk inhibition), PP2 (Src inhibition) an analysed as in A. Data are means + SD of three independent experiments.

C Purified neutrophils of wild-type or Clec12a$^{-/-}$ mice were incubated with medium, 100µg Curdlan (as negative control), 500µg MSU or 500µg aluminiumhydroxide in the presence of 50µM Luminol. Data are means + SD of two independent experiments.

4.5 Clec12a plays a role in the activation of APCs

One of the first steps associated with the activation of the adaptive immune system is the activation of antigen-presenting cells (APCs). Dendritic cells (DCs) are among the most efficient APCs, presenting processed antigen directly to T-Cells. *In vitro*, DC differentiation and proliferation in bone marrow cultures can be induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) or *fms*-like tyrosine kinase 3 ligand (Flt3L). Flt3L is reported to generate three different types of steady-state DC (Flt3L-DC) equivalents *in vivo*: conventional $CD8^+$ and $CD8^-$ (lymphoid tissue-resident) DCs and plasmacytoid pre-DCs (Naik et al., 2005). GM-CSF derived DCs (GM-DC), however, are suggested to represent a more inflammatory DC phenotype normally found *in vivo* (Xu et al., 2007). Despite the fact GM-CSF-derived DCs exhibit low Clec12a protein expression compared to macrophage colony-stimulating factor (M-CSF) MPs (Neumann et al., 2014), flow cytometry reveal high levels of Clec12a at surfaces of both types of DCs by {Figure 4.10A}.

Since dendritic cells and macrophages act as mediators between the innate and adaptive immune responses, determining how they respond to the activation of Clec12a stimulation was important. After the stimulation of GM-DCs with alum and MSU, $Clec12^{-/-}$ cells produced elevated ROS levels compared to wild-type cells {Figure 4.10B}. As already observed in bone marrow cells, the impact of Clec12a on ROS production induced by alum stimulation was stronger than its impact on MSU-induced ROS production. Flt3L-DCs, however, did not appear to produce ROS after stimulation with either MSU or alum (data not shown).

Another important type of immune cells able to produce ROS after stimulation are macrophages. ROS production in macrophages also increased after stimulation with MSU or alum. Here, a significant difference in ROS production was only observed following alum stimulation in $Clec12a^{-/-}$ cells compared to wild-type cells but not after MSU stimulation {Figure 4.10C}.

These data indicated that Clec12a acts as a negative regulator of the alum-induced activation of neutrophils, dendritic cells, and macrophages, which may influence the adaptive immune response.

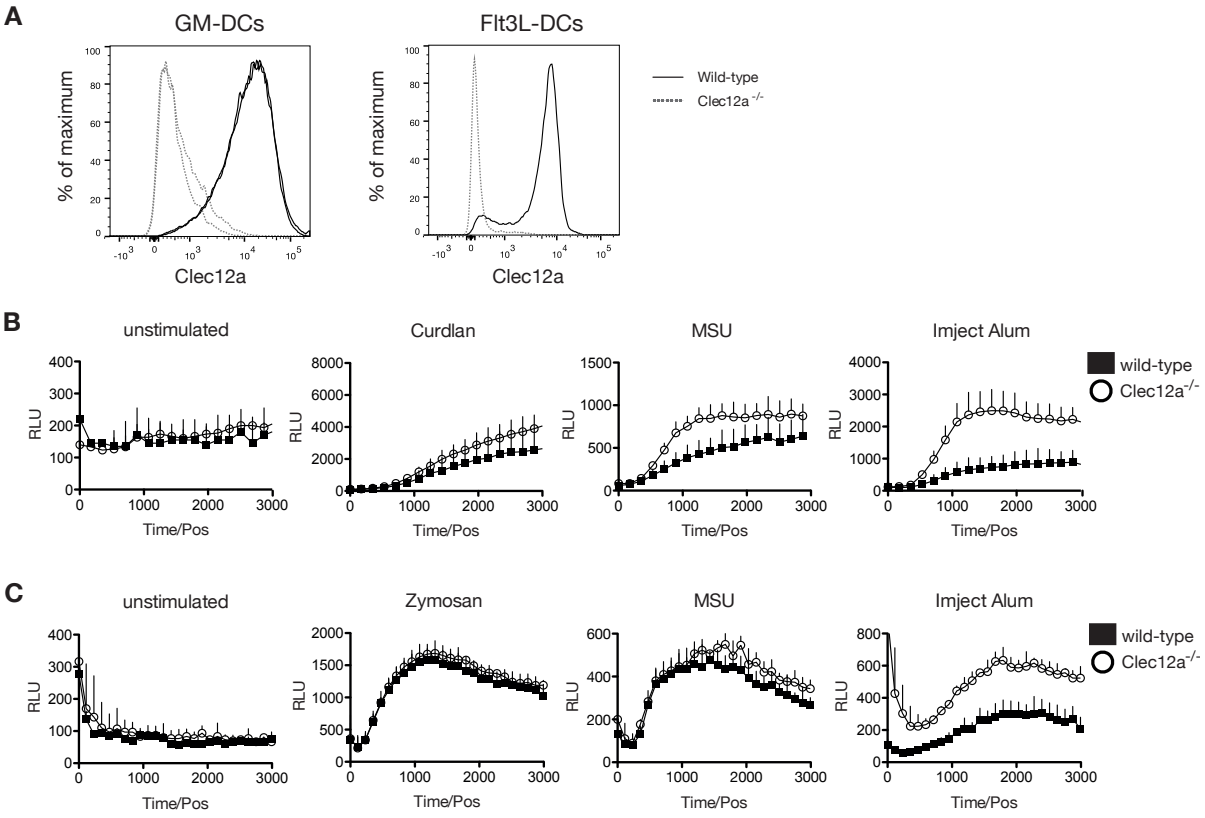

Figure 4.10 Clec12a also inhibits ROS production in dendritic cells and macrophages

A GM-DCs and Flt3L-DCs were stained for α-mClec12a (PE) and analysed with flow cytometry

B GM-DCs of wild-type or Clec12a$^{-/-}$ mice were incubated with medium, 500µg Curdlan (as negative control), 500µg MSU or 500µg Imject Alum in the Presence of 50µM Luminol. Emitted light was recorded in 2 minutes intervals for 50 minutes. Data are means + SD of three mice representative for two independent experiments.

