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Generation and Analysis of Card9-Deficient Mice

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Du bereitest vor mir einen Tisch
im Angesicht meiner Feinde.
Psalm 23,5

Index

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Zusammenfassung

Das angeborene Immunsystem der höheren Organismen erkennt mikrobielle Pathogene an molekularen Strukturen die typisch sind für spezielle mikrobielle Komponenten. *Toll-like* Rezeptoren (TLRs) stellen eine Hauptgruppe dieser *pattern-recognition* Rezeptoren (PRRs) dar, aber auch andere PRRs haben essentielle Funktion für die Erkennung bestimmter Pathogene. Die Mechanismen, mit Hilfe derer diese PRRs inflammatorische Signalwege aktivieren sind noch unzureichend verstanden. In dieser Arbeit wurde die Funktion des Proteins *Caspase-recruitment-domain* Protein 9 (Card9) untersucht. Mit Hilfe Card9 defizienter Mäuse konnte ein neuer nicht-TLR abhängiger Signalweg definiert werden, der für die angeborenen Immunantwort sowie die Aktivierung der erworbenen Immunantwort gegen Pilzinfektionen notwendig ist. Card9 ist strukturell verwandt mit dem CARD-MAGUK (*membrande-associated guanylate kinase*) protein 1 (Carma1). Carma1 vermittelt *Nuclear Faktor kappa B* (NF-κB) Aktivierung über Bcl10 (*B-cell lymphoma 10*) und Malt1 (*mucosa-associated-lymphoid-tissue lymphoma-translocation gene 1*) nach B Zell und T Zell Rezeptor Aktivierung. Card9 bindet an Bcl10 und kann in Überexpressionsexperimenten ebenfalls NF-κB aktivieren. Die physiologischen Funktionen von Card9 waren zu Beginn dieser Arbeit unbekannt.

Hier konnte gezeigt werden, das T und B Zell Funktion in Card9 defizienten Mäusen der in wildtyp Tieren entspricht, Card9 also nicht am Carma1/Bcl10 Signalweg beteiligt ist. Am Modell von aus Knochenmark generierten Dendritischen Zellen (*bone-marrow derived dendritic cells*, BMDCs) wurde gezeigt, dass die Zytokin-Antwort nach Stimulation mit verschieden TLR-Liganden ebenfalls durch Card9 nicht beeinflusst wird. Jedoch produzieren Card9 defiziente Dendritische Zellen im Vergleich zum Wildtyp deutlich weniger Zytokine nach Stimulation mit dem Hefe-Zellwandbestandteil Zymosan oder inaktivierten *Candida albicans* Zellen. Zudem sind Card9 defiziente Tiere extrem Anfällig für Infektionen mit *Candida albicans*. Auf molekularer Ebene wurde nachgewiesen, dass Card9 spezifische Signale vom β-Glucan Rezeptor Dectin-1, dem wesentlichen PRR für Zymosan leitet. Hierbei kooperiert Card9 mit Bcl10 in der Aktivierung von NF-κB.

In weiteren Arbeiten konnte gezeigt werden, dass die Aktivierung adaptiver T-Zell Antworten gegen Pilzinfektionen durch Dendritische Zellen ebenfalls durch den Dectin-1/Card9 Signalweg kontrolliert wird. Durch Infektionsexperimente konnte gezeigt werden, dass wildtyp Mäuse nach *Candida albicans* Infektion antigenspezifische T-Zell Antworten aktivieren. In Card9 defizienten Tieren bleibt diese Reaktion aus. Dabei ist von besonderem Interesse, dass durch diesen Signalweg präferentiell T_H17 T-Helferzellantworten induziert werden.

Diese Daten definieren Card9 als unentbehrlichen Teil eines neuen, TLR-unabhängigen Signalwegs der angeborenen Immunität, der Dectin-1 nach Pilzerkennung mit Bcl10 verbindet und NF-κB aktiviert und an die Aktivierung adaptiver T_H17 Immunantworten koppeln kann.

Abstract

Fungal infections are increasing worldwide with the dramatic rise in immunodeficiencies including AIDS. However, the immune responses to such infections remain poorly understood. Dectin-1 is the major mammalian pattern recognition receptor for the fungal component zymosan. Dectin-1 represents the prototype of innate non-Toll like receptors (TLRs) containing immunoreceptor tyrosine-based activation motifs (ITAMs), which are related to those of adaptive antigen receptors. In this work, Card9 was identified as a key transducer of Dectin-1 signalling. Although dispensable for TLR/MyD88-induced responses, Card9 controls Dectin-1 mediated myeloid cell activation, cytokine production and innate anti-fungal immunity. Card9 couples to Bcl10 and regulates via Bcl10/Malt1 zymosan-induced NF-κB activation. Yet, Card9 is dispensable for antigen receptor signalling that utilizes Carma1 as a link to Bcl10/Malt1. Thus, these results define a novel innate immune pathway and indicate that evolutionarily distinct ITAM receptors in innate and adaptive immune cells employ diverse adaptor proteins to selectively engage the conserved Bcl10/Malt1 module.

Figure List

Table list

Abbreviations

AIDS	acquired immune deficiency syndrome
Alum	aluminium hydroxide
AP	adaptor protein complex
APC	antigen presenting cell
APG	acylpolygalactoside
ATF	activating transcription factor
B cell	B lymphocyte
Bcl	B cell lymphoma associated protein
BCR	B cell receptor
BMDC	bone marrow derived dendritic cells
BMDM	bone marrow derived macrophages
C	complement component
CARD	caspase recruitment domain
Carma	CARD-MAGUK protein
CCL	CC (cystein-cystein) chemokine ligands
CD	cluster of differentiation
CFU	colony forming units
IAP	inhibitors of apoptosis
Clec	C-type lectin domain family member
CLR	C-type lectin like receptor
CR	complement receptor;
CRD	carbohydrate recognition domain
CRP	C-reactive protein
C-type	calcium-dependent type
Dap	DNAX-activation protein
DC-SIGN	dendritic cell-specific ICAM3-grabbing nonintegrin
DD	death domain
Dectin	dendritic cell-associated C- type lectin
DNA	desoxyribocucleic acid
EAE	experimental autoimmune encephalomyelitis
EMCV	encephalomyocarditis virus
EMSA	electromobility shift assays
Erk	extracellular signal-regulated kinase

Fc	crystalisable fragment
FEEL	fasciclin EGF-like, laminin-type EGF-like, and link domain containing SR
FOX	forkhead box
gp340	glycoprotein 340
GTP	guanosyl triphosphate
GUK	guanylate kinase
HIV	human immunodeficiency virus
HSV	herpes simplex virus
ICAM	intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
Iga/b	B cell receptor immunoglobulin a and b chain
IkB	inhibitors of NF-kB
IKK	IkB kinase
IL	interleucin
IL-1R	interleucin-1 receptor
iNOS	nitic oxide synthetase
IRAKs	IL-1R-associated kinases
IRF	Interferon Regulatory Factor
ITAM	immunreceptor tyrosine-based activation motifs
Jnk	c-Jun N-terminal kinase
kDa	kilo Dalton (molecular mass)
LBP	lipid binding protein;
Lck	lymphocyte cell-specific protein-tyrosine kinase
LOX	lectin-like oxidized LDL receptor
LPG	lipophosphoglycan
LPS	lipopolysaccharide
LRRs	leucin rich repeats
LTA	lipoteichoic acid
Lyn	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MAGUK	membrande-associated guanylate kinase
MAIR	myeloid-associated Ig-like receptor
Malt	mucosa-associated-lymphoid-tissue lymphoma-translocation gene
MAP	mitogen-activated protein
MAPK(KKK)	mitogen-activated protein kinase (kinase kinase kinase)
MARCO	macrophage receptor with collagenous structure;

MAVS	mitochondrial antiviral-signaling protein
MBL	mannose-binding lectin
MCMV	murine cytomegalovirus
MDA	melanoma-differentiation-associated gene
MDP	muramyl dipeptide
MHC	mayor histocompatibility complex
MRR	macrophage mannose receptor
Mx	myxovirus resistance protein
MyD	myeloid differentiation primary-response protein
NACHT	nucleoside triphosphatase protein domain contained in NAIP, CIITA, HET-E and TP1
NAIP	neuronal apoptosis inhibitor protein
NDV	newcastle disease virus
NF-AT	nuclear factor of activated T cells
NF-kB	nuclear factor kappa-B
NK cells	natural killer
NLR	NACHT–leucine-rich repeat
NO	nitic oxide
NOD	nucleotide-binding oligomerization domain
OSCAR	osteoclast-associated receptor
P	phosphate group
p38	protein of 38 kDa
p65	protein of 65 kDa
PAMP	pathogen associated molecular pattern
PCR	polymerase chain reaction
PGRP	peptidoglycan-recognition protein
PKC	phosphotyrosin kinase C
PLCg	phospholipase C gamma
PDZ	perfect dark zero
PRR	pattern recognition receptor
PTX	pentraxin
PYD	pyrin N-terminal homology domain
R	receptor
Rel	v-rel avian reticuloendotheliosis viral oncogene homolog
RIG-I	retinoic-acid-inducible gene I
RIP	receptor interacting protein

RLH	RIG like helicases
RNA	ribocucleic acid
ROS	reactive oxygen species
RSV	respiratory-syncytial virus
RT	reverse transcriptase
SAP	serum amyloid protein
SD	standard deviation
SH	Src homology domain
SIGN	specific ICAM3-grabbing non-integrin
SIGNR	IGN-related
SMAD	SMA and MAD homolog
snRNP	small nuclear Ribonucleoprotein
SP	surfactant protein
SR	scavenger receptor
Src	v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
STAT	signal transduction and activator of transcription
Syk	splenic tyrosine kinase
T cell	T lymphocyte
TAD	transcription activation domains
TAK	TGF-beta activated-kinase
TCR	T cell receptor
TH	helper T cell
TIR	Toll/IL-1R domain
TIRAP	Toll-interleukin 1 receptor (TIR) domain-containing adapter protein
TLR	Toll like receptor
TNF	tumor-necrosis factor
TNFR	tumor-necrosis factor receptor
TRAF	TNF-receptor-associated factor
Treg	regulatory T cell
TREM	triggering receptor expressed on monocytes
TRIF	tiled raster interchange format
ub	ubiquitin
VSV	vesicular stomatitis virus
WNV	west nile virus
Y	tyrosine, ITAM
ZAP-70	zeta-chain asociated protein of 70 kDa

1 Introduction

Long-lived, multi-cellular organisms are under the constant attack of microbial pathogens. In order to deal with these intruders, multiple defence mechanisms have developed in vertebrates and invertebrates and are collectively known as the immune system. It consists of a plethora of different cell types, each utilising distinct approaches to defend the organism. In mammals, most of these cells derive from stem cells, located in the bone marrow. They float through the blood and lymph and accumulate in lymphoid organs, such as the thymus, spleen, and lymph nodes or reside in the tissues they protect (Fu and Chaplin 1999). Defence is activated through the recognition of pathogens-specific foreign structures by receptors of the immune system. There are two main classes of receptors, carried by different cells types, which discriminate the two major branches of the immune system: innate and adaptive immunity. Whilst the receptors of innate immune cells are germ line encoded (Janeway and Medzhitov 2002), the receptors of adaptive immune cells are produced by somatic chromosomal rearrangements in the genes encoding the antigen-specific parts of the receptors (Fugmann, Lee et al. 2000).

1.1 The Innate Immune Response

Innate immune responses are evolutionarily much older than adaptive responses and are not restricted to vertebrates but also present in other phyla like insects (Medzhitov, Preston-Hurlburt et al. 1997) or plants (Deyoung and Innes 2006). These responses rely on a limited spectrum of so-called pattern recognition receptors (PRRs, Figure 1) that recognize specific pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov 2002). Such molecules are indispensable for pathogen cellular function but are absent in the host. Through the detection of PAMPs by their PRRs, phagocytes, like neutrophils and macrophages can immediately react to pathogen invasion without time delay due to clonal expansion. Following phagocytosis, pathogens are killed by the exposure to reactive oxygen species, nitric oxide and lytic enzymes (Underhill and Ozinsky 2002). In addition, the release of

distinct cytokines and other mediators by tissue-resident macrophages and dendritic cells in the vicinity of pathogen entrance is the starting point of local inflammation. The inflammatory response encompasses recruitment of lymphocytes and serum proteins like complement components from the blood to the site of infection as well as phagocyte activation.

1.1.1 The Cellular Basis of Innate Immunity

Several cell types perform innate immune reactions. These cell types differ in the mechanisms and kinetics of anti-pathogen responses. The most prominent cells to promote innate responses are neutrophils and macrophages, which make up the phagocyte family. Neutrophils are the first cell type to respond to an infection and upon recognition they ingest and kill microbes. Following pathogen uptake, neutrophils die after a few hours (Eyles, Roberts et al. 2006). They are the most abundant type of leukocyte in the blood. During inflammation, they rapidly cross the vessel endothelium and invade the infected tissue. Macrophages on the other hand, reside in tissues or float in the blood where they are termed monocytes. Monocytes also migrate into inflamed tissues but unlike neutrophils, they don't die after activation but survive in the tissue for long periods of time, even after pathogen encounter (Gordon and Taylor 2005).

Tissue-resident macrophages produce messenger molecules, so-called cytokines in response to the recognition of invading pathogens. Amongst the expressed cytokines are tumour necrosis factor alpha (TNF-α), Interleukin-1 (IL-1) and chemoatractant cytokines, termed chemokines (Brown 2006). They stimulate the endothelial cells of capillary vessels close to the site of infection, which in turn produce adhesion molecules and loosen their cell-cell contacts. Phagocytes then bind to the adhesion molecules and according to the chemokine milieu produce high-affinity ligands for the adhesion molecules on the endothelial cells. By this mechanism, phagocytes are recruited from the bloodstream and enter the tissue. They now travel along the chemokine gradient through the tissues to the very site of pathogen presence. Activated lymphocytes enter inflammatory tissues by the same mechanisms (Abbas and Lichtman 2006).

1.1.2 Mechanisms of Pathogen Killing

Some of the PRRs work as phagocytosis activating receptors. In addition to PRRs, phagocytes also recognize antibody coating of pathogens that the adaptive immune system had already encountered earlier, by a special receptor. These so-called Fcγ receptors (Figure 1) bind to the constant region (Fc region) of antibody molecules of the IgG isotypes, but only when they are bound to antigen (Sedlik, Orbach et al. 2003). It also mediates phagocytosis and cell activation. Upon recognition, pathogens are engulfed by the cell membrane and are eventually taken up by membrane vesicles called phagosomes. These vesicles fuse with lysosomes (lytic vesicles) forming phagolysosomes, in which pathogens are killed. To perform this fuction, lysosomes contain several microbicidal agents, such as reactive oxygen species (ROS), which are produced by the enzyme phagocyte oxidase. Basically the enzyme converts oxygen into superoxide anions and free radicals, which are toxic to the pathogens. Another example is the inducible nitic oxide synthetase (iNOS) enzyme that produces toxic nitric oxide from arginine. In addition, lysosomes contain proteolytic enzymes that degrade pathogen proteins. All these enzymes are produced or activated in the lysosomes after PRR pathogen recognition (Gordon 2003). Localization of pathogen killing in this special membrane compartment protects the host cell from the toxic effects of the microbicidal agents that would harm them as well (Sansonetti 2006).