C MPs of wild-type or Clec12a$^{-/-}$ mice were incubated with medium, 100µg Zymosan (as negative control), 500µg MSU or 500µg Imject Alum in the Presence of 50µM Luminol. Emitted light was recorded in 2 minutes intervals for 50 minutes. Data are means + SD of three mice.

4.6 Clec12a in adaptive immune response

As described above, Clec12a influences activation of DCs and macrophages activation by alum. Macrophages and DCs play important roles in activating the adaptive immune system and alum is widely used as an adjuvant in vaccines to enhance antibody production. Thus, in addition to regulating ROS in APCs, Clec12a may also regulate alum-enhanced antibody responses. To test this hypothesis, wild-type and Clec12a-deficient mice were immunized with Ovalbumin (OVA) using alum as adjuvants.

A previous study demonstrated that the basal levels of serum immunoglobulins were not altered in Clec12$^{-/-}$ mice (Vilariño, 2014). Since alum was found to induce a persistent T_h2-response, possibly by inducing uric acid, an experimental design based on immunisation by intraperitoneal injection was used for this dissertation (Kool et al.,

2008).

4.6.1 Immunisation of mice with OVA and alum

To induce an antibody response, mice were immunised with OVA and alum. Therefore, a well-established protocol developed in our laboratory for immunising mice with OVA and a different kind of adjuvant was used. Mice were bled weekly to determine the OVA-specific antibody response. To identify the optimal amount of adjuvant, wild-type mice were immunised with 20µg OVA and different volumes of alum {Figure 4.11A}. A dose of 400µg alum was found to induce a high level of OVA-specific IgG1 by day 14 after injection and was therefore used in the fallowing experiment. After 14 and 21 days, OVA-specific IgG1 levels were slightly increased in Clec12a$^{-/-}$ mice compared with those in wild-type mice. The differences in antibody levels, however, were not significant {Figure 4.11B}.

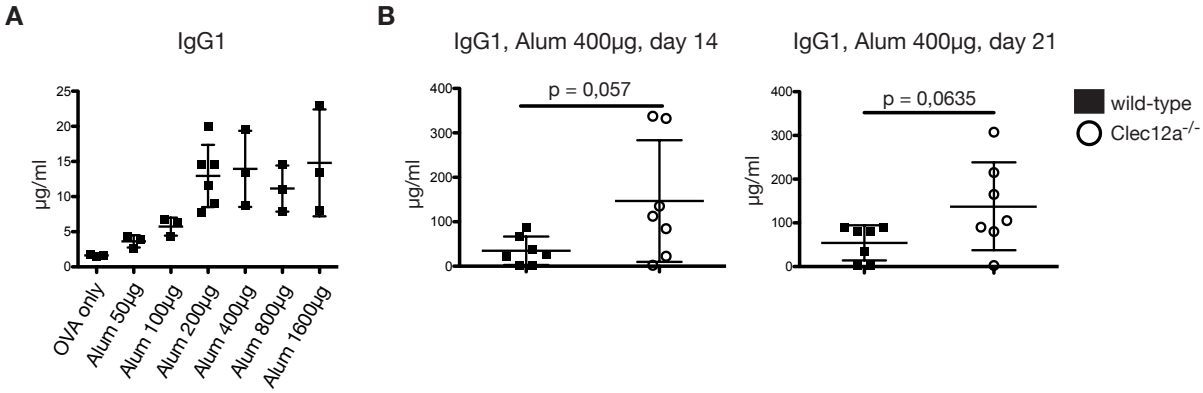

Figure 4.11 OVA IgG1 production after immunisation with OVA plus alum

A Mice sera were analysed for IgG1 with ELISA at day 14 after immunisation with 20µg OVA plus different doses of alum. Sera were diluted 1:50. All mice were female and 8.5-9 weeks old. Data are means + SD of one to two mice per test.

B Mice sera were analysed for IgG1 with ELISA at day 14 and 21 after immunisation with 20µg OVA plus 400µg alum. Sera were diluted 1:50. All mice were male and 8.5-10 weeks old. Data are means + SD of seven mice per group.

Repeating this experiment using 200µg alum observed no difference in OVA-specific IgG1 response. The immunoglobulin production was less in total and the quantitative difference was even weaker {Figure 4.12A}. On day 28, the protocol provided the option of a booster injection of OVA, similar to the protocol used for human immunisations. After boosting the total amount of OVA-specific IgG1 had increased {Figure 4.12B}. The OVA-specific IgG1 response in wild-type compared to Clec12$^{-/-}$ mice, however, was similar.

Mice were then also immunised using 800 µg alum as adjuvant. Although the total IgG1 response increased, the experiment also failed to identify any Clec12a

dependent effect on either timepoint {Figure 4.12C+D}.

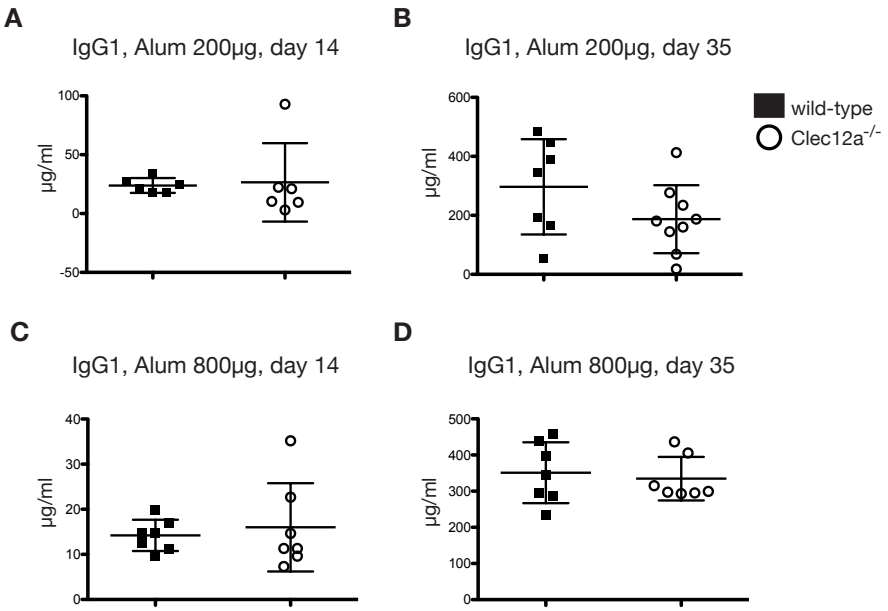

Figure 4.12 αOVA IgG1 production after immunisation with different doses of OVA + alum

A Mice sera were analysed for IgG1 with ELISA at day 14 after immunisation with 20µg OVA plus 200µg alum. Sera were diluted 1:40. All mice were female and 10-13 weeks old. Data are means + SD of eight mice per group.

B Mice sera were analysed for IgG1 with ELISA at day 35 after immunisation with 20µg OVA plus 200µg alum and boost with 20µg OVA on day 28. Sera were diluted 1:40. Data are means + SD of eight mice per group.

C Mice sera were analysed for IgG1 with ELISA at day 14 after immunisation with 20µg OVA plus 800µg alum. Sera were diluted 1:40. All mice were male and 8.5-9.5 weeks old. Data are means + SD of seven mice per group.

D Mice sera were analysed for IgG1 with ELISA at day 35 after immunisation with 20µg OVA plus 800µg alum and boost with 20µg OVA on day 28. Sera were diluted 1:40. All mice were male and 8.5-9.5 weeks old. Data are means + SD of seven mice per group.

To determine whether the antibody production by B-cells may have been quantitatively different between the two groups before the class switch, IgM was measured in all immunised mice {Figure 4.13}. However, this again failed to identify any consistent Clec12a-dependent effect.

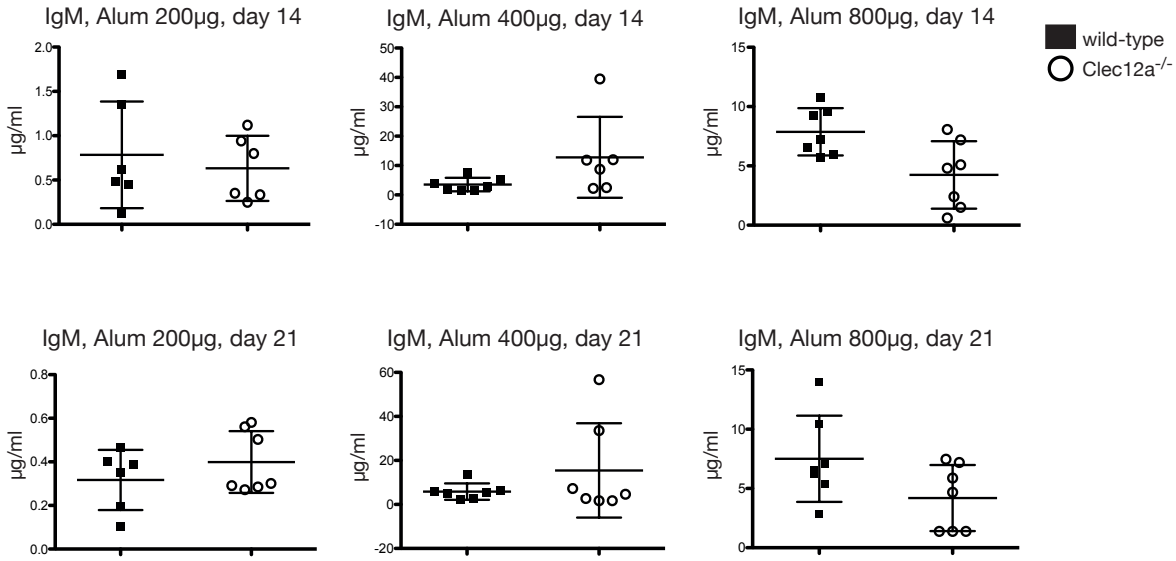

Figure 4.13 αOVA IgM production after immunisation with different doses of OVA + alum

Mice sera were analysed for IgM with ELISA at day 14 and 21 after immunisation with 20µg OVA plus the amount of alum displayed. Sera were diluted 1:20. Data are means + SD of six or seven mice per group.

Finally, as alum is a well-known inducer of T_h2 immune responses, it was hypothesised that differences in IgE levels may be observed between Clec12a$^{-/-}$ mice and wild-type mice. To obtain measurable results, due to the overall low levels of αOVA antibodies, the OVA-specific IgE response was only monitored in mice injected with 800 µg alum as an adjuvant {Figure 4.14}. This failed to demonstrate any Clec12a-dependent effect on this T_h2 response indicating Clec12a plays no role in the adjuvant effect of alum. Another antibody that is produced by the T_h1 response is IgG2a (Bretscher et al., 1992). However, IgG2a levels were too low to measure at any concentration (data not shown).

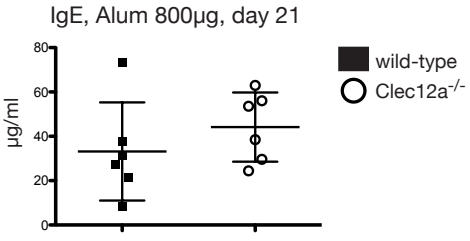

Figure 4.14 αOVA IgE production after immunisation with 800µg of OVA + alum

Mice sera were analysed for IgE with a special ELISA-kit at day 21 after immunisation with 20µg OVA plus 800µg alum. Sera were diluted 1:25 and 1:50. Data are means + SD of six mice per group.