In the case of pathogens too big to get phagocytosed like helmith parasites, ROS, nitic oxide (NO) and proteolytic enzymes are secreted from specialised cells called eosinophils. Due to the fact that release of microbicidal agents can cause massive tissue damage, these cells are tightly controlled. They are only weakly activated by PRR stimulation and actually require an additional activating signal from the adaptive immune system to become fully activated. This signal comes from the Fcε receptor that binds, in contrast to Fcγ, free antibody molecules of the IgE isotype (Kraft and Kinet 2007). IgE is specifically produced by B-cells against antigens derived from large, multicellular, non-phagocytosable pathogens. Thereby, by producing distinct types of antibodies, B-cells participate in the decision whether macrophages or eosinophils are preferentially activated. T-cells also contribute to the macrophage and eosinophil activation by production of the cytokine interferon gamma (IFN-γ) (Gordon 2003).

Using these pathways, the function of innate immune cells is controlled by the adaptive immune system in order to orchestrate the response and to avoid over-reactions and host tissue damage.

1.1.3 The Interface between Innate and Adaptive Immunity.

For the activation and shaping of T-cell and B-cell responses, another tissue-resident cell type carrying PRRs plays a major role, namely dendritic cells (DCs) (Reis e Sousa 2006). Like macrophages, dendritic cells reside in all tissues of the body. In some tissues, they carry different names, such as langerhans cells in the epidermis, stern cells in the liver and microglia in the brain. After recognition and phagocytosis of a pathogen, these cells move from the site of pathogen-encounter to the closest "draining" lymph node. Here they present antigen patterns of the pathogen to T cells and activate T cell clones that carry receptors with high affinity for those antigens (Reis e Sousa 2006). According to the composition of PRR stimuli received from the pathogen, dendritic cells produce a varying pattern of cytokines - a process poorly understood. This DC derived cytokine cocktail contains, among others, IL-12, IL-6, TNF-α, IL-10, IL-2 and IL-23. Apparently, the composition of this mixture provides information for the T-cell about the nature of the pathogen from which the antigen was derived. T helper cells now differentiate into different types that in turn stimulate other immune cells, like macrophages, eosinophils and B cells. Humoral responses are thereby also tuned to produce either preferentially IgG or IgE (Calame, Lin et al. 2003). Collaboration of innate and adaptive immunity is therefore essential for a strong, directed and sustained immune response.

1.2 The Adaptive Immune Response

B lymphocytes and T lymphocytes (B and T cells) constitute adaptive immunity. Their receptors are the B cell and T cell receptors respectively, which are summarised as antigen receptors (Figure 1). Each naïve T or B cell clone carries a receptor with a unique specificity (Bassing, Swat et al. 2002). Upon recognition of their specific ligand, the antigen, the cells are activated, start to proliferate and carry out their specific

function whilst being inactive in the absence of antigen. The enormous variety of antigen receptors originates from genetic changes in the genes encoding them (Jung and Alt 2004). This genetic rearrangement process constantly generates new B and T cells with new specificities throughout long time periods. New T cells are generated during childhood and adolescence, while new B cells are produced throughout the whole lifetime (Muljo and Schlissel 2000). Due to the fact that these changes occur randomly, newly generated cells are tested before release into the periphery. Any receptors that are auto-reactive are deleted as their receptor is activated by contact with a self-antigen expressed by special antigen presenting cells, a process called negative selection. Failure to delete these self-responding cells leads to autoimmune diseases. During maturation, receptor function is also tested in a process termed positive selection. Cells with receptors that are unable to mount a signal via their receptor die. Therefore only functional but not auto-reactive B and T cells reach the stage of mature, naïve lymphocytes (Nemazee 2000). The term T cells originates from the thymus, in which this process is carried out (Starr, Jameson et al. 2003). B cells selection occurs in the bone marrow (Niiro and Clark 2002).

1.2.1 B-Lymphocytes and the Humoral Immune Response

B cells are the source of antibodies, soluble proteins mainly found in the blood and lymph. Due to this feature, B cell mediated immunity is called the humoral response, which is directed against extracellular pathogens (Calame, Lin et al. 2003). Antibodies carry two or more antigen recognition sites, binding to their respective antigen. Recognition of antigen by the B-cell receptor that is, in fact, a membrane-bound antibody molecule (Figure 1), triggers the B cell to proliferate and initiates production of soluble antibodies. Clonal expansion leads to a greater number of activated B cells, producing huge amounts of antibodies, which in turn coat the pathogen, toxin or any other foreign molecule. The presence of more than one binding site on one antibody molecule, numerous molecules of the same antigen and of several different B cell clones, producing antibodies specific for different antigens on a pathogens surface interlinks them, hindering their vital processes, like entering host cells (DeFranco 2000). The antibody coating also marks the foreign component, i.e.

pathogen or toxin, for destruction by phagocytes, mainly macrophages. To mount this activity, phagocytes carry a receptor for the non-antigen-specific part of the antibody molecule, the so-called Fc-part (Fc-receptor, see Figure 1) that recognises antigen-bound antibody, as mentioned above. In addition, recognition of antigen by antibody also induces the activation of the complement system against the antigen (Heyman 2000). Complement is a system of proteins, recruited to the pathogen surface not only via bound antibodies but also by direct recognition of foreign structures. Complement forms pores in the microbe's cell wall, relieving its membrane potential and eventually killing it. Complement components in turn are recognized by phagocyte receptors. The coating of a foreign particle such as a pathogen both with antibodies and complement is summarised as opsonisation (Carroll 2004).

1.2.2 T-Lymphocytes Interact with MHC Molecules on Host Cells

In contrast to the humoral response, the cellular immune response is carried out by T cells and is directed against intracellular pathogens, that is, infected host cells. While B cells recognize any present antigen, T cells only recognize antigen after it is presented to them on the surface of host cells by a special molecule, the mayor histocompatibility complex (MHC) (Rammensee and Bevan 1984). The MHC presents short peptides of all proteins present in a cell. While healthy host cells only present self-peptides, infected cells also present peptides from the bacteria or viruses infecting them (Ackerman and Cresswell 2004). These peptide-MHC complexes trigger antigen-specific T cell clones to expand and react. Two different classes of T cells react on two different forms of MHCs. Cytotoxic T cells interact with MHC class I that is present on every host cell. Specific binding to MHC class I is facilitated by the co-receptor molecule CD8 on cytotoxic T cells. Upon recognition of foreign antigen on a host cell, indicating infection, cytotoxic T cells induce apoptosis within than cell und thus prevents further reproduction of the pathogen and exposes them to a humoral response. A second effect of antigen recognition is the induction of proliferation of the T cells, ensuring a broad systemic killing of infected host cells (Hewitt 2003).

The second class of T cells is the helper T cells that are rendered by the expression of the coreceptor CD4. $CD4^+$ T cells are the main regulators of adaptive

immunity. They interact with MHC class II, which is only present on professional antigen presenting cells (APCs), like dendritic cells and macrophages (Bryant, Lennon-Dumenil et al. 2002). Full activation of $CD4^+$ T cells requires three stimuli by the APC. Besides stimulation of the antigen receptor (signal 1), so-called costimulatory receptors, such as CD28 and CD40L, must also be triggered (signal 2). Their ligands are molecules of the B7 family, namely CD80 and CD86 in mice and humans and CD83 in humans alone, and CD40, upregulated on the APC (Sharpe and Freeman 2002). As already mentioned in the discussion of dendritic cells, the third signal consists of cytokines, soluble proteins released by the APC (Kapsenberg 2003).

Helper T cells fall into diverse classes that are distinguishable by their production of different cytokines upon stimulation. Responses by interferon IFN-γ-producing $CD4^+$ T helper type 1 (T_H1) cells promote immunity to viruses, intracellular bacteria and protozoan parasites mainly through hyperactivation of macrophages. T_H2 cells make interleukin 4 (IL-4), IL-5 and IL-13 and direct immunity to metazoan parasites. T_H2 responses do this mainly by modulating B cell responses in order to produce special immunoglobulin isotypes like IgE, that in turn activate eosinophils, as mentioned above (Sher and Coffman 1992) (Abbas, Murphy et al. 1996). Whether a naïve T helper cell (T_H0) differentiates into a T_H1 or a T_H2 cell depends on the cytokine milieu accompanying the antigen receptor and co-receptor stimulation (Murphy and Reiner 2002).

Another class of proinflammatory helper T cells, T_H17 cells, has also been recently added to the spectrum. They are named after their most prominent feature, the production of IL-17 upon stimulation. IL-17 and/or its receptor have been linked to resistance to infection by extracellular bacteria such as *Klebsiella pneumoniae* and fungi such as *Candida albicans* (Ye, Rodriguez et al. 2001; Huang, Na et al. 2004). Whereas T_H1 cells are usually induced by the production of IL-12 by the APC, the closely related cytokine IL-23 appears important for the production of T_H17 cells. Their specific function and antigen receptor specificities remain largely unknown to date.

Regulatory T cells (T_{regs}) on the other hand don't induce but mainly suppress immune reactions, preventing over-reactions and excessive tissue damage (Mills 2004). Antigen presenting cells, T helper cells and regulatory T cells all contribute to the orchestration and balance of immune reactions, ensuring the right kind and strength of

response. Together, they optimise the immune response for maximum pathogen killing and minimum host tissue damage.

1.2.3 NK Cells Participate in Viral and Cancer Defence

Loss of MHC expression in any vertebrate cell is critical due to the fact that an infection of such a cell would be obscured. Many viruses hide from cytotoxic T cell action by downregulating MHC expression of their host cells. Therefore another cell type, the natural killer (NK) cells kill any cell that doesn't carry MHC class I on its surface (Kim, Poursine-Laurent et al. 2005). Although NK cells are a part of innate immunity, since they don't carry antigen receptors, they are introduced here due to their interaction with MHC and their relationship to T cells in terms of development and mechanism of target cell killing. NK cells carry a wide spectrum of activating and inactivating receptors (Yokoyama and Plougastel 2003). Any healthy cell of the body carries proteins, mostly MHC alleles, on its surface that inactivate NK cells. If a cell fails to inactivate a passing NK cell, it is killed. This is also an important mechanism for the prevention of cancer. Developing cancer cells might loose expression of MHC class I or other molecules important for the inactivation of NK cells and are thus eventually killed (Pardoll 2003). NK cells also participate in the immune responses to viruses. They recognise and kill virus-infected cells mostly by the action of receptors of the C-type lectin family. In addition, they produce large amounts of class I interferons upon recognition of viral infecton. These soluble messenger molecules locally or systemically reduce host cell proliferation, thereby also slowing down virus production in host cells. Interferons also help shape T cell responses for optimal virus defence (Katze, He et al. 2002).

1.3 Immune cell activation

Cells usually perceive their environment and obtain signals from it through membrane-bound receptors. These receptors typically consist of an extracellular recognition domain, a transmembrane domain and an intracellular signalling domain. The recognition domain binds to its specific ligand, ideally just one chemical

compound or a small group of structurally related molecules, thereby facilitating ligand specificity. Binding of the ligand leads to changes in the receptor molecule conformation and/or modifies the cellular distribution relative to other membrane bound proteins. Thereby, proper cellular processes are initiated in response to the ligand recognition. The receptors of the immune system recognize foreign structures and activate the immune cells to engage defence. The fundamental difference between innate and adaptive immunity is the nature of their receptors, as outlined in the following sections. In this section, the main focus lies on the introduction of the receptors of innate immunity and their ligands. Nevertheless, some information about further signal transduction is also included. A more general as well as more detailed discussion of signal transduction and gene regulation follows in the next chapter.

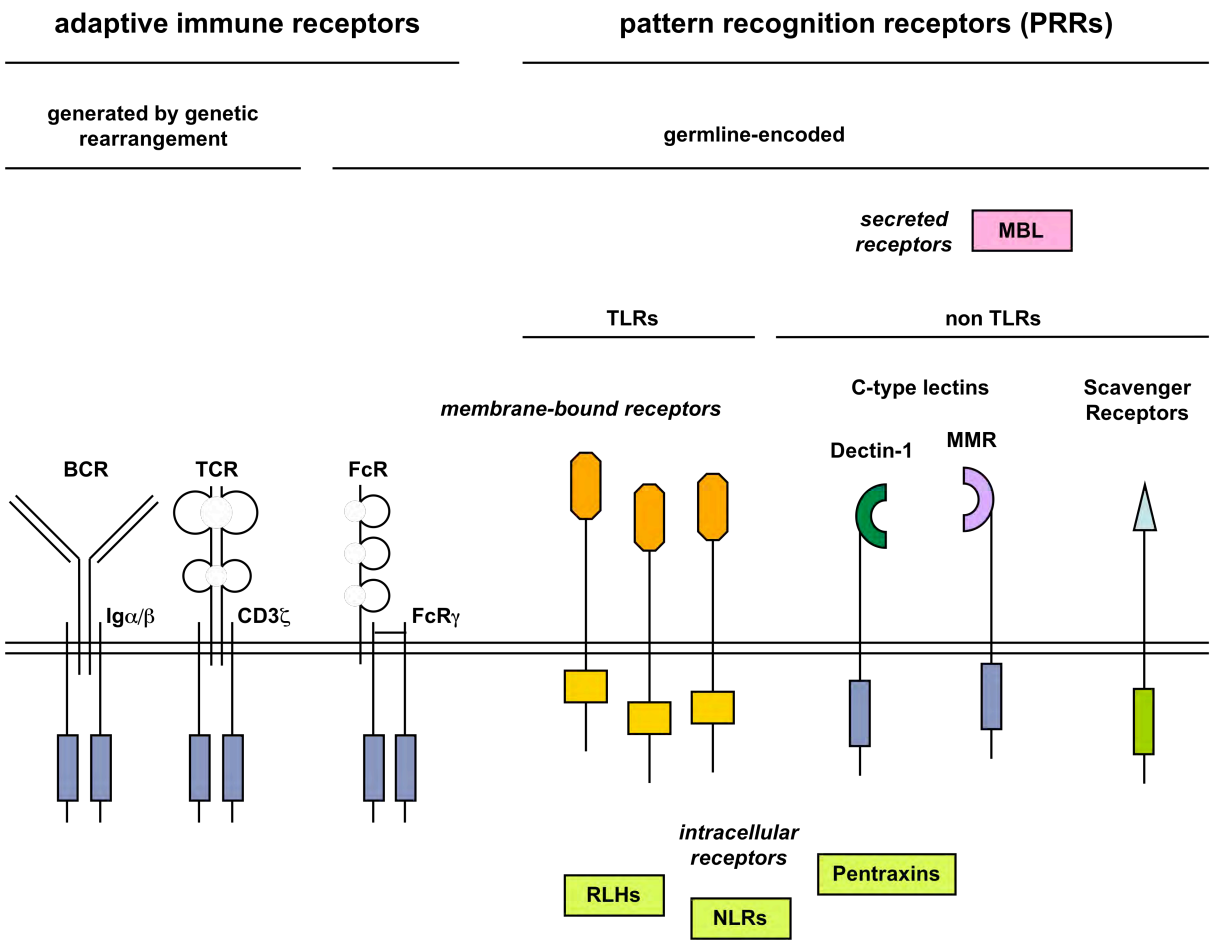

Figure 1: Receptors of innate and adaptive immunity. For abbreviations, see page 13ff.