4.6.2 Immunisation of mice with OVA and MSU

The data presented above indicate that although alum is a ligand for Clec12a this plays no significant role in mediating the adjuvant effects of alum. To determine if this was also the case for another Clec12a ligand, mice were immunised with OVA and MSU. Although MSU is primarily known to induce "antigen-specific CD8 T cell responses", MSU has also been suggested to induce T_h2 response (Kool et al., 2008; Kool et al., 2011). However, MSU clearly acted as an adjuvant this effect was also Clec12a-independent, both with respect to the primary IgG1 response to OVA and the secondary response induced following a booster injection {Figure 4.15}.

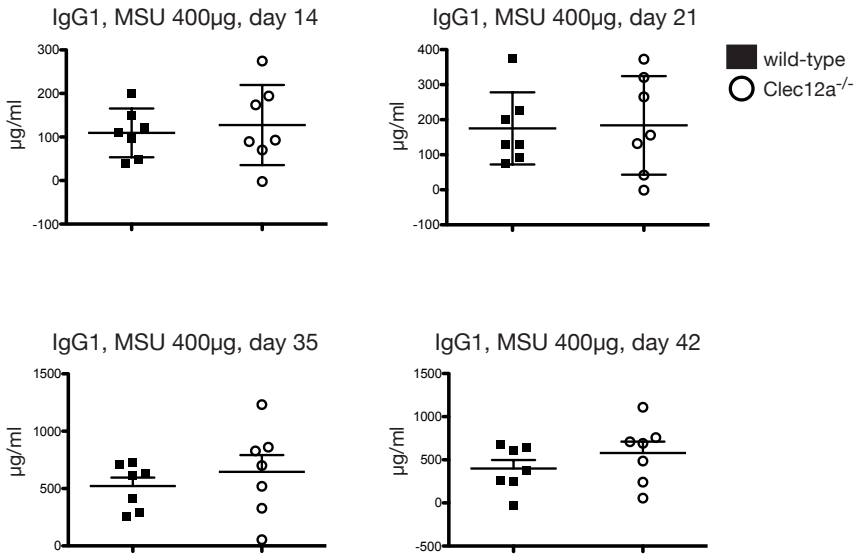

Figure 4.15 αOVA IgG1 production after immunisation with 400µg of OVA + MSU

Mice sera were analysed for IgG1 with ELISA at day 14, 21, 35 and 42 after immunisation with 20µg OVA plus 400µg MSU. Sera were diluted 1:20. All mice were male and 8-11 weeks old. Data are means + SD of seven mice per group.

5 Discussion

This dissertation investigated alum as a potential ligand for Clec12a, a CLR previously demonstrated before to recognise certain types of necrotic cells such as freeze-thawed kidney cells and MSU crystals. Clec12a, in principle, can act as a negative regulator of neutrophil influx following cell death via a mechanism involving recognition of MSU crystals (Neumann et al., 2014). This dissertation extended these findings by investigating the impact of Clec12a on antibody production following vaccination using alum or MSU as an adjuvant.

5.1 No elevated cytokines in Clec12a-deficient mice without insult

Before this work was started our laboratory demonstrated Clec12a$^{-/-}$ mice displayed no disease phenotype when housed under specific pathogen-free conditions; their weight, appearance, behaviour and number of major types of immune cells in blood are not differing significantly from wild-type mice (Neumann et al., 2014). Additionally, cytokine levels in the blood of wild-type and Clec12a$^{-/-}$ mice were also investigated under homeostatic conditions. All ten tested chemokines or cytokines were present at detectable levels in both wild-type and Clec12$^{-/-}$ mice. However, only small differences were observed between wild-type and Clec12a$^{-/-}$ donors; IL-1β, IL-6, and IL-17 all being present at slightly lower concentration in Clec12a$^{-/-}$ mice compared to wild-type controls. These results indicated that Clec12a does not significantly influence inflammatory cytokine production under homeostatic conditions. In addition, Clec12a-deficient mice breed and develop normally, indicating this CLR is not involved in clearance of apoptotic cells during normal embryonic and neonatal development (Neumann et al., 2014). A study by another research group subsequently confirmed Clec12a does not appear to have any obvious role under homeostatic conditions (Redelinghuys et al., 2016).

5.2 Clec12a in sterile inflammation

5.2.1 Clec12a reduces the neutrophil influx

Clec12a was previously demonstrated to inhibit neutrophil influx in response to the intraperitoneal injection of dead (freeze-thawed) kidney cells, indicating that Clec12a, in principle, can inhibit sterile inflammation in response to dead cells (Neumann et al). This dissertation extended these observations to investigate the role of Clec12a in models of X-ray and dexamethasone-meditated thymocyte death. In both models of X-ray and dexamethasone-meditated thymocyte death neutrophil flux into the thymus was enhanced in Clec12$^{-/-}$ mice compared to wild-type controls. These observations confirm that Clec12a acts as a negative regulator of neutrophil

recruitment in these models of sterile inflammation.

Because both, low-dose X-ray exposure and dexamethasone injection, induce apoptosis in thymocytes, whereas Clec12a only recognises necrotic cells, the thymocytes likely cannot be cleared und secondary necrosis occurs. These models were chosen because inflammation in these models strongly depends on the CLR Mincle. Mincle has a similar binding specificity for damaged cells as observed for Clec12a but harbours an intracellular ITAM motif instead of an ITIM (Yamasaki et al., 2008). Given that ITIM motifs are always paired to and inhibit the response of ITAM-coupled receptors, Mincle may represent the opposing receptor for Clec12a in response to cell death. Intriguingly Mincle is upregulated by the cellular activation of TLR, whereas Clec12a is downregulated by TLR ligands (Marshall et al., 2006).

5.2.2 Cytokine regulation of Clec12a

IL-1β is a highly relevant cytokine during inflammation and is involved in the development of inflammatory diseases. ProIL-1β is processed by the NLRP3 inflammasome, an NLR that can be activated by DAMPs, such as extracellular adenosine triphosphate (ATP), MSU (Martinon et al., 2006) or even alum (Eisenbarth et al., 2008). Furthermore, ROS production has also been suggested to modulate NLRP3 although the mechanism remains unclear (Haneklaus et al., 2013).

Data generated in the course of this dissertation demonstrate that ROS production was significantly higher in Clec12a$^{-/-}$ cells compared to wild-type cells after stimulation in MSU and alum. However, no differences were observed among MSU and alum-induced IL-1β production *in vitro*. Similarly, Gagne et al could not detect any increase in IL-1β levels after the MSU stimulation of human neutrophils treated with a Clec12a blocking antibody (Gagne et al., 2013). Nonetheless, a significant increase in IL-1β production was observed after the induction of sterile inflammation *in vivo* in Clec12a$^{-/-}$ mice compared to wild-type mice. These results suggest that the transcription of proIL-1β *in vivo* is (directly or indirectly) modulated by Clec12a only, whereas the activation of NLRP3 is not. This is supported by the notion that IL-1α and TNF are not regulated by the inflammasome, while the distribution of those is increased by the absence of Clec12a.

Another cytokine with increased expression in Clec12a$^{-/-}$ mice after sterile inflammation is TNF, which is expressed at high levels during many auto-inflammatory diseases. Mutations in TNF have been linked to various diseases, including rheumatoid arthritis (Tang et al., 2014) and atherosclerosis (Poirier et al., 2004). TNF has long been known to be involved in malignancies, such as myelodysplastic syndrome (Shetty et al., 1996) and acute myelogenous leukaemia (AML) (Gao et al., 1998), for which Clec12a has also been shown to be involved (Bakker et al., 2004). Interestingly, several studies have identified correlations between the incidence of autoimmune disorders and myelodysplastic syndrome or AML (de Hollanda et al., 2011; Enright & Miller, 1997).

Joint regulation of TNF and IL-1β is a cytokine signature of nuclear factor (NF)-κB pathways, which can be activated by ITAM receptors. The upregulation of these cytokines in Clec12a$^{-/-}$ mice, therefore, probably reflects loss of inhibitory signalling mediated by Clec12a resulting in a corresponding increase in signalling associated with activation of ITAM receptors, suggesting that Clec12a likely plays a role in diseases associated with sterile inflammation, such as gout.

However, IL-1α was only elevated in the thymus following low dose irradiation and not following dexamethasone-mediated thymocyte cell death. This discrepancy could be due to the different kinetics of cell death in the two models, which in turn may be related to the intensity of the local inflammatory response, Clec12a being downregulated after immune cell activation (Marshall et al., 2006). One may speculate that irradiation results in rapid thymocyte death whilst the effects of dexamethasone are somewhat slower and presumably involves different mechanisms.

Relatively subtle changes in the intensity and cellular kinetics of inflammation in these models might result in different rates of cytokine down regulation. If this is the case, local concentrations of neutrophil chemo attractants may drop to baseline levels before there is any significant decrease in neutrophil numbers. To test this hypothesis, cellular changes and neutrophil recruitment in the thymus could be analysed at earlier time points and correlated with cytokine/ chemokine expression in these models. Kool et al., for example, measured elevated cytokines, including IL-4, IL-5, IL-10 IL-13 and IFNγ, after alum injection in the peritoneal supernatant after only two hours (Kool et al., 2011).

Similarly to obtain additional information regarding the possible influence of alum on MSU release and thereby Clec12a activation, uricase could be injected prior to injecting alum to inhibit MSU formation (Kool et al., 2011). This would help determine whether the higher level of Clec12a activation induced by alum is a specific effect, rather than being due to amplification of Clec12a activation by MSU generated in response to alum-induced cell death. However, it should be noted that both models of sterile inflammation showed enhanced inflammation in Clec12a$^{-/-}$ mice, and their cytokine profiles indicate enhanced neutrophil recruitment involved classical inflammatory responses.

5.3 Clec12a ligates to MSU and alum

5.3.1 MSU and alum activate Clec12a

The activation of Clec12a reporter cells was examined following alum administration, which showed activation of both mouse and human Clec12a reporter cells. However, human Clec12a-expressing cells showed stronger responses than mouse Clec12a reporter cells. The expression was comparable after MSU stimulation, whereas the activation of mouse Clec12a reporter cells after alum stimulation was considerably weaker compared to human Clec12a, which may indicate a higher affinity for human Clec12a to alum. However, potential differences in expression of

Clec12a in these reporter cell lines could also affect the activation kinetics. This requires reinvestigation to make sure the observed differences in the ability of alum to activate human and mouse Clec12a reporter cell lines is not a technical artefact. Nonetheless, these experiments clearly demonstrate MSU and alum recognise and activate Clec12a *in vitro*. The mechanistic basis of these interactions is discussed below.

5.3.2 Regulation of surface proteins

Alum and MSU are both ligands for Clec12a and Clec12a is now known to inhibit neutrophil influx and cytokine production in models of sterile inflammation *in vivo*. It therefore seems reasonable to assume expression and induction of neutrophils activation markers might differ between Clec12a$^{-/-}$ and wild-type cells. However, in neutrophils there were no significant differences in upregulation of CD11b, which is an adhesion molecule, or CD11c, which is important for cell junction, observable by either ligand. Similarly, the presence or absence of Clec12a in bone marrow derived dendritic cells (BMDCs) had no effect on expression of CD86 and MHC class II, which are important for T-cell activation, in response to alum or MSU.

On first sight these data indicate Clec12a plays no role in regulating expression of these activation markers on immune cells. Still, it should be noted that whilst *in vitro* studies detected no effect of Clec12a on IL-1α expression (Vilariño, 2014), this is not the case *in vivo.* This might be due to involvement of other cells or circulating proteins that may modulate cytokine expression indirectly at the site of inflammation. It is possible this involves ROS dependent mechanisms, as ROS production is triggered rapidly following Clec12a activation by MSU and alum and has the potential to mediate and often differential effects on cytokine production and immune cell maturation (Sies & Jones, 2020). Upregulation of activation markers takes much longer and may depend on processes after phagocytosis, where Clec12a may be less important.