1.3.1 Receptors of Innate Immunity

As already mentioned, innate immunity relies on a vast amount of different germline-encoded receptors to recognize pathogens. Every cell of innate immunity carries lots of different receptors, all recognizing different non-self structures, thereby allowing one cell to recognize many different pathogens. These receptors belong to different protein families and differ in their ways of action (Gordon 2002). Many of them, like toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are membrane-bound, either on the cell surface or in intracellular vesicles. Other receptors, like mannose-binding lectin (MBL) are secreted or, like nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs) or retinoic-acid-inducible gene I (RIG-I)-like RNA helicases (RLHs) are present in the cytoplasm. Table 1 lists the most prominent PRR families (Medzhitov 2001; Figdor, van Kooyk et al. 2002; Inohara and Nunez 2003; Takeda, Kaisho et al. 2003; Brown 2006; Fritz, Ferrero et al. 2006; Honda and Taniguchi 2006; Klesney-Tait, Turnbull et al. 2006; Meylan, Tschopp et al. 2006; Robinson, Sancho et al. 2006).

Family	Receptors	localization	Ligand or Pathogen
Complement	C3, C1q	Serum or tissue fluid	Microbial surfaces
Toll-like receptors	TLR1-11	Cell surface or Endosome	PGN, LPS, dsDNA, dsRNA, ssRNA,...
Lipid transferases	LBP	Serum or tissue fluid	LPS
Collectins	SP-A, SP-D, MBL	Serum or tissue fluid	Influenza A virus, herpes simplex virus, HIV, *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Neisseria meningitidis, Haemophilus influenzae, E. coli,Pneumocystis carinii, Cryptococcus neoformans, Aspergillus fumigatus, Candida albicans*
Pentraxins	PTX3, SAP, CRP	Serum or tissue fluid	Zymosan, *S. aureus, E. coli, Streptococcus pyogenes, N. meningitidis, A. fumigatus, C. albicans, Plasmodium falciparum*, influenza A virus, LPS, Phosphorylcholine on mimrobial membranes
RLHs	RIG-I, MDA5	Cytosol	3P-RNA
Leucine-rich repeat proteins	CD14	Cell surface	LPS, LTA, peptidoglycan, *E. coli*
Scavenger receptors	SR-A, SRCL-I, SR-PSOX, FEEL-1, FEEL-2, LOX1, gp340, SR-BI, MARCO, LTA, CD36	Cell surface	*E. coli, S. aureus, L. monocytogenes, Mycobacterium tuberculosis, Enterococcus faecalis, N. meningitidis, Streptococcus* spp., *Helicobacter pylori*, LPS, bacterial DNA
Classical C-type lectin	Mannose receptor, DC-SIGN, L-SIGN, SIGNR1	Cell surface	*C. albicans, P. carinii, M. tuberculosis, K. pneumoniae, Leishmania donovani*, HIV, zymosan, Ebola virus
Non-classical C-type lectin	Dectin-1	Cell surface	*Saccharomyces cerevisiae, C. albicans, P. carinii, Coccidioides posadasii, A. fumigatus*
Integrins	CR3, CR4	Cell surface	Complement-coated microorganisms, LPS, LPG, APG, *C. albicans, M. tuberculosis, C. neoformans*
Long PGRP	PGRP-L, PGRP-I, PGRP-I	Cell surface	Peptidoglycan, *Bacillus subtilis, Micrococcus luteus*
Other	Lactosylceramide	Cell surface	Glucan, *P. carinii, C. neoformans, C. albicans, H. pylori*
Interferon-induced proteins	RNA-activated protein kinase, 2', 5'-oligoadenylate synthetase, Mx protein GTPases, p65 guanylate-binding proteins	Cytosol	dsRNA, viral protein complexes
NLR	NOD1, NOD2, NAIP	Cytosol	Muramyl dipeptide, *Shigella flexneri*
Integrins	Mac-1	Cell surface	

Table 1. Pattern recognition receptor (PRR) families with their localisation and recognised ligands. For abbreviations, see page 13ff. Modified from (Brown 2006)

Toll-like receptors are structurally and presumably phylogenetically related to Toll, an insect protein that also, among other ontogenetic properties, participates in the insect immune response (Hoffmann and Reichhart 2002). TLRs consist of extracellular leucin-rich repeats (LRRs) a transmembrane domain and an intracellular Toll/IL-1-receptor (TIR) domain. TLR-7, -8 and -9 are localized in membranes of the endosome, while the others are found on the cell surface. TLR-1, -2, -4, -5, -6, and -11 recognize components of microbial surfaces, like lipopolysaccharide (TLR-4) in the cell wall of gram-negative bacteria, peptidoglycan (TLR2) in gram-positive ones or flagellin (TLR5), a component of the flagella of some motile bacteria. TLR-3, -7, -8 and -9 recognize nucleic acids, i.e. double-stranded RNAs of viruses (TRL3) or CpG motifs, special DNA sequences that are absent in mammals (TLR9) (Medzhitov 2001). The further events of signalling and altered gene expression induced by TLRs upon PAMP recognition are discussed later.

Toll-like receptors are membrane bound and directed to the extracellular space, thereby detecting external or phagocytosed pathogens. Another class of membrane-bound PRRs, the C-type lectin like receptors (CLRs) are the subject matter of section 1.4.4. In contrast, many pathogens, viruses and some bacteria reside in the cytoplasm and soluble cytoplasmatic receptors are responsible for their perception. The most prominent family of intracellular molecules monitoring bacteria is the NOD-(Nucleotide binding and oligomerisation domain) like receptors (NLRs). This family currently consists of 22 members (Meyland, Nature 2006), NOD2 being its most prominent and best-studied member. NLRs typically contain an N-terminal CARD or pyridine effector domain (PYD), a nucleotide binding and oligomerization (NACHT) domain and a variable number of C-terminal leucin rich repeats (LRRs). NLRs are synthesized in an autorepressed form, in which their LRRs are folded back to the rest of the protein. This inhibits oligomerization by NACHT domains. Upon ligand recognition by the LRRs, the protein unfolds and homooligomerizes. The CARD or PYD recruits downstream effectors into the complex activating further signalling. NOD2 is involved in the perception of intracellular muramyl dipeptide (MDP), a breakdown product of bacterial peptidoglycan. It is unclear though, whether it is the receptor of MDP or interacts with the actual receptor or a downstream effector of it. Like the TLRs, NOD2 activates mitogen-activated protein kinases (MAPK) and nuclear factor κB (NF-κB)

signalling cascades by CARD dependent binding of receptor interacting protein 2 (RIP2). NOD2 is indicated to be involved in immunity to *Streptococcus*, *Mycobacteria* and *Listeria* species. Mutations in the LRRs of NOD2 have been associated with chronic inflammatory disorders like Crohn's disease. This is an inflammatory bowel disease associated with increased production of proinflammatory cytokines such as TNF-α and IL-1β and hyperactivation of NF-κB in gut associated lymphoid cells (Kaparakis, Philpott et al. 2007).

Viruses also give away their presence in the host by carrying special PAMPs. One of the most prominent of these is single- or double-stranded RNA (ssRNA or dsRNA). Although TLR3 can recognize extracellular dsRNA, it is most important for the infected cell to recognize intracellular viral RNA being produced in the cytoplasm upon viral replication. Two members of the RIG like helicases (RLHs), RIG-I and melanoma-differentiation-associated gene 5 (MDA5) recognize intracellular viral RNA. The closely related proteins consist, like NOD2, of two N-terminal CARDs and, unlike NOD2 of a C-terminal helicase domain. These receptors distinguish between host and virus RNA by the presence of 5′ triphosphate groups in viral RNA that is absent in host RNA (Thompson and Locarnini 2007). Upon binding of 5′ triphosphate RNA, they interact by CARD-CARD interaction with MAVS, a protein located in the mitochondrial membrane. Further signalling activates the transcription factors NF-κB and interferon regulatory factor (IRF) 3 and 7, eventually leading, amongst other effects, to the production and release of the antiviral cytokines interferon (IFN) α and IFNβ (type-I interferons). These two receptors have been shown to be important for immune responses to Japanese encephalitis virus, Newcastle disease virus, vesicular stomatitis virus, Sendai virus and influenza virus (RIG-I), as well as encephalomyocarditis virus (MDA5).

The different receptors and receptor families of innate immunity utilize distinct signal transduction pathways to trigger diverse cellular reactions like activation, proliferation, cytokine production, phagocytosis and oxidative burst. Many of these reactions are based on altered gene transcription. Table 2 summarizes the signalling mechanisms and induced actions of some membrane-bound receptors or receptor families.

PRR	Localization	Adaptor	Action	Pathogens or ligand
Toll-like Receptors (TLRs)				
TLR2	Cell surface	MyD88	NF-κB	Peptidoglycan (PGN), Gram-positive bacteria
TLR3	Cell surface (on epithelial cells) or endosomes	TRIF	NF-κB, IRFs	RNA, Reovirus (dsRNA virus) WNV (positive-sense ssRNA virus) RSV (negative-sense ssRNA virus) MCMV (dsDNA virus) *Leishmania donovani* and *Schistosoma mansoni* Virus-infected dying cells
TLR4	Cell surface	MyD88, TIRAP, TRIF	NF-κB, IRFs	LPS, Gram-negative bacteria, RSV (negative-sense ssRNA virus)
TLR7	Endosome	MyD88	NF-κB, IRFs	RNA, VSV and influenza virus (negative-sense ssRNA viruses), Loxoribine, Immune complexes of snRNP and IgG
TLR9	Endosome	MyD88	NF-κB, IRFs	Unmethylated CpG-motifs, HSV and MCMV (dsDNA viruses) Immune complexes of host DNA and IgG
C-Type Lectins (CLRs)				
Mannose receptor	Cell surface		Phagocytosis	Endogenous and exogenous ligands bearing mannose, fucose, *N*-acetyl glucosamine and sulphated sugars via cysteine rich domain
DC-SIGN	Cell surface		Phagocytosis, T-cell interaction, migration	HIV and other pathogens due to mannose type CRD. ICAM-2 and -3 via CRD.
L-SIGN	Cell surface			Similar to DC-SIGN
SIGNR1	Cell surface			Mannose-type CRD, dextran, *Streptococcus pneumoniae*, *Candida albicans*, HIV, ICAM-3
Langerin	Cell surface		Formation of Birbeck granules	Mannose, fucose, *N*-acetylglucosamine
Dectin-1	Cell surface	Syk	NF-κB, Phagocytosis, T-cell interaction, migration	Zymosan, Curdlan, *Candida albicans* yeast form
Dectin-2	Cell surface	FcRγ, Syk	NF-κB	Zymosan, *Candida albicans* hyphae
Clec4c	Cell surface		Phagocytosis	
NOD-Proteins (NLRs)				
NOD1	Cytosol	RICK, Caspase-1	NF-κB, MAPK	Muramyl dipeptide
NOD2	Cytosol	RICK, Caspase-1	NF-κB, MAPK	Muramyl dipeptide
NAIP	Cytosol		NF-κB, MAPK	Muramyl dipeptide
Card-Helicases (RLHs)				
RIG-I	Cytosol	MAVS	NF-κB, IRFs	VSV, NDV, Sendai virus and influenza virus (negative-sense ssRNA viruses)
MDA5	Cytosol	MAVS	NF-κB, IRFs	EMCV, Theiler's murine encephalomyelitis virus and Mengo virus (positive-sense ssRNA viruses)

Table 2. Selected PRRs from prominent families with their localisation, way of action and recognised ligands. For abbreviations, see page 13ff. Modified from (Honda and Taniguchi 2006); (Medzhitov 2001; Takeda, Kaisho et al. 2003) (TLRs); (Figdor, van Kooyk et al. 2002; Brown 2006; Robinson, Sancho et al. 2006) (CLRs); (Inohara and Nunez 2003; Fritz, Ferrero et al. 2006) (NLRs); (Meylan, Tschopp et al. 2006) (Card-Helicases).

1.4 Signal Transduction for Immune Cell Activation

Signal transduction is summarized as the intracellular events of information processing following receptor engagement, which triggers instant cascade pathway responses and leads to cellular responses including gene activation. Proteins that, after activation, bind to special DNA motifs and activate or suppress genes are termed transcription factors. Information is transduced from one molecule in a signalling cascade to another by a number of different mechanisms. Basically, one molecule passes information to the next molecule in the signalling cascade by modifying it, which allows the modified molecule in turn to influence the next member of the chain. These modifications include the addition or removal of phosphate groups, ubiquitin

molecules or other components. Its interaction partner can also induce conformational changes of a molecule. A third mechanism is the change of local molecule concentrations, for example by oligomerisation, which influences the molecules activity or by bringing interaction partners into closer proximity by binding to the same large scaffold protein.

Mitogen-activated protein kinase (MAPK) pathways are a classical example of a signalling cascade that is important in a lot of different cellular processes in presumably all eukaryotic cells. MAPK pathways typically consist of a chain of proteins phosphorylating the next protein in line after having been phosphorylated itself. This leads to a nomenclature, were a MAP-Kinase (MAPK) gets phosphorylated by a MAPK-Kinase (MAPKK) that in turn is phosphorylated and thereby activated by a MAPKK-Kinase (MAPKKK) and so on (Elion 2000). Important MAP kinases in immunity are extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (Jnk) and p38 (protein of 38 kDa) that activate, amongst others, the transcription factor adaptor protein complex 1 (AP-1) (Ashwell 2006). Further important transcription factors in both innate and adaptive immunity are nuclear factor κB (NF-κB), nuclear factor of activated T cells (NF-AT) and the IRF family (Honda and Taniguchi 2006).

1.4.1 NF-κB Signal Transduction

The transcription factor NF-κB is of remarkable importance in the immune system. A vast number of processes in innate and adaptive immunity rely on the activation of NF-κB. Upon PAMP recognition; virtually all PRRs activate NF-κB, resulting in the production of proinflammatory cytokines and other responses. These cytokines, i.e. TNF-α can in turn induce further NF-κB activation in their target cell by the action of their specific receptors like the TNF-α receptor. NF-κB is also of central importance in the activation of B- and T-cell through the antigen receptor (Karin and Greten 2005).