5.3.3 Cellular reaction upon Clec12a activation

Previous findings demonstrate neutrophils bind to MSU crystals leading to production of ROS by NADPH oxidase (Gaudry et al., 1993). In this dissertation, the impacts of Clec12a on ROS production in response to MSU and alum were examined in bone marrow cells, which contain mostly neutrophils. As described previously (Neumann et al., 2014), Clec12a limited ROS production in response to MSU. More profoundly, the induction of ROS production by MSU was almost completely blocked by Syk or Src inhibition. This indicates that Clec12a inhibits Syk by recruiting Src, which in turn deactivates presumably another MSU recognising ITAM.

Surprisingly, the inhibitory impacts of Clec12a on alum-induced ROS production were overall greater than those in response to MSU. The reasons for these effects remain unclear. However, in the experiment with purified neutrophils,

aluminium hydroxide gel was used and the difference in ROS production between Clec12a$^{-/-}$ and wild-type mice was similar after MSU or aluminium hydroxide stimulation. This discrepancy to the other experiments presumable is due to different structures in these aluminium hydroxide compounds (Exley et al., 2010).

This response phenotype was also observed when BMDCs were used as targets. Clec12a does not show any impact on MSU-induced ROS production in bone marrow derived macrophages (BMDMs), but this CLR clearly inhibits ROS production in response to alum. These results indicate that in principle Clec12a signalling is able to limit ROS production in BMDMs, but not when these cells are exposed to the Clec12a ligand MSU. This indicates that alum mediated responses in Clec12a$^{-/-}$ mice could be the more relevant in terms of regulation of DC and MP responses and concomitant adaptive immune responses than those induced by MSU. Why Clec12a has these differential effects on the ability of alum and MSU to induce ROS production remains unclear, but one possibility is that these ligands exhibit differential binding to ITAM containing receptors. In this scenario MSU initiates a very strong activating signal that overrides the immunomodulatory potential of Clec12a activation. In contrast, recognition of alum by these as yet “hypothetical” ITAM containing receptors is minimal and the response is dominated by its activation of Clec12a.

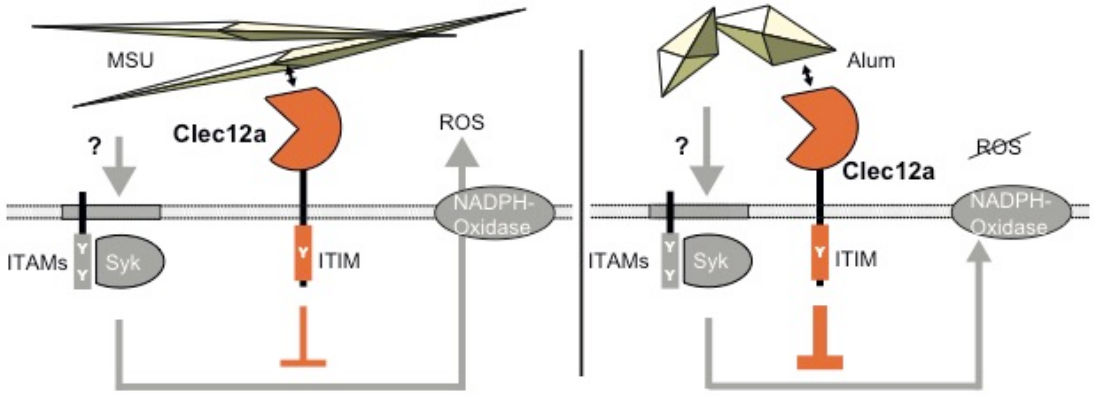

Figure 5.1 Model for Clec12a-mediated inhibition

Both MSU and alum activate immune cells by inducing phosphorylation of yet undefined ITAM-coupled receptors by either receptor dependent or receptor independent mechanisms. This leads to Syk activation, which activates NADPH-oxidase to produce ROS. Clec12a limits the NADPH-oxidase activation. The inhibitory effect is stronger after recognition of alum, which nearly completely blocks ROS production. Adapted from the graphical abstract from our manuscript (Neumann et al., 2014)

5.4 Clec12a in the adaptive immune response

Alum is the oldest and most frequently used adjuvant in human vaccines, although the mechanism through which it enhances the immune response is still not well understood. Clec12a is probably the first transmembrane receptor reported to

recognise alum. Additionally, not only in neutrophils but also in dendritic cells and macrophages the ROS production was significantly elevated in Clec12a$^{-/-}$ cells compared to wild-type cells after stimulation with alum. Hence, it was important to investigate whether these Clec12a-depentent effects the adjuvanticity of alum in Clec12a$^{-/-}$ mice, as compared to wild-type controls.

The first experiment using 400µg Alum as adjuvant indicated Clec12a$^{-/-}$ mice mounted a slightly higher but not statistically significant anti-OVA antibody response compared to wild-type mice. The amount of adjuvant used was then modified to determine whether this might provide a clearer result as whether a Clec12a-dependent mechanism might regulate the OVA-specific antibody response. However, the reduction of the adjuvant to eradicate the possibility of an OVA over dose (200µg) showed no difference in antibody production. Also, the increased amount of alum (800µg) on the hypothesis, that the amount of 200µg alum dose may have been eliminated too rapidly, did not show any significant differences in antibody production. The slight differences might also be explainable by subtle differences in the mouse groups used. Any possible impact of Clec12a on alum-induced antibody responses appears to be biologically irrelevant.

MSU is known to induce cytotoxic T cells (Sakamaki et al., 2008), but has also been shown to induce antibody production (Behrens et al., 2008). Furthermore, uric acid is released upon alum injection *in vivo* and induces T_h2 cell adjuvant effects that do not depend on NLRP3 (Kool et al., 2011). Still, the amount of MSU induced OVA-antibodies showed no difference in Clec12a$^{-/-}$ mice compared to wild-type mice.