In mammals, the term NF-κB designates a family of five transcription factors, p50, p52, RelA (p65) c-Rel and RelB. Their common feature is the N-terminal DNA-binding/dimerization domain (Rel homology domain), through which they can form homo- and heterodimers. This domain facilitates also binding to distinct DNA motifs,

called κB sites, to alter gene expression. RelA, c-Rel and RelB also contain C-terminal transcription activation domains (TADs) for the activation of target gene expression. These sites are not contained in p50 and p52, which makes them negative regulators of gene expression. These two family members can also form heterodimers with the other members, in these cases they can act as positive regulators of gene expression. During absence of a stimulatory signal, NF-κB is sequestered in the cytoplasm by inhibitors of NF-κB proteins (IκBs). After stimulation, IκB proteins become phosphorylated and are then ubiquitinated and thereby designated for proteasomal degradation. The destruction of IκB allows NF-κB to translocate into the nucleus, subsequently activating its target genes (Bonizzi and Karin 2004). In most cases the IκB proteins are phosphorylated by the IκB kinase (IKK) complex. It consists of two catalytic subunits, IKKα and IKKβ and a regulatory unit, NEMO. In the more abundant canonical pathway, IKKβ and NEMO are required for gene activation, while IKKα is not essential but might also contribute to the IKK complex. In the alternative pathway, IKKα alone is responsible for the activation of NF-κB complexes inhibited by the IκB protein p100. Activating signals and target genes of these two distinct pathways might overlap. Upstream of IKK, individual receptors utilise different pathways for NF-κB activation. NF-κB, dependent on the cell type, activates many different genes. These include genes enhancing proliferation and survival in lymphocytes and cytokine and costimulatory molecules in myeloid cells. The statement that NF-κB is of extraordinary importance in immunity is underlined by its conservation throughout evolution. In fact, it is even the primary regulator of innate immunity in insects like *Drosophila* (Govind 1999).

1.4.2 Antigen Receptor Signalling for NF-κB Activation

B and T cell receptor signalling activates many transcription factors including NF-κB, for cell activation and proliferation (Figure 2). Membrane-bound subunits of the antigen receptor complexes, Igα/β for BCR and CD3ζ for TCR contain immunreceptor tyrosine-based activation motifs (ITAMs). Upon receptor activation, the tyrosines become phosphorylated by SRC family kinase like lck or lyn.

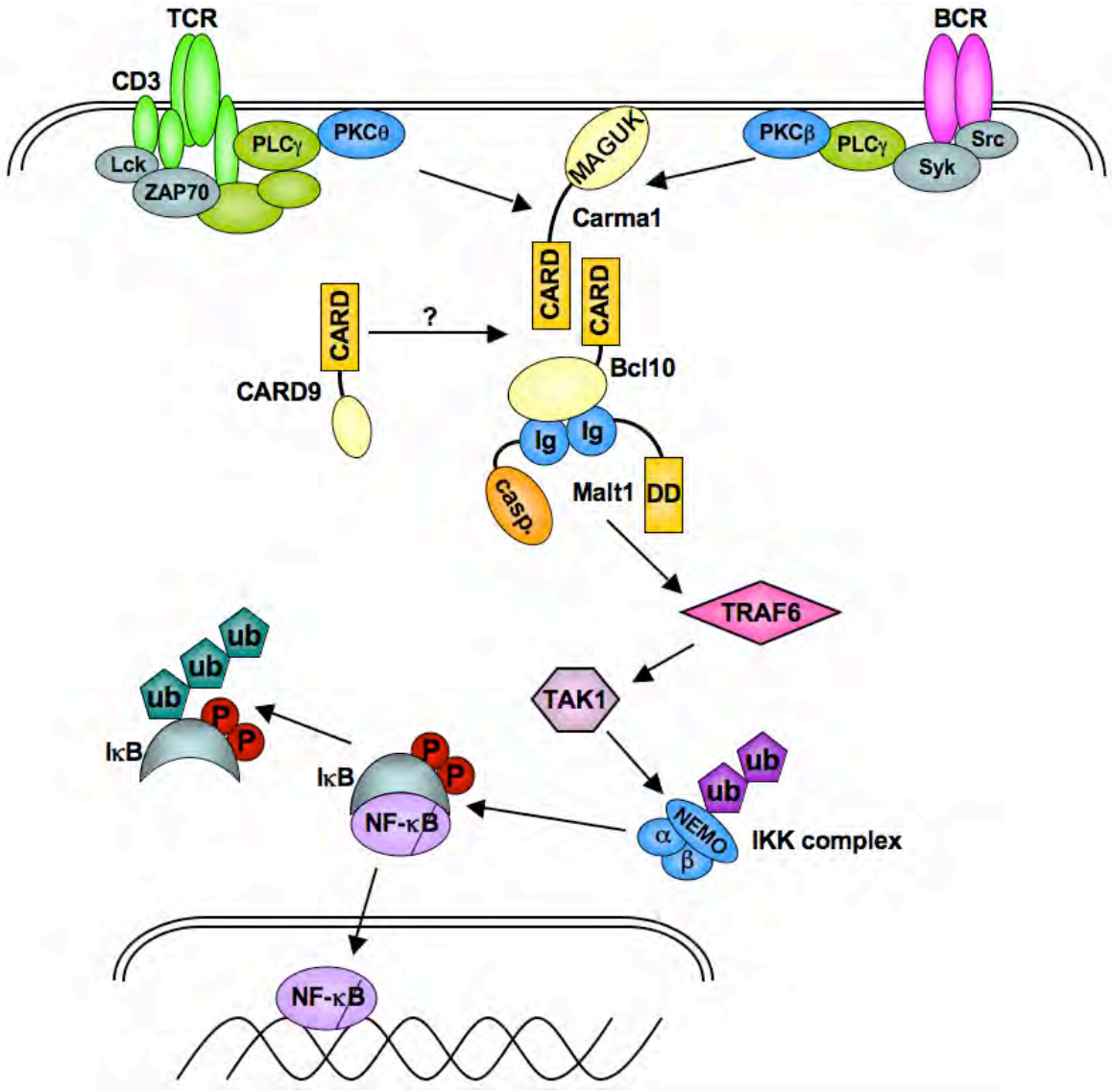

Figure 2: Signal transduction from B and T cell receptor to NF-κB via Bcl10. Abbreviations see abbreviation index on page 13. Modified from Thome and Tschopp (Thome and Tschopp 2003).

These phosphorylated ITAMs bind to and activate splenocyte tyrosine kinase (Syk) family kinases (Ruland and Mak 2003).Syk recruitment triggers a cascade in which activation of kinases, adaptors and phospholipases leads to the activation of phosphotyrosin kinase C (PKC). PKCβ or -θ can phosphorylate Carma1 in its linker region, connecting the membrane-associated guanylate kinase (MAGUK) and coiled-coil domain of Carma1 (Lin and Wang 2004). Carma1 and its two homologues Carma2 and -3 are large scaffold proteins consisting of a caspase recruitment domain (CARD), a coiled-coil domain and a MAGUK domain (Figure 4). Carma1, -2 and -3 share very high sequence homology but are expressed in different tissues (Thome 2004). The

coiled-coil and MAGUK domains of Carma1 mediate homo-oligomerisation of many Carma1 molecules with each other. In this way, many molecules of the same signalling components downstream of Carma1 are brought into close proximity with one another (Rawlings, Sommer et al. 2006). It has been shown that homo-oligomerisation of some downstream components of Carma1 is sufficient to activate NF-κB. Oligomerisation of one of the components can often be achieved by simple overexpression. The phosphorylation by PKC allows refolding of Carma1 (Matsumoto, Wang et al. 2005), making the CARD accessible for interaction with Bcl10 (Figure 4). As implicated by the name, CARD-containing proteins were initially identified as mediators of caspase activation and apoptosis (Bouchier-Hayes and Martin 2002). The discovery of Bcl10s role in antigen receptor activation has been the first report of an antiapoptotic/proproliferative role of a CARD containing protein (Ruland, Duncan et al. 2001). Bcl10 binds to Malt1 (Ruland, Duncan et al. 2003), which is thereby also included into the signalling complex (Figure 2). Recently, Klemm *et al.* demonstrated an involvement of Bcl10 and Malt1 in myeloid cells, namely mast cells, for NF-κB activation and cytokine production after stimulation of the ITAM receptor Fcε (Klemm, Gutermuth et al. 2006). This implies that the Bcl10/Malt1 pathway is not restricted to lymphoid cells and antigen receptors but also used in myeloid cells and might well be engaged by further receptors and pathways. Downstream of Malt1 the pathway converges with those of other receptors such as TNF receptor or TLRs by activating TNF-receptor-associated factor 2 (TRAF-2) and TRAF-6. Finally, the signal is transduced to the IKK complex via TGF-beta activated-kinase 1 (TAK1) (Figure 2).

1.4.3 The Toll-like Receptor Pathway for NF-κB

As outlined above, the 11 members of the Toll-like receptor family bind various ligands derived from many pathogens. Most of these receptors use a common signalling pathway to induce the activation of NF-κB. TLRs are type 1 transmembrane proteins and consist of extracellular leucin rich repeats (LRRs) and an intracellular Toll/IL-1R (TIR) domain (Medzhitov, Preston-Hurlburt et al. 1997). Variations in the LRRs of the different TLR family members are responsible for the binding specificity to their specific PAMPs. The intracellular TIR domain that the TLRs share with the interleucin-1 receptor

(IL-1R) mediates signalling by binding to the adaptor protein myeloid differentiation primary-response protein 88 (MyD88) (Medzhitov, Preston-Hurlburt et al. 1998). This binding is induced by dimerization of the receptors due to conformational changes upon ligand recognition. MyD88 recruits IL-1R-associated kinases (IRAKs) 1 and 4 by the death domain (DD) mediated protein-protein interaction (Akira and Takeda 2004). IRAK4 then phosphorylates IRAK1, which in turn recruits tumor-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6), and eventually leads to nuclear translocation of NF-κB.

1.4.4 Non-TLR Signalling in Innate Immunity: Dectin-1

In addition to TLRs, other receptors also contribute to immune cell activation by gene regulation in response to innate pathogen recognition, as summarised in Table 1. Compared to antigen-receptor and TLR pathways, little is known about these pathways (Figure 1, Table 2). Prototypic for such non-TLR PRRs is dendritic cell-associated C-type lectin 1 (Dectin-1) (Taylor, Brown et al. 2002). The recognition of fungal infections is primarily through this type II transmembrane receptor of the C-type lectin family.

Dectin-1 recognizes insoluble β-Glucans, which are present in the cell wall of yeast cells. In its cytoplasmic tail, Dectin-1 carries an immunoreceptor tyrosine-based activation motif (ITAM) (Brown 2006) by which it forwards activating signals. Receptors and receptor interacting signalling molecules throughout innate and adaptive immunity utilise ITAMs to forward signals (Table 3) (Barrow and Trowsdale 2006). As mentioned above, the ITAM-carrying receptor associated molecules Igα/β and CD3, found in B and T cell receptors, respectively signal via Syk (or its homologue ZAP-70) and Bcl10 (Ruland and Mak 2003) for lymphocyte activation and proliferation (Figure 3). The ITAM of Dectin-1, like FcRγ, also signals via Syk for phagocytosis, oxidative burst, and activation of gene transcription (Underhill, Rossnagle et al. 2005). Dectin-1s ITAM is extraordinary when compared to those of other receptors due to the fact that it carries only one YxxL motif, while usually two of these are required to interact with the two SH2 domains of Syk (Table 3). It has been shown though, that this one tyrosine is required and sufficient for Syk activation (Brown 2006). However, the pathways in which Dectin-1/Syk mediate their responses remains largely elusive.

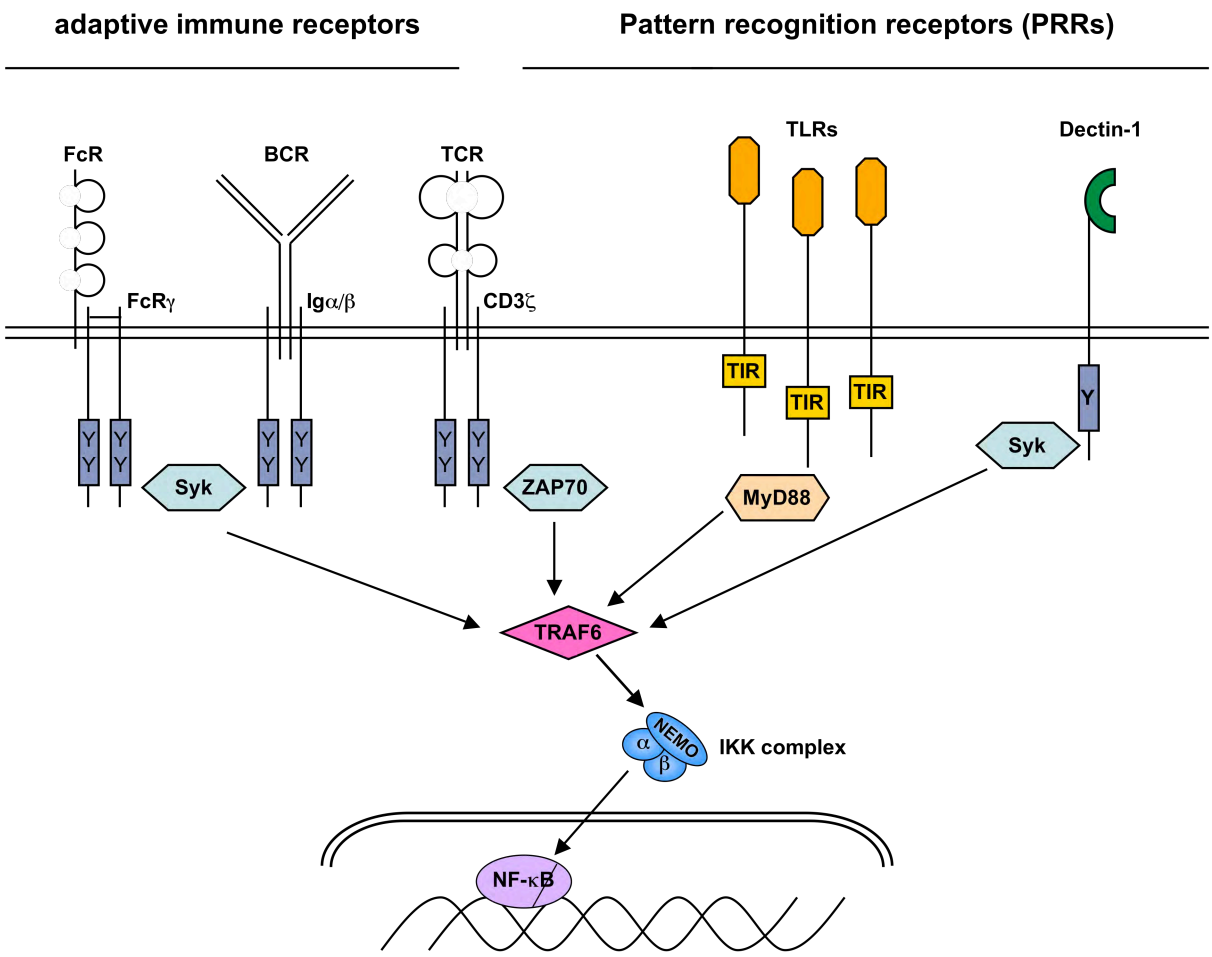

Figure 3: Various receptors engage common downstream signalling components for NF-κB activation **Y**, ITAM. For abbreviations, see page 13ff.

C-type lectins are a heterogenous family of receptors that are defined by the presence of a C-type lectin domain. The term C-type derives from the calcium dependent binding of the first discovered receptors, but not all of the receptors carrying this domain are calcium dependent. C-type lectins are primarily expressed on cells of the immune system, from both lymphoid and myeloid lineage. The Dectin-1 gene is located in the NK-cell receptor locus on chromosome 12 in humans and chromosome 6 in mice. Many other structurally related immune receptors are found on these loci. The bigger proportion of these C-type lectin like receptors are expressed mainly on NK-cells and contribute to the activation or inactivation of NK-cells. The activating

receptors of this family interact with stress or virus induced proteins on the surface of host cells (Yokoyama and Plougastel 2003). They also utilize ITAMs for cellular activation, either by carrying an ITAM in their cytoplasmatic portion or by utilizing adapter proteins like DNAX-activation protein 12 (DAP12) or FcRγ, which both carry ITAM motifs (Table 3).

Molecule	Amino Acid Sequence
CD3γ	DQL**YQPL**KDREDDQ**YSHL**
FcRγ	DGV**YTGL**STRNQET**YETL**
Igβ	DHT**YEGL**DIDQTAT**YEDI**
DAP12	ESP**YQEL**QQQRSDV**YSDL**
Dectin-1	MEYHPDLENLDEDG**YTQL**

Table 3: Immunreceptor tyrosine-based activation motifs (ITAMs) in signalling adaptors and receptors. The amino-acid sequence of a portion of the N-terminal intracellular part of several signalling molecules is shown. ITAM consensus sequences (YxxI/Lx(6-12)YxxI/L) in bold. Modified from (Barrow and Trowsdale 2006).

1.5 Card9

Caspase recruitment domain protein 9 (Card9) is a protein composed of 536 amino acids (AA) with a molecular weight of 59 kDa. Besides the caspase recruitment domain, from AA1 to 104 it consists of a coiled-coil domain ranging from AA140-420 (Figure 4). The CARD is structurally related to the death domain, death effector domain and pyrin domain that all form a module of six or seven antiparallel α–helices, termed the death-domain fold. Coiled-coil domains have been reported to facilitate homophilic interaction, while death-domain fold proteins mediate heterophilic interaction of different proteins carrying the same type of domain (Bouchier-Hayes and Martin 2002).

In previous work, Card9 was identified as a Bcl10-interacting protein (Bertin, Guo et al. 2000). The interaction was first identified in a two-hybrid screening in which CARD containing proteins were tested for interaction with Card9. Interaction of endogenous as well as overexpressed Bcl10 and Card9 was demonstrated. In addition, Card9 and Bcl10 were coprecipitated in a cell free system. Cellular colocalization was also demonstrated using immunofluorescence staining. NF-κB activation was demonstrated in reportergene assays in which overexpressed Card9 activated κB site controlled luciferase expression. For NF-κB activation the CARD was sufficient, while the coiled-coil domain was dispensable. Interaction with Bcl10 was also CARD dependent and could be diminished by mutation of Leucin 41 (L41R) in the CARD of Card9.

Card9 shows a strong resemblance to the N-terminus of Carma1/2/3 that also consist of a CARD and a coiled-coil domain, but its physiological function was unknown until recently.

The goal of this work was to identify the function of Card9 by generating Card9 deficient mice.

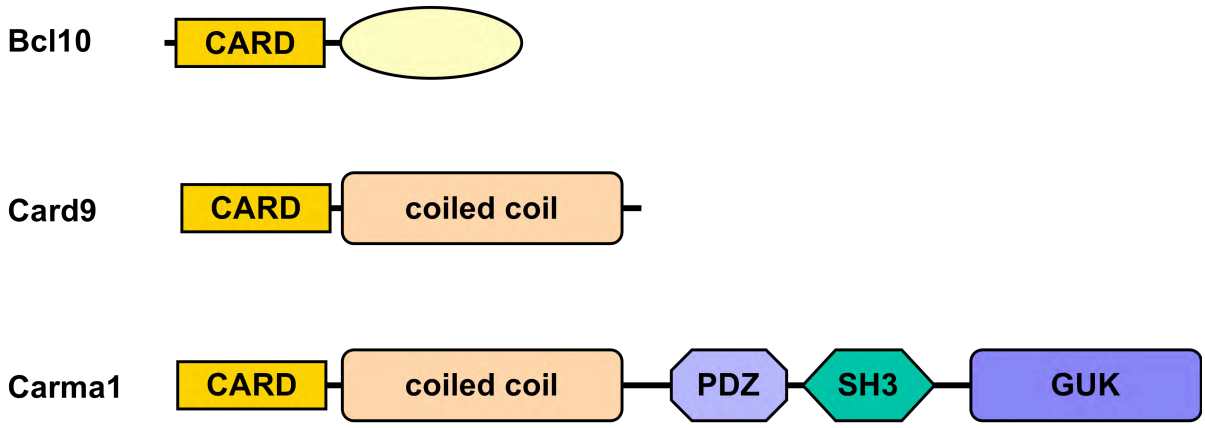

Figure 4: Protein structure of Carma1, Card9 and Bcl10. For abbreviations, see page 13ff.

2 Materials and Methods

2.1 Material

2.1.1 Reagents

If not otherwise stated, all chemicals were from Sigma. All cell culture materials were from Invitrogen; stem cell serum was from PAN biotech; serum with very low endotoxin levels was from Perbio (Hyclone).

PCR and RT-PCR reagents were from Invitrogen; primers were designed using Gene Construction Kit software and synthetized by MWG; DNA constructs were sequenced by GATC.

2.1.2 Primer list

Primers for construction of the targeting construct:

Card9_SA_3_XbaI	GCT CTA GAC AGC CCC ACA GCC ACA TGG TCC
Card9_SA_5_NotI	ATT GCG GCC GCG ACC AAG CTT TGT CCC ACT GCA C
C9_SA_Scr_5_NotI	ATT GCG GCC GCG GGA AGC TTG CAG CTC ATA G
Card9_LA_XhoI_4.5kb	GAG CTC GAG GTG TGT TAG TCT AAG AGT TC
Card9_LA_ClaI+	CCA TCG ATG GCC TGA TGA TAC TCA CAG GCT

Primers for PCR screening of stem cells and mice:

Card9_SA_ScPr_3	CCA TAG AGG ACT ATA GCT GCC TAC AG
NEO Scr. (N73)	GGG TGG GAT TAG ATA AAT GCC TGC TC
Card9_WT_ScPr_1	TGG TTG ACC CAG TGG ACA GAC ATT TC

Primers for cloning Card9 cDNA into pcDNA3.1 vector:

hsCard9-JR15	CGC GGA TCC GTT ATG TCG GAC TAC GAG AAC
hsCard9-JR16	CCG GAA TTC CTA CTA GGA GCC CTC AGT GTC

Primers for RT-PCR:

Card9_fwd5_E3	TGA GAA TGA CGA CGA GTG CTG
Card9_rev4_E4	CTC CAA CGC CAT CAT AGA AGC

2.2 Methods

2.2.1 Generation of Card9-Deficient Mice

Gene targeting was performed by standard methods (Thomas and Capecchi 1987). A genomic *Card9* clone was isolated from a 129/J bacterial artificial chromosome (BAC) library (CHORI) and used as a PCR template to construct a pBluescript (Stratagene) based targeting vector (Figure 5) that was electroporated into E14K ES cells. Homologous recombination replaced a 2 kilobase (kb) genomic fragment containing the exons encoding the CARD-domain of Card9 by a neomycin-resistance gene. Clones resistant to G418 were selected and recombinants were identified by PCR. Injection of three independent clones into C57/BL6 blastocysts generated chimeric mice and Card9$^{+/-}$ mice. Germline transmission was confirmed by PCR and Southern blot analysis of tail or thymus DNA. Card9$^{+/-}$ mice were intercrossed to generate homozygous Card9$^{-/-}$ animals. All animals were housed at the facilities of the Institut für Medizinische Mikrobiologie, Immunologie und Hygiene der Technischen Universität München at the Klinikum rechts der Isar in accordance to standard protocols and German and European laws and regulations. Littermate controls were used in all experiments.

2.2.2 Flow Cytometric Analysis

Surface marker expression of thymocytes, splenocytes, lymph nodes, bone marrow cells or bone marrow derived myeloid cells was analyzed using a flow cytometer (FACScalibur, Becton Dickinson), CellQuest software and FlowJo Software (Tree Star, Inc.) according to standard protocols. Flourescently labeled antibodies were from Becton Dickinson, eBioscience, Caltag or Serotech.

2.2.3 Measurement of Serum Immunoglobulin Concentrations

Ig isotypes of 8-12 week old Card9$^{+/+}$ and $^{-/-}$ mice were analyzed by ELISA on serially diluted serum samples and anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, or IgM antibodies, as recommended by the manufacturer (Southern Biotechnology).

2.2.4 *In Vivo* Immunizations

Mice were immunized intraperitoneally (i.p.) with either 200 µg NP-Ova (Calbiochem) in Alum (Sigma) as a T cell-dependent antigen or 25 µg TNP-Ficoll (Biosearch Technologies) as a T cell-independent type II antigen. Blood from mouse tails was collected at various time points. ELISA for the determination of antigen-specific Ig isotypes was performed utilizing plate-bound TNP-Ficoll or NP-BSA instead of capture antibodies to bind immunoglobulin (Tafuri, Shahinian et al. 2001).

2.2.5 Proliferation Assays

T and B cells were purified using magnetic beads (Dynabeads, Dynal) according to manufacturers protocols. T cells were stimulated in 96 well U-bottom cell culture plates (Becton Dickinson) with phorbol-12 myristate 13-acetate ester and calcium ionophore (PMA + Iono, 10 ng/ml each), soluble anti-CD3 (0,1 – 10µg/ml), and soluble anti-CD28 (2 µg/ml) in the presence or absence of IL-2 (50 U/ml). B cells were stimulated with anti-IgM (1 - 10 µg/ml) and/or anti-CD40 (5 µg/ml). Cells were harvested with a Skatronic 96 well harvester onto glass fibre membranes at 24 or 48 h after an 8 h pulse with [^{3}H]thymidine (1 µCi/well). Incorporation of [^{3}H]thymidine was measured with a scintillation Matrix 96 direct β-counter system (Canberra Packard).

2.2.6 Generation and Stimulation of Bone Marrow Derived Dendritic Cells (BMDC)

Dendritic cells were differentiated from bone marrow (BM) as previously described (Sparwasser, Koch et al. 1998) by rinsing femora and tibiae bones of Card9 knock-out or control mice with culture medium using a syringe and 22-gauge needle to elucidate bone marrow and erythrocytes were were lysed with RBC lysis buffer (eBioscience). BM cells ($4x10^6$) were seeded into a 100 mm standard petri dish in 10 ml complete RPMI 1640 medium (Invitrogen), supplemented with 10% very low endotoxin fetal bovine serum (Hyclone) and 2% GM-CSF containing cell culture supernatant (Lutz, Kukutsch et al. 1999) or 20 ng/ml recombinant GM-CSF (Peprotech). Cells producing GM-CSF were provided by Christian Meyer zum Büschenfelde. 10 ml

medium was added on day three and 10 ml (50%) were exchanged for fresh medium on day six after spinning down half the culture volume at 300xg for 5 minutes. On day six to eight, immature dendritic cells ($CD11c^+$, $MHCII^{+low}$), representing at least 50% of the population, were transferred into 24 well tissue culture plates at a density of 2-5 $x10^5$ per well in 500 µl of GM-CSF containing medium. On day seven to nine, cells were challenged with 500 µl two-fold stimuli or 50 µl 10-fold stimuli, diluted in GM-CSF containing medium. Supernatants were harvested after a period of 5 to 48 hours for further analysis of cytokine production. For analysis of activation marker, cells were harvested on day 4 and plated on non-tissue culture treated 6 well plates with medium without GM-CSF. Cells were stimulated after 3 h and activation marker expression was measured by FACS after 16 h.

R848 and CpG were kind gift of Stefan Bauer and Tim Sparwasser, respectively. All other TLR Ligands and MDP were purchased from Invivogen. Zymosan was either from Sigma or Invivogen. Curdlan (Wako) was suspended in PBS at a concentration of 10 mg/ml. *Candida albicans* strain SC5314 was obtained from Rudolf Rupec. *Candida albicans* cells for stimulation were grown on blood-agar or Sabourand plates at 30°C, suspended and washed three times with cold PBS. Boiling for 1 h inactivated yeast cells.

In order to inhibit Syk activity, Piceatannol and Syk-Inhibitor (Calbiochem) were used. MyD88 deficient animals were kindly provided by Bernhard Holzmann.

2.2.7 Generation of Bone Marrow Derived Macrophages (BMDM)

BMDMs were prepared as previously described (Hammer, Mages et al. 2005). In brief, femora and tibiae bones from mice ($Card9^{-/-}$ or heterozygous) were flushed with medium to elucidate bone marrow and erythrocytes were were lysed with RBC lysis buffer (eBioscience). Cells were cultured in complete Dulbecco's modified Eagle Medium (DMEM, Invitrogen), containing 10% fetal bovine serum (Hyclone), and 10% L-cell-conditioned medium (LCCM) as a source of M-CSF and incubated overnight in 10 cm cell culture dishes. Nonadherent cells were counted and replated at a density of 3 x 10^5 cells/ml in DMEM with 10% LCCM in tissue culture plates. After 6-7 days of differentiation, cultures were nearly confluent and the cells were used for experiments

after removal of nonadherent cells, washing with DMEM and resting overnight in DMEM without M-CSF.

2.2.8 Cytokine Production

The amount of secreted TNF-α, IL-2, IL-6, IL-10, IL-12p40, IL-12p70, IL-23, IFN-γ and IL-17 in culture supernatants was quantified using ELISA (Becton Dickinson or eBioscience) according to manufacturers recommendations. To increase sensitivity, supernatants were incubated on the ELISA plates (Nunc maxisorp) overnight at 4°C. Values were measured with a TECAN Sunrise platereader.

2.2.9 Survival and Clearance of *Candida albicans* Infection

Card9-deficient and heterozygous control mice were infected with 10^3 – 10^6 cfu *Candida albicans* (strain SC5314) per mouse in PBS via the tail vein. Infected mice were monitored daily for signs of disease, which is shaggy fur, decreased motility and tearing eyes. In order to determine pathogen load, mice were sacrificed on day four post infection and the right kidney, the lung and the liver of these mice were disintegrated by mashing them through a 100 µm cells strainer. Serial dilutions of the organs in PBS were plated on 'Candida ID2' diagnostic plates (Biomerieux) at various dilutions. After 36h of incubation at 30°C, colonies were counted and pathogen load was calculated.

2.2.10 Restimulation of T-cells after Infection and Cytokine Measurement

Card9$^{-/-}$ mice and Card9$^{+/-}$ littermate control mice were infected intravenously with 4x10^3 to 1.2x10^4 colony-forming units of *Candida albicans* strain SC5314. On days 6–8 after infection, mice were sacrificed and either total splenocytes or splenocyte samples depleted of CD4$^+$ cells (2x10^6 cells per well) were restimulated in 96-well U-bottomed plates with heat-inactivated *Candida albicans* (1x10^6 to 1x10^8 per ml). After 48 h, IFN-γ and IL-17 in the supernatants were measured by sandwich ELISA.