Although Clec12a specifically recognises MSU and alum, which is the most widely used adjuvant in vaccines, Clec12a does not appear to regulate this adjuvanticity. These missing alterations in alum-induced antibody production in Clec12a$^{-/-}$ mice compared to wild-type mice may be due to the interactions between alum and proteins in the host environment (Morefield et al., 2005) that might block those sites on the crystal surface recognised by Clec12a. Alternatively, the inflammatory response induced by alum may downregulate Clec12a (Marshall et al., 2006), or inflammatory monocytes that take up alum may not express Clec12a, or finally Clec12a simply plays no physiological role in determining the ability of alum to act as an adjuvant in antibody production.

However, pro-inflammatory cytokines, such as IL-1α, also indicate a potential role for Clec12a in the cytotoxic T-cell responses. Because increased IL-1 levels were observed in Clec12a$^{-/-}$ mice in response to cell death, a similar response to alum may occur, which could lead to enhanced cytotoxic CD8$^+$ T-cell responses (Ben-Sasson et al., 2013). Thus, further experiments examining the CD8$^+$ T-cell responses after alum or MSU immunisation may be warranted. One possibility would be to perform immunisations in the presence of OT I cells, which are T-cells extracted from transgenic mice possessing the specific T-cell receptor against ovalbumin, followed by the extraction of draining and non-draining lymph nodes (Leibundgut-Landmann et al., 2008), or the direct flow cytometric analysis of antigen-specific CD8$^+$ T-cells.

5.5 Clec12a recognises the crystalline surface of MSU and alum

Three lines of evidence identify Clec12a as a receptor for MSU. First, two independent recombinant versions of human and mouse Clec12a specifically bind to MSU, whereas recombinant versions of similar CLRs do not bind to MSU. Second, reporter cells expressing human or mouse Clec12a, but not reporter cells expressing other CLRs were activated by MSU. Lastly, Clec12a-deficient mice showed enhanced neutrophil influx after the intraperitoneal injection of MSU.

However, the mechanism by which the receptor Clec12a specifically recognises MSU crystals remains unclear. Since this interaction should be blocked by an antibody against the receptor-binding site, the impact of a monoclonal antibody recognising the extracellular domain of human Clec12a to interfere with crystal binding was examined. This antibody strongly reduced the binding of MSU to Fc-hClec12a and Strep-hClec12a recombinant proteins confirming that MSU binds to a relevant site in the extracellular domain of Clec12a. Interestingly, this antibody has also been shown to enhance the responses of human neutrophils following MSU stimulation (Gagne et al., 2013), which could be a result of direct inhibition of the MSU-Clec12a interaction. During these experiments, alum, another crystalline structure that was small enough to be analysed by a flow cytometer, was examined for its ability to bind to Clec12a. Intriguingly, alum bound to mouse and human Strep-Clec12a, and like MSU, was not recognised by Clec7A or Clec9A. Furthermore, the same monoclonal Clec12a antibody that blocked binding of MSU to Clec12a also blocked alum binding to Clec12a. These experiments confirm MSU and alum both bind to the extracellular domain of Clec12a but lack the spatial resolution to determine if these two ligands bind to the same site on this domain.

The binding mechanism, however, between Clec12a and crystal structures remains unknown. One possibility is that this is determined by the ligands surface charge. However, MSU crystals are negatively charged and alum has a positively charged surface, yet both bind to the extracellular domain of Clec12a. Furthermore, silica crystals also have a negatively charged surface and do not bind to Clec12a. These findings suggest that a binding mechanism based solely on the surface charge is unlikely.

Crystal-binding proteins are a well-known biological phenomenon and include a group of proteins known as antifreeze proteins, which are widely expressed in grass, bacteria, and cold-water fish. One group of piscine antifreeze proteins identified in fish strongly resembles CTLDs (Zelensky & Gready, 2005). However, whether the binding mode used by these antifreeze CTLDs is similar to that used by Clec12a to recognise MSU and/or alum remains unclear. However, CTLDs may be especially suited for the recognition of crystalline structures, which could mean the mammalian immune system may have evolved more CLRs capable of recognising crystalline

structures. In line with this notion, the activating CLR MINCLE has been demonstrated to recognise cholesterol crystals (Kiyotake et al., 2015).

Several studies have determined that MSU can be coated with 25 different proteins, such as IgG, fibronectin, or complement components (Giclas et al., 1979; Jaques & Ginsberg, 1982; Terkeltaub et al., 1983). Barabe et al. showed that antibodies directed against CD16 or CD11b could block MSU-induced neutrophil responses (Barabe et al., 1998), whereas Scott et al. showed that CD14 is required for inflammatory responses by macrophages, and Ng et. al. demonstrated the direct interaction between MSU and cholesterol in the plasma membrane (Ng et al., 2008). Yet, whether any of these other molecules constitute bona fide receptors for uric acid like Clec12a remains unclear. However, these data suggest that multiple recognition mechanisms for crystals may exist, which may or may not be regulated by Clec12a.

5.6 Clec12a and its role in disease

5.6.1 Clec12a in autoimmunity and autoinflammation

Clec12a has become a receptor of interest for research in rheumatoid arthritis, a disease that results in synovial inflammation and deformation, especially in the joints, and diverse systemic impairments. Its pathogenesis, however, remains unknown although it is now generally considered the outcome of a complex interplay between genetic and environmental factors. However, Clec12a is itself not genetically associated with rheumatoid arthritis (Redelinghuys et al., 2016). Nonetheless, several lines of evidence indicate it plays an important role in helping to limit tissue damage in inflamed joints. In collagen antibody-induced arthritis, a commonly used murine model of rheumatoid arthritis, Clec12a appears to be “essential to control inflammation during collagen antibody-induced arthritis“ (Redelinghuys et al., 2016). Additionally, this work group has translated these observations to the human disease by identifying a subset of rheumatoid arthritis patients who develop autoantibodies against hClec12a, which may be associated with “more severe and prolonged disease”. Presumably, these autoantibodies may act by inhibiting functions of Clec12a that are necessary to control inflammation in rheumatoid arthritis. However, this has not been formally demonstrated.