2.2.11 *Staphylococcus aureus* Infection

Mice were infected with $5x10^5$ colony-forming units (cfu) *Staphylococcus aureus* (ATCC 29219) as described before (Takeuchi, Hoshino et al. 2000) and monitored daily for signs of disease. To determine pathogen titers, serial dilutions of homogenized spleens were plated on CNA diagnostic plates (Biomerieux), incubated over night at 37°C and colonies counted.

2.2.12 Western Blot Analysis and Antibodies

Protein lysates from unstimulated or stimulated BMDC or BMDMs were subjected to Western blott according to standard protocols (Ruland, Duncan et al. 2001) using antibodies against Syk, phospho-Syk (NEB), β-actin (Sigma) or Card9 (Santa Cruz) and using equipment from Bio-RAD.

2.2.13 Zymosan Uptake

BMDCs were incubated with FITC-conjugated zymosan particles (100 µg ml^{-1}, Invitrogen) and internalization of zymosan particles was measured by FACS on a 15 minute basis. Cells were kept in a 37°C water bath between measurements. Cells were counterstained and gated for CD11c.

2.2.14 Immunofluorescence

For immunofluorescence, BMDCs were seeded on 20 mm cover glasses in 6-well plates at a density of $5x10^5$ cells/well. After stimulation for 1 h with 100 µg/ml zymosan or 200 ng/µl LPS, the cells were washed once with PBS, fixed in ice cold methanol for 5 min, and then blocked and permeabilized for 30 min at RT in blocking buffer (BB), consisting of 10% FCS, and 0.1% Triton-X100 in PBS. Cells were stained with anti-p65 polyclonal antibodies (C-20, Santa Cruz, 1:200 in BB, 1h at RT), followed by a short incubation in BB supplemented with 2 µg/ml DAPI. Cells were then washed twice with BB and incubated with a secondary antibody (Alexa Fluor 594, goat anti-rabbit, Molecular Probes, 1:500 in BB, 1h at RT). After a further three washes with BB,

the samples were mounted in Vectashield embedding medium (Vector Laboratories) and observed under a LSM confocal microscope (LSM510, Zeiss). Images were evaluated with the Image Browser Software (Zeiss).

2.2.15 Gel Mobility Shift Assay

Nuclear protein extracts were prepared from unstimulated and stimulated cells as previously described (Ruland, Duncan et al. 2001). Extracts (4 µg protein) were incubated in 20 µl binding buffer with radio-labelled, double-stranded DNA probes (NF-κB, 5′-ATC AGG GAC TTT CCG CTG GGG ACT TTC CG-3′; AP-1, 5′-CGC TTG ATG ACT CAG CCG GAA-3′; NF-Y, 5′-AGA CCG TAC GTG ATT GGT TAA TCT CTT-3′) and fractionated on a native 5% polyacrylamide gel. NF-κB and NF-Y binding buffer: 5 mM HEPES (pH 7.8), 50 mM KCl, 0.5 mM dithiothreitol, 2 µg poly (dI-dC), and 10% glycerol. AP-1 binding buffer: 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 0.5 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiothreitol, 2 µg poly (dI-dC), and 10% glycerol.

2.2.16 NF-κB Reporter Assay

Bcl10$^{+/-}$ and -/- MEFs were plated at 1 x 10^5 cells/well in a 96 well flat bottom cell culture plate 24 h prior to transfection. The MEFs were co-transfected with 500 ng Bcl10, Card9 or Carma3 (kindly provided by Gabriel Nunez, University of Michigan) or Nod1 expression plasmids plus 6xκB-luciferase (250 ng) and pRL-TK (250 ng, Promega). Transfectin (Bio-Rad) was used to transfect MEFs according to manufacturers protocols. 293T cells were transfected with calcium phosphate according to standard protocols or with Lipofectamin (Invitrogen) with Dectin-1 (50 ng / well in 96 wells) and/or a combination of Card9 and Bcl10 expression vectors (5 ng each / well in 96 wells). Medium was renewed and cells were stimulated with 100 µg/ml zymosan after 24h. After 48 hrs, the cells were lysed and luciferase activity was determined using a commercial assay kit (Promega) and a Berthold Luminometer.

Expression vector pUNO-Dectin-1 was from Invivogen, inserting Card9 cDNA into pcDNA3.1 from Invitrogen generated Card9 expression vector.

3 Results

3.1 Gene-Targeting of Card9

In order to investigate the function of Card9, Card9 deficient (Card9$^{-/-}$) mice were generated by gene targeting (Figure 5). The murine Card9 gene consists of 12 exons with a coding sequence of 1611 bp encoding a protein of 536 amino acids with a molecular weight of about 59 kDa. It is located on chromosome 2; location 2 A3. The original data base record XM_285326 from 30-OCT-2003 described two additional exons ahead of exon 1 that were absent in the human gene. Since then the file has been updated (27-APR-2006) and now shows a structure very similar to the human gene (86% exact protein sequence match), which is located on chromosome 9, location 9q34.3, with a coding sequence of also 1611 bp (NM_052813). The knock-out strategy was based on the older sequence, depicted in Figure 5, which contained 14 exons.

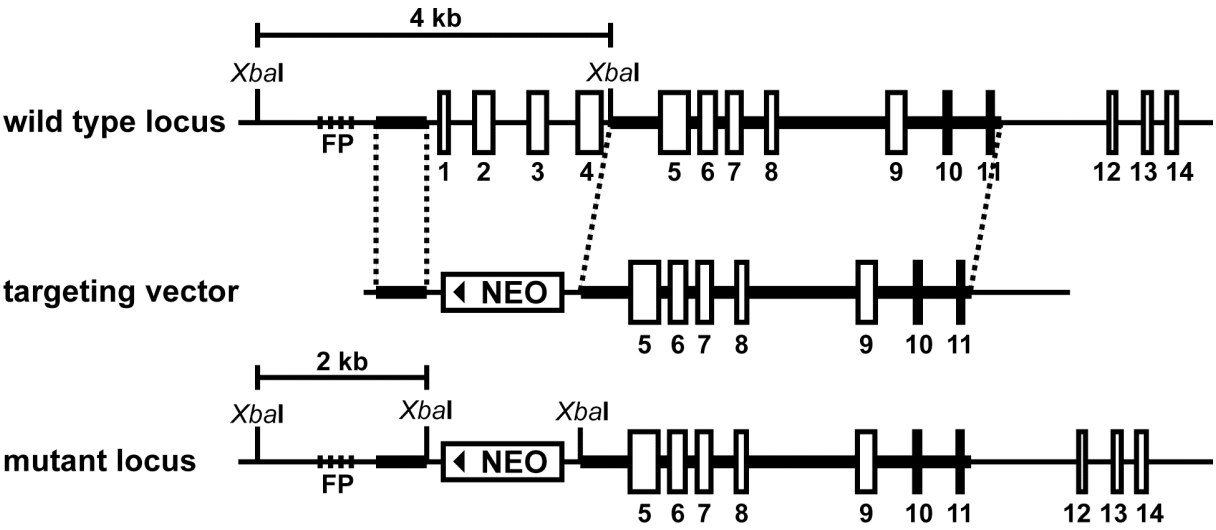

Figure 5: Gene targeting strategy for the generation of Card9-deficient mice. Shown is a schematic representation of a portion of the WT murine Card9 locus (top) showing relevant restriction sites and their resulting fragment lengths. Exon 1-14 (E1-E14) are shown as open boxes. The position of the 5' flanking probe (FP) is indicated. The structure of the targeting vector (middle) and the resulting mutant locus (bottom) are also shown.

To obliterate protein function, exons 1-2 (1-4), encoding the CARD, responsible for protein-protein interaction and presumably protein function, were replaced by a neomycin resistance gene. Germline transmitting chimeras were obtained from three independent cell lines from two independent electroporations. Homologous recombination was verified by Southern blot analysis using thymus DNA (Figure 6a). Card9$^{-/-}$ mice were born at mendelian ratios from heterozygous (+/-) intercrosses (Figure 6b) without gross anatomical abnormalities. RT-PCR and Western blotting from the splenocytes of Card9-deficient and wildtype mice confirmed the absence of Card9 mRNA transcript and protein (Figure 6c, d).

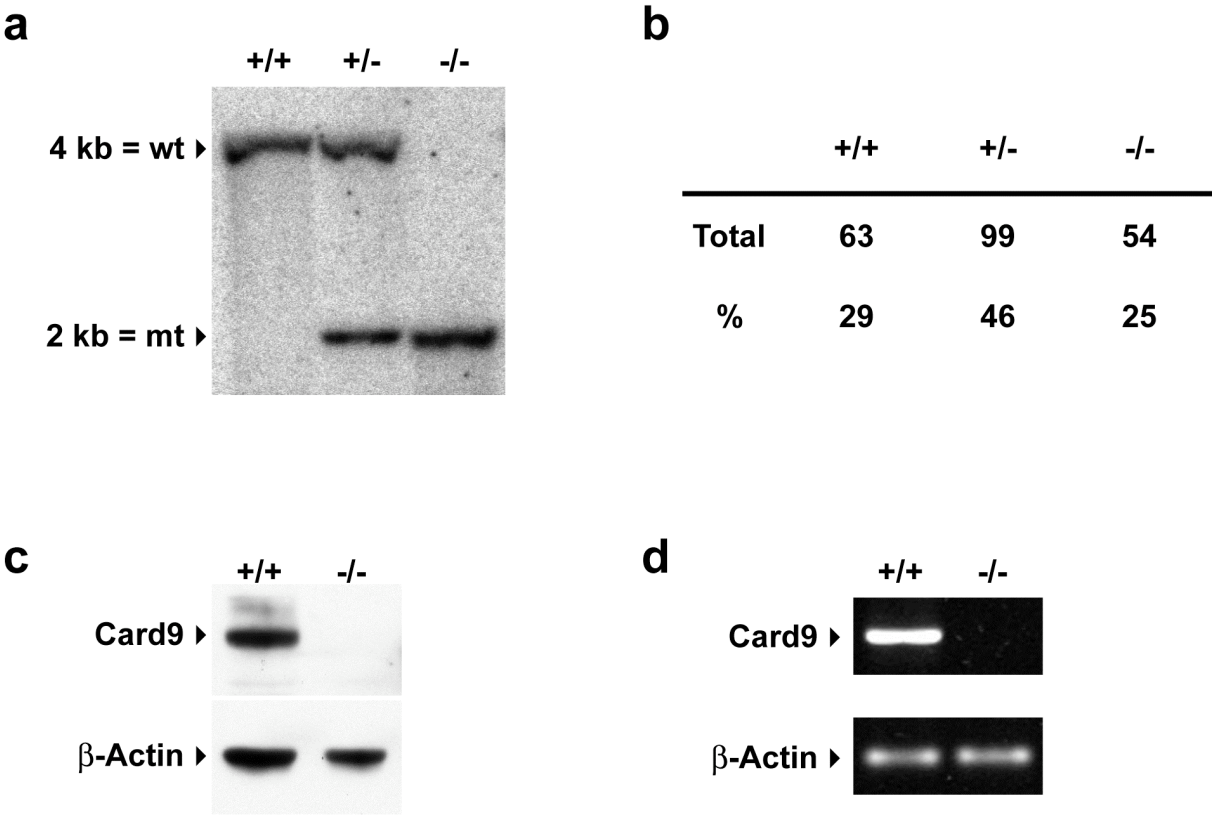

Figure 6: Verification of the deletion of Card9 on the DNA, RNA and protein level and Mendelian Analysis. **a** Southern blot analysis of thymus DNA isolated from WT (+/+), heterozygous (+/-) and Card9-deficient (-/-) mice. Genomic DNA was digested with *Xba*I and hybridized with the flanking probe indicated in Figure 5. **b** Mendelian analysis of the offspring of Card+/- parents. **c** BMDM lysates from Card9+/+ and -/- mice were analysed for Card9 protein content by Western blot. **d** Splenocyte RNA isolated from Card9+/+ and -/- mice was subjected to RT-PCR analysis using primer pairs specific for Card9 or β-actin.

3.2 Regular Adaptive Immune Responses without Card9

Bcl10 deficient mice show severe defects in adaptive immunity. Since Card9 binds to Bcl10 and represents a homologue of Carma1, the requirement of Card9 for adaptive immunity was analyzed first. Flow cytometric analysis of the thymus, lymph nodes, bone marrow and the spleen revealed regular T and B cell development in Card9$^{-/-}$ mice (Figure 7).

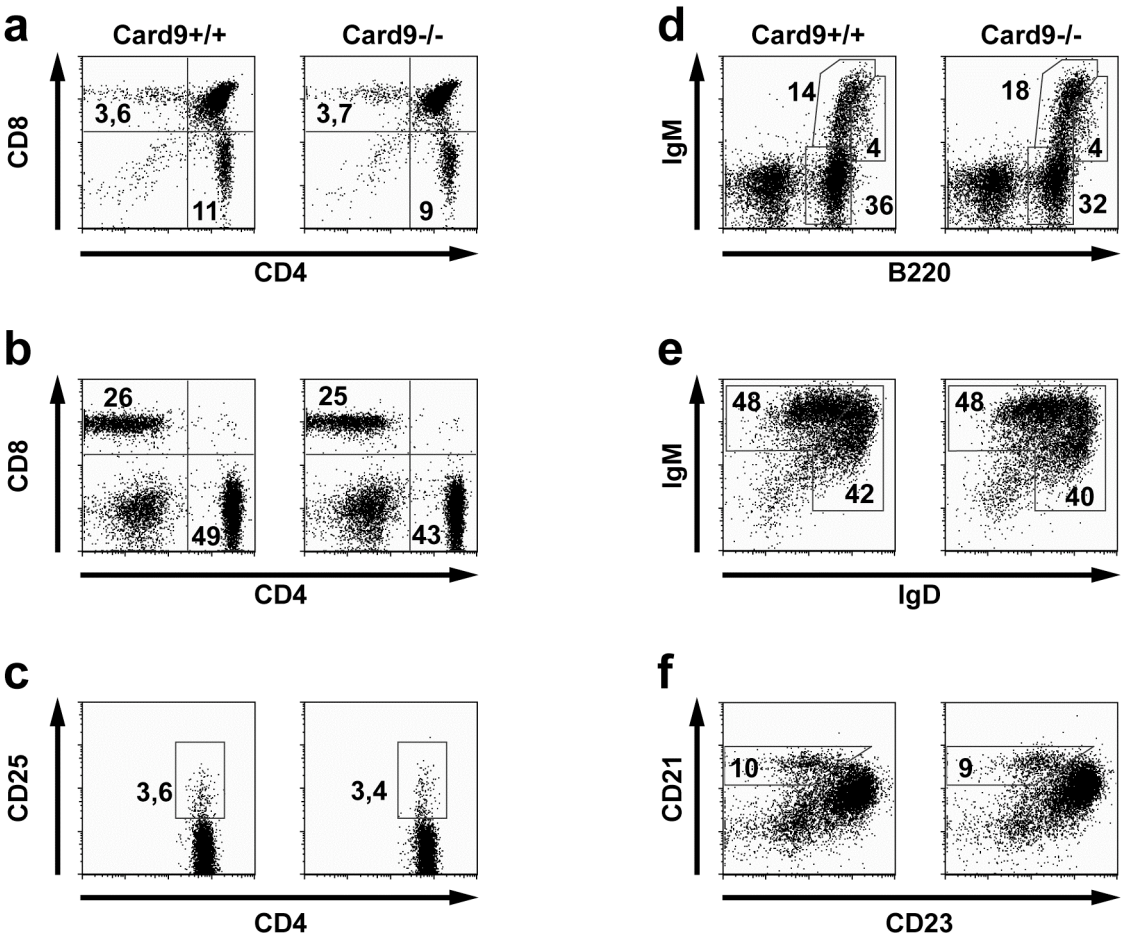

Figure 7: Flow cytometric analysis of lymphocyte development in Card9$^{+/+}$ and -/- mice. Specificity of fluorescently labeled antibodies and proportion of distinct populations as indicated. **a** T-cell development in the thymus, cells stained for CD4 and CD8; **b** Ratio of helper T cells (CD4^{+}) and cytotoxic T cells (CD8^{+}) in the lymph nodes; **c** Regulatory T-cell fraction in lymph nodes cells were stained for CD4 and CD25, gated on CD4; **d** B-cell development in bone marrow, cells stained for B220 and IgM; **e** Immature und mature B-cell in the spleen, cells gated on B220 and stained for IgD and IgM; **f** Marginal zone B-cells in the spleen, cells gated on B220 and analysed for CD21 and CD23 expression. Numbers in % indicate proportions of cells in gates.

All tested lymphocyte subsets including $CD4^+CD25^+$ regulatory T cells, recirculating B cells, transitional type 1, transitional type 2, mature follicular and marginal zone B cells were detected at normal frequencies and numbers (Figure 7).

To directly study antigen receptor mediated lymphocyte activation, T and B cells from $Card9^{-/-}$ mice and control littermates were isolated and *in vitro* stimulated with antibodies against their respective antigen receptors either alone or together with co-receptor ligation or with phorbol ester and calcium ionophore. Regular signal induced proliferation was detected in both B and T lymphocytes from $Card9^{-/-}$ mice (Figure 8). In addition, no considerable differences in TCR- or BCR-induced NF-κB activation between $Card9^{-/-}$ and control cells were detected (Figure 9).

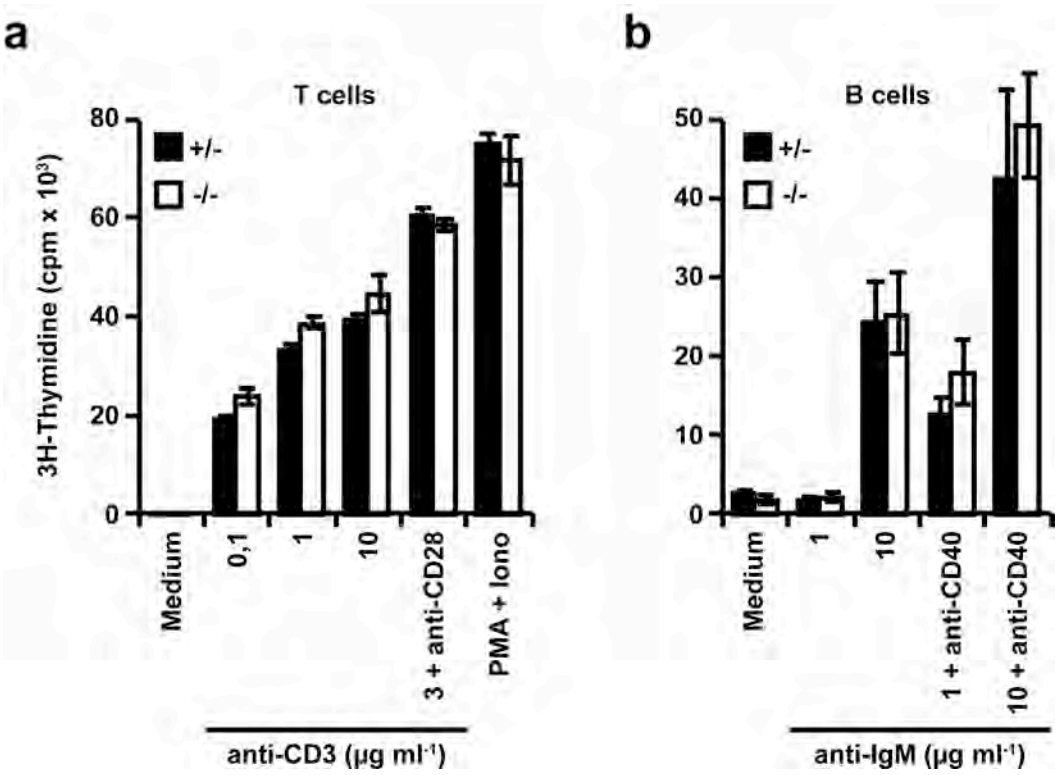

Figure 8: Signal induced lymphocyte proliferation. $CD4^+$ T cells (**a**) and $B220^+$ B cells (**b**) were stimulated for 48 h with agonistic antibodies (anti-IgM or anti-CD3, as indicated; anti-CD28, 2 µg ml^{-1}; anti-CD40, 5 µg ml^{-1}) or PMA + Iono (10 ng ml^{-1} each) and assessed for proliferation by measuring [^{3}H]-thymidine incorporation. Data are representative for four independent experiments with a total of n=18 Card9-/- and n=18 control mice. Indicated values are means ±SD of triplicates.

To study alterations in the function of Card9$^{-/-}$ lymphocytes *in vivo,* basal immunoglobulin (Ig) levels between wild type (wt) and Card9$^{-/-}$ mice were compared. No significant differences were detected (Figure 10). The mice were then immunized with either the T cell independent antigen 2,4,6-trinitrophenyl (TNP) conjugated to ficoll or the T cell dependent antigen 4-hydroxy-3-nitrophenylacetyl (NP) conjugated to ovalbumin (Figure 10b). Both, T cell dependent as well as independent IgM and IgG responses were comparable in wt and Card9$^{-/-}$ animals. Thus, it was concluded that Card9 is unlikely to be critically involved in the Carma1/Bcl10 signaling pathway that mediates T and B cell differentiation and activation for adaptive immunity.

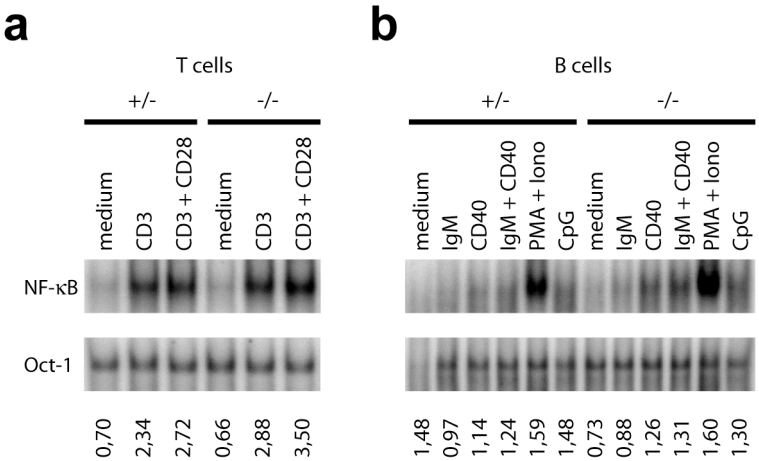

Figure 9: NF-κB activation after antigen receptor stimulation. Nuclear proteins were extracted 4h after stimulation and subjected to electromobility shift assay (EMSA). ^{32}P-labeled oligonucleotide probes represented the recognition site of NF-κB or Oct-1, used as a loading control. **a** Total lymph node cells from Card9 deficient and control littermates were stimulated with final concentrations of 10 µg/ml anti-CD3 and/or 2 µg/ml anti-CD28. **b** Total splenocytes from Card9 deficient and control littermates were stimulated with final concentrations of 10 µg/ml anti-IgM and/or 5 µg/ml anti-CD40, 10 ng/ml PMA + Iono each and 1µM CpG.

3.3 Card9 Controls Zymosan-Induced Cell Activation

Since Card9 is also expressed in myeloid immune cells (Bertin, Guo et al. 2000) including dendritic cells and macrophages (Figure 11), Card9 was also considered to have a role in innate immunity. To test this hypothesis bone marrow derived dendritic cells (BMDCs) from Card9$^{-/-}$ mice were generated and stimulated with various TLR

ligands as well as ligands for non-TLR PRRs. As an initial readout, ligand-induced production of tumor necrosis factor-α (TNF-α) was measured (Figure 12). No reproducible differences were detected between wild type and Card9 deficient BMDCs in response to TLR ligands Pam3CSK4 (TLR2), LPS (TLR4), R848 (TLR7), CpG DNA (TLR9) or upon stimulation with the NOD2 agonist muramyl dipeptide (MDP) alone or in combination with LPS.

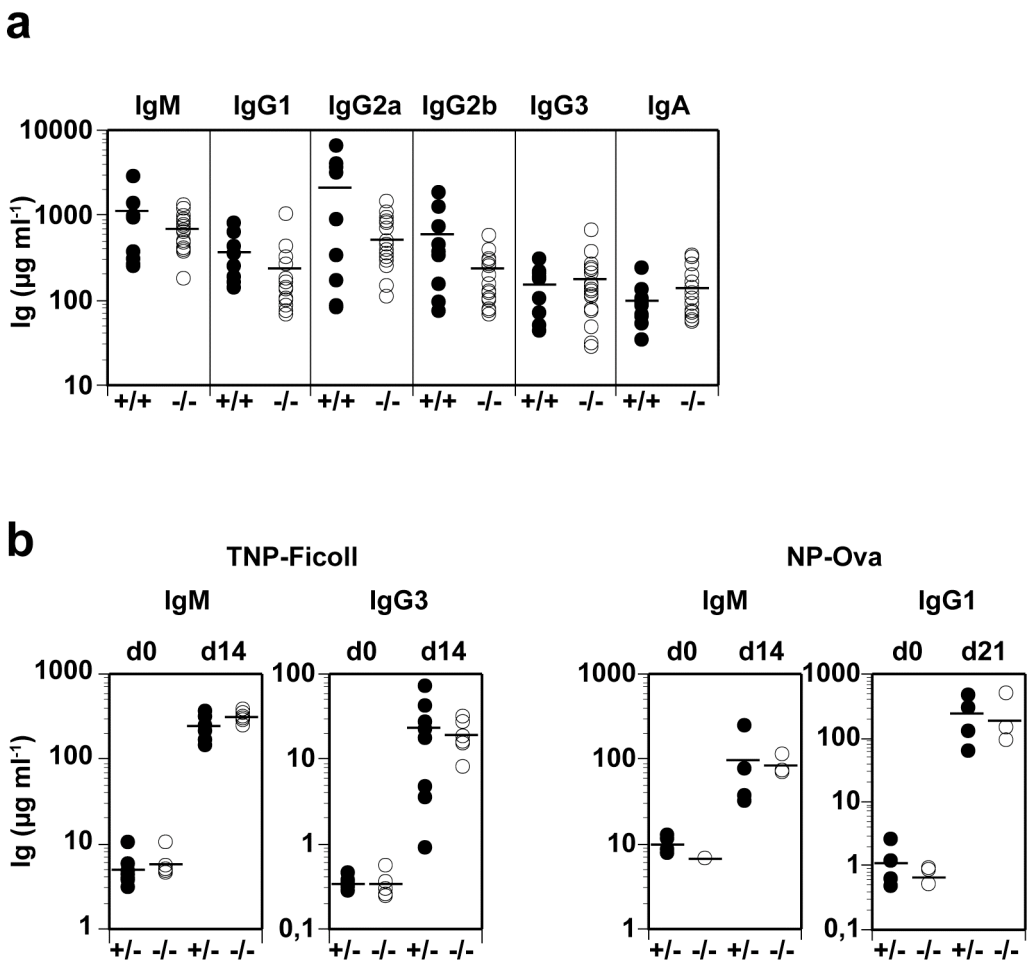

Figure 10: Humoral immune response. **a** Basal immunoglobulin (Ig) concentrations. Ig isotypes in sera of 8-12 week old wt (+/+, n=9) and Card9$^{-/-}$ (n=18) mice were determined by ELISA. **b** Regular antibody responses. Left: control (+/-, filled symbols, n=8) and Card9-/- mice (open symbols, n=6) were immunized with 25 µg TNP-ficoll for T cell-independent immune responses. Concentrations of TNP specific IgM and IgG3 were determined before and after immunization. Right: for T cell-dependent antibody responses, control (+/-, filled symbols, n=4) and Card9$^{-/-}$ mice (open symbols, n=3) were injected with 200 µg NP-Ova absorbed on alum. NP-specific IgM and IgG1 concentrations were measured before and after immunization. Results are representative for two independent experiments.

It has also been demonstrated that the β-glucan receptor Dectin-1 signals via recruitment and phosphorylation of Syk to induce activation of dendritic cells (Rogers, Slack et al. 2005). This is mediated by an ITAM motif in the intracellular domain of Dectin-1. B- and T-cell receptor complexes also utilize ITAM motifs and Syk family kinases for cell activation, e.g. by activation of the Carma-1/Bcl10/Malt1 pathway for NF-κB activation. These correlations in receptor activation motifs and the similarities between the C-terminus of Carma-1 and Card9 led to the idea that Card9 might be involved in Dectin-1 signalling. To test this hypothesis, wild type and Card9 deficient BMDCs were stimulated with zymosan, the common stimulus for Dectin-1. Zymosan is a major yeast cell wall component that is principally composed of β-glucan polymers and additionally contains α-mannan and mannoproteins (Di Carlo and Fiore 1958). In sharp contrast to TLR stimulation, TNF-α production induced by zymosan is strongly reduced in the absence of Card9.

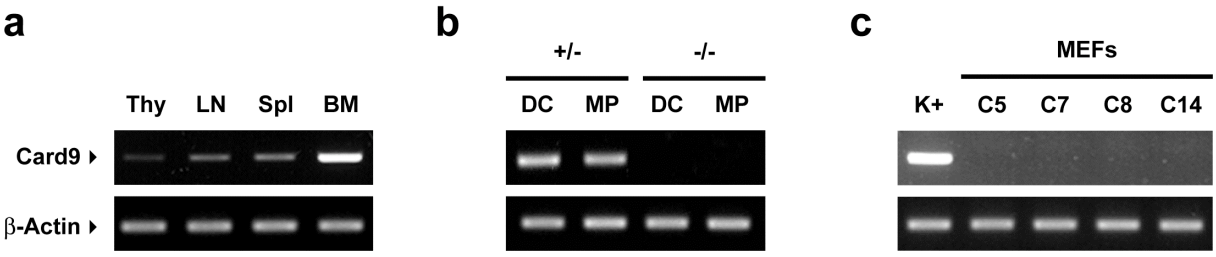

Figure 11: Card9 expression analysis by RT-PCR of the Card9 transcript in in tissues and cell lines. **a** Total thymus (Thy), lymph node (LN), spleen (Spl) and bone marrow (BM); **b** Bone marrow derived dendritic cells (DC) and bone marrow derived macrophages (MP) from Card9$^{+/-}$ or -/- mice; **c** Mouse embryonic fibroblast (MEF) cell lines (lines C5, C7, C8 and C14; all Card9 wild type). Macrophage RNA was utilized as a positive control (K+).