Conversely, in experimental autoimmune encephalitis (EAE), an experimental counterpart for multiple sclerosis in mice, Clec12a appears to play another role as blocking Clec12a reduces migration of DCs across the blood-brain barrier and demyelination, resulting in reduced disease progression (Sagar et al., 2017). In this case the interpretation is Clec12a dependent mechanisms actively contribute to disease development and tissue damage. Although this is difficult to equate with immunomodulatory activity demonstrated in this dissertation and this report, uric acid and by inference MSU, plays a protective role in EAE (Kean et al., 2000). Yet despite these possible controversies these results identify Clec12a as a regulating or dysregulating target molecule involved in the pathogenesis autoinflammatory

diseases that may provide a promising target for clinical interventions.

5.6.2 Clec12a in cancer

The concept that Celec12a is of clinical significance is much further advanced in cancer biology. Bakker et al. found Clec12a is highly expressed on primary acute myeloid leukaemia (AML) blasts and have discussed its potential use as a diagnostic and therapeutic target in this disease (Bakker et al., 2004). Furthermore, expression of Clec12a was stable from disease onset and during treatment making it a promising marker for diagnosis and follow-up, even in cases with minimal residual disease (Roug et al., 2014). Other research groups have now developed either bispecific or monoclonal antibodies against Clec12a as treatment tool. An artificial bispecific αClec12a-αCD3 antibody was found to increase the number of T-cells (Leong et al., 2016). Zhoa et al. demonstrated a monoclonal antibody directed against Clec12a was cytotoxic to human pro-myelocytic leukaemia cells, *in vitro*, and showed significant growth delays in one-third of all cases (Zhao et al., 2010).

These investigations raise the question why Clec12a is more highly expressed on different types of AML cells. Possibly, Clec12a functions as an inhibitor of the immune system in leukaemic cells, which, in turn reduces the elimination of leukaemic cells. In contrast to this hypothesis, αClec12a antibodies activate Clec12a and, thus, inhibit tumour growth. Therefore, examining the influence of Clec12a on T-cell proliferation, cytotoxicity, or tumour growth in response to the binding of MSU or alum would be interesting.

Furthermore, in solid tumours Clec12a likely influences tumour progression. Wang et al. injected alum into mice with hepatocarcinoma and detected increased IL-1β and TNF levels, which resulted in the enhancement of tumour specific $CD8^+$ T-cells and alum-induced tumour suppression (B. Wang et al., 2015). Therefore, the blockade of Clec12a prior to alum injection may improve the tumour reduction results.

5.7 Conclusion

CLRs are a group of PRRs, which require further investigation regarding their ligands, pathways and collaboration with other receptors. Clec12a is a receptor belonging to this family that is primarily expressed on myeloid cells such as neutrophils and DCs. Furthermore, Clec12a negatively regulates immune responses in response to MSU or alum, but nonetheless does not modulate the ability of these compounds to act as adjuvants. This binding mechanism utilised by alum and MSU, and the as yet "hypothetical" activating receptors (or co-receptors) that may recognise these ligands remain to be elucidated. To examine the involved co-molecules or co-receptors, the selective blockade of CD14, CD11b, or CD16 could be performed, *in vitro,* followed by stimulation with alum or MSU and the measurement of ROS production.

Additionally, *in vivo*, Clec12a showed the inhibition of sterile inflammation, in response to cell death. However, cytokines during this inflammatory process were mostly measured at a single time point, when neutrophil recruitment was already visible. The results of this study, combined with the findings that human patients with rheumatoid arthritis develop antibodies against Clec12a, indicate the clinical importance of Clec12a in disease conditions involving sterile inflammation.

In conclusion, this dissertation demonstrates that alum represents another crystalline ligand for Clec12a, revealing that Clec12a recognises certain crystalline surfaces, independent of uric acid. Clec12a further regulates innate immune responses but does not seem to play a role in the adjuvant effect of both MSU or alum.

Table of Figures

Danksagung

Mein außerordentlicher Dank gilt meinem Doktorvater Prof. Dr. med. Jürgen Ruland, der mir die Möglichkeit gegeben hat in seinem ehrgeizigen Team mitzuarbeiten und seine Unterstützung über all die Jahre. Sie werden mir in einzigartiger Erinnerung bleiben.

Ebenso möchte ich mich bei meinen Prüfern PD Dr. med. Veit Buchholz und Prof. Klaus-Peter Janssen bedanken, dass sie dank schneller Korrektur und Terminfindung es mir ermöglicht haben meine Dissertation noch zwölf Tage vor Geburt meiner Tochter zu verteidigen.

Besonders möchte ich auch Dr. rer. nat. Konstantin Neumann, meinen Betreuer im Labor danken, der mich während meiner Dissertation begleitet und immer wieder ermutigt hat. Er hat mich in das Gebiet der Immunologie eingeführt, meine Sichtweise erweitert und mir geholfen, unabhängiger zu denken.

Ich möchte auch der gesamten Forschungsgruppe von Prof. Dr. med. Jürgen Ruland für die konstruktive Diskussionen und das Teamwork danken. Hervorzuheben ist mein Dank an Nicole Prause, die mich zum einen mit Tipps und Anleitung bei den Experimenten und zum anderen als Freundin mental unterstützt hat. Und ich möchte mich bei Prof. Dr. rer. nat. Olaf Groß bedanken, der mir geholfen hat, die Dinge aus einem anderen Blickwinkel zu sehen.

Ein ganz besonderer Dank gilt auch Prof. Hermann Schlüsener und Prof. Christopher Linington, die meine Doktorarbeit am Ende auf formale und Englisch-Fehler überprüft haben.

Mein Dank geht natürlich auch an meinen lieben Michael, der die letzten intensiven Meter mit mir mitgefiebert und mich unterstützt hat. Und zum Schluss möchte ich sehr meinen Eltern, Monika und Richard, danken, die mich mit Liebe, Finanzen, Nervennahrung und immer wieder guten Worten über diese lange Zeit zum Durchhalten motiviert haben. Ohne Sie wäre mir die Beendigung dieser Arbeit nicht möglich gewesen!

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