The C-type lectin like receptor Dectin-1 is the main mammalian PRR for zymosan (Brown and Gordon 2001) but zymosan also co-stimulates TLR2 (Underhill, Ozinsky et al. 1999). To characterize the requirement of Card9 in zymosan-induced DC activation in more detail, dose response curves for zymosan-induced TNF-α, IL-6, IL-2, IL-10 and IL-12 production were obtained. A strong Card9 gene dose dependency was detected for the production of all five cytokines (Figure 13).

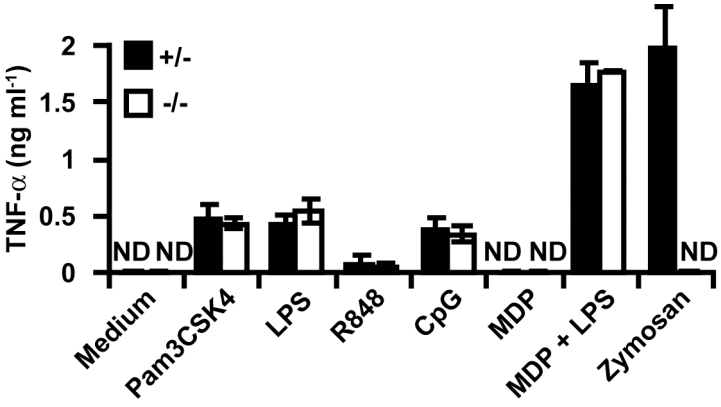

Figure 12: Cytokine production in Card9 deficient dendritic cells in response to PRR stimulation. TNF-α production in Card9-/- compared to Card9+/- BMDCs after stimulation with various TLR-ligands, Muramyl dipeptide (MDP) and zymosan. BMDC were treated with 200 ng/ml Pam3CSK4, 100 ng/ml Lipopolysaccharide (LPS), 10 µg/ml MDP, 1 µM CpG, 200 ng/ml R848 or 3 µg/ml zymosan or unstimulated (medium). TNF-α concentration in the medium was determined after 24h by ELISA. ND, not detected. Data are means ±SD of triplicates and representative of three independent experiments.

In contrast, TLR4 ligation with LPS induced regular dose dependent TNF-α or IL-6 synthesis in Card9$^{-/-}$ BMDCs (Figure 14) whereas LPS induced IL-2 production in neither Card9$^{-/-}$ nor control cells (data not shown).

It is believed that the recognition of zymosan or β-glucans triggers immune responses that are primarily designed for the control of fungal pathogens (Brown and Gordon 2003). Therefore, the role of Card9 in immune responses to whole fungal cells was studied using *Candida albicans* as a model (Villamon, Gozalbo et al. 2004). Consistent with a role of Card9 in zymosan recognition signalling, Card9$^{-/-}$ BMDCs exhibited severe defects in *Candida albicans*-induced TNF-α, IL-2, IL-6 and IL-12 production (Figure 15). To determine the relevance of these findings *in vivo,* the susceptibility to infection in Card9$^{-/-}$ mice and heterozygous littermates was compared after injection of live *Candida albicans* (Figure 16). In several sets of experiments, mice were infected with varying doses of *Candida albicans* cells, ranging from 10^3 to 10^6 colony forming units (cfu) (Villamon, Gozalbo et al. 2004). While Card9 deficient animals survived for a maximum of nine days, control animals cleared the infection or survived significantly longer, depending on the dose (Figure 17). Homozygous wildtype control mice survived an infection even longer than Card9 heterozygous animals, indicating a gene-dosage effect (Figure 17b).

In order to assess intravital fungal growth, the animals were sacrificed 4 days after injection with $3x10^4$ *Candida albicans* cells. It was macroscopically apparent that the kidneys of all Card9$^{-/-}$ animals were massively infiltrated by the fungus (Figure 17a). Compared to control mice, Card9$^{-/-}$ had a 100 fold higher load of *Candida albicans* in the kidneys and livers (Figure 17b). In addition high *Candida albicans* titers were observed in the lungs of infected Card9$^{-/-}$ mice, whereas lungs of control animals were pathogen free. In contrast, there was no significant difference in the clearance of the gram positive bacterial pathogen *Staphylococcus aureus* between Card9$^{-/-}$ mice and control littermates (Figure 17c). Together, these results demonstrate a critical requirement for Card9 in zymosan-induced DC activation and innate anti-fungal immunity.

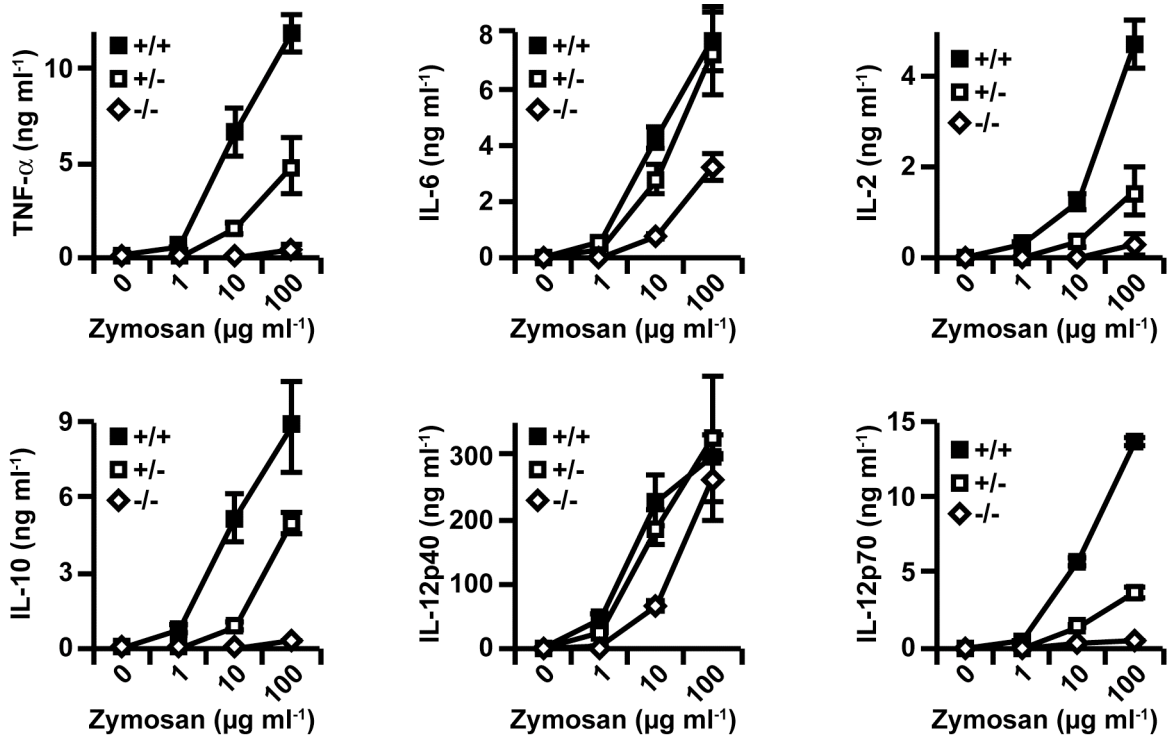

Figure 13: Dose dependent TNF-α, IL-6, IL-2, IL-10 or IL-12 production from Card9+/+, +/- or -/- BMDCs after 24 h of zymosan stimulation. The experiment was performed three times with comparable results. Indicated values are means ±SD of triplicate samples. Cytokine concentrations were determined after 24h by ELISA.

3.4 Card9 is Essential for Dectin-1/Syk Signalling

Zymosan stimulates Dectin-1 and TLR2 which cooperate for full cell activation and optimal cytokine production (Underhill, Ozinsky et al. 1999; Brown and Gordon 2001; Gantner, Simmons et al. 2003; Rogers, Slack et al. 2005; Brown 2006). Zymosan-induced Dectin-1 signalling depends on an intact ITAM as well as direct binding to and downstream activation of Syk, whereas zymosan-induced TLR signalling is transduced via the conserved MyD88 pathway (Rogers, Slack et al. 2005). To selectively investigate the involvement of Card9 in the Dectin-1 independent TLR2 pathway, two non-related pure TLR2 agonists, Pam3CSK4 and peptidoglycan (PGN) were used for a dose dependent stimulation of Card9$^{-/-}$ BMDCs (Aliprantis, Yang et al. 1999; Takeuchi, Hoshino et al. 1999). Both TLR2 ligands induced regular TNF-α and IL-12 production in the absence of Card9 (Figure 18), which is consistent with the regular TLR2-dependent clearance of *Staphylococcus aureus* (Takeuchi, Hoshino et al. 2000) in Card9$^{-/-}$ mice.

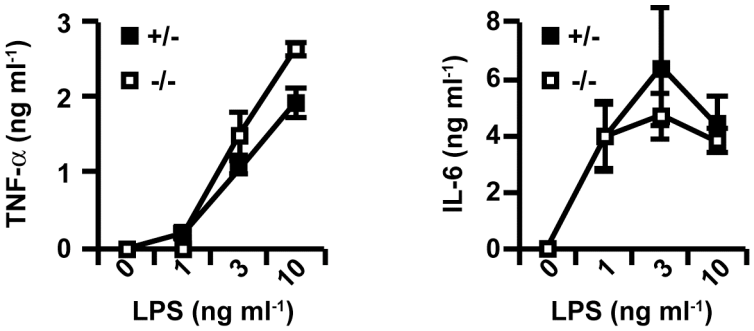

Figure 14: Dose dependent TNF-α or IL-6 production from Card9+/- or -/- BMDCs after 24 h of LPS stimulation. Indicated values are means ±SD of triplicate samples and representative of three independent experiments.

Since there are no single agonistic Dectin-1 ligands available, it is more intricate to study Dectin-1/Syk signalling without considering TLR2 costimulation. However, recent work has shown that the Dectin-1/Syk axis selectively controls zymosan-induced IL-10 production entirely independent of TLR/MyD88 signalling (Rogers, Slack et al. 2005). Thus, IL-10 production in response to zymosan stimulation was used as a specific readout for the Dectin-1/Syk pathway. In order to provide suitable controls for TLR/MyD88 and Dectin-1/Syk signalling, BMDCs from MyD88$^{-/-}$ mice (Kawai, Adachi

et al. 1999) and wild type dendritic cells that were pre-incubated with the small molecule Syk inhibitor piceatannol were now also included (Figure 19a). Consistent with published data (Rogers, Slack et al. 2005), zymosan stimulation induced a strong IL-10 production in wildtype BMDCs. This production was MyD88 independent but robustly blocked by inhibiting Syk function. The Card9 deletion also completely abolished zymosan-induced IL-10 synthesis, suggesting a role for Card9 in Dectin-1/Syk dependent, TLR/MyD88 independent signalling events. Compared to zymosan, the TLR ligands LPS and CpG induced regular albeit lower amounts of IL-10 in Card9$^{-/-}$ BMDCs (Figure 19b). Next, zymosan-induced production of TNF-α, IL-6, IL-2 and IL-12 in wild type BMDCs, wild type BMDCs that were preincubated with Syk inhibitor and in Card9$^{-/-}$ BMDCs was compared (Figure 19c). Indeed, zymosan-induced TNF-α and IL-2 production are highly dependent on Syk and Card9.

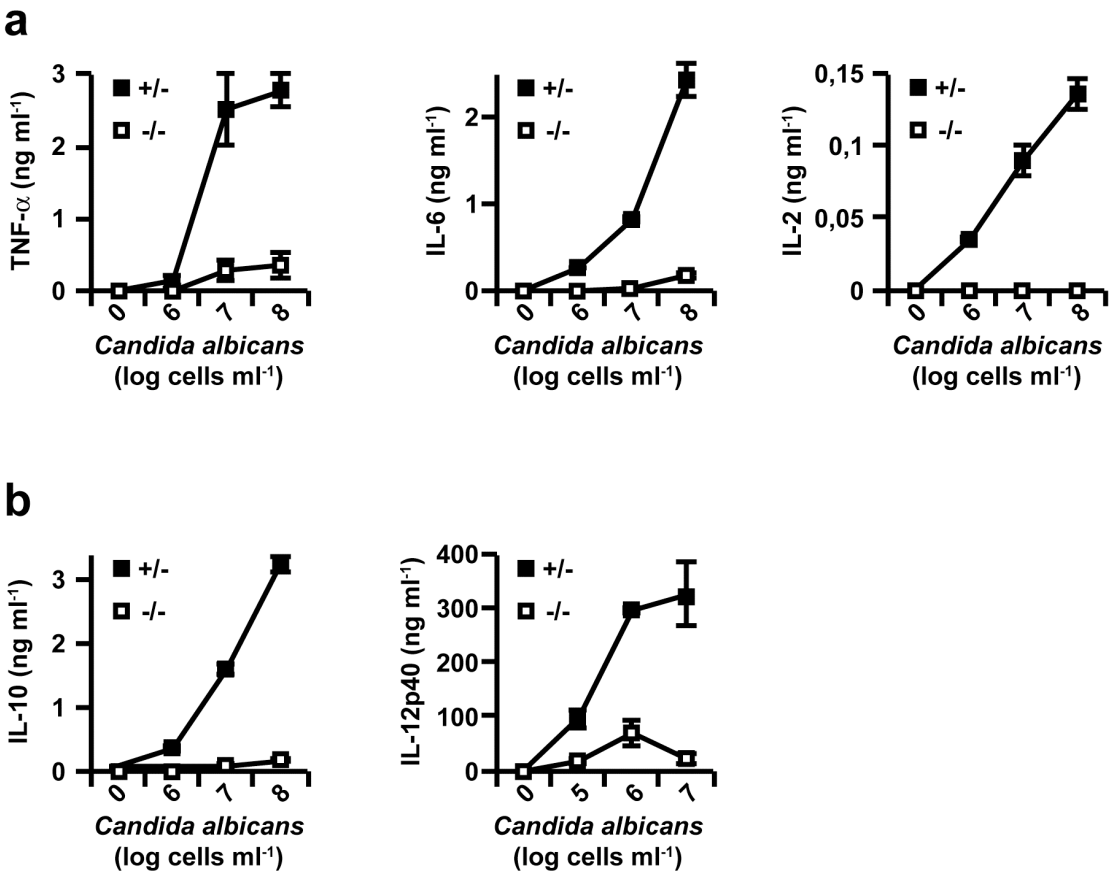

Figure 15: Dose dependent *Candida albicans* (strain SC5314) induced TNF-α, IL-6, IL-2, IL-10 or IL-12 production in Card9-/- BMDCs. Cells were stimulated for 24 h with indicated concentrations of heat-inactivated *Candida albicans* cells. Cytokine production was measured by ELISA. Results are means ±SD of triplicates and representative for three independent experiments.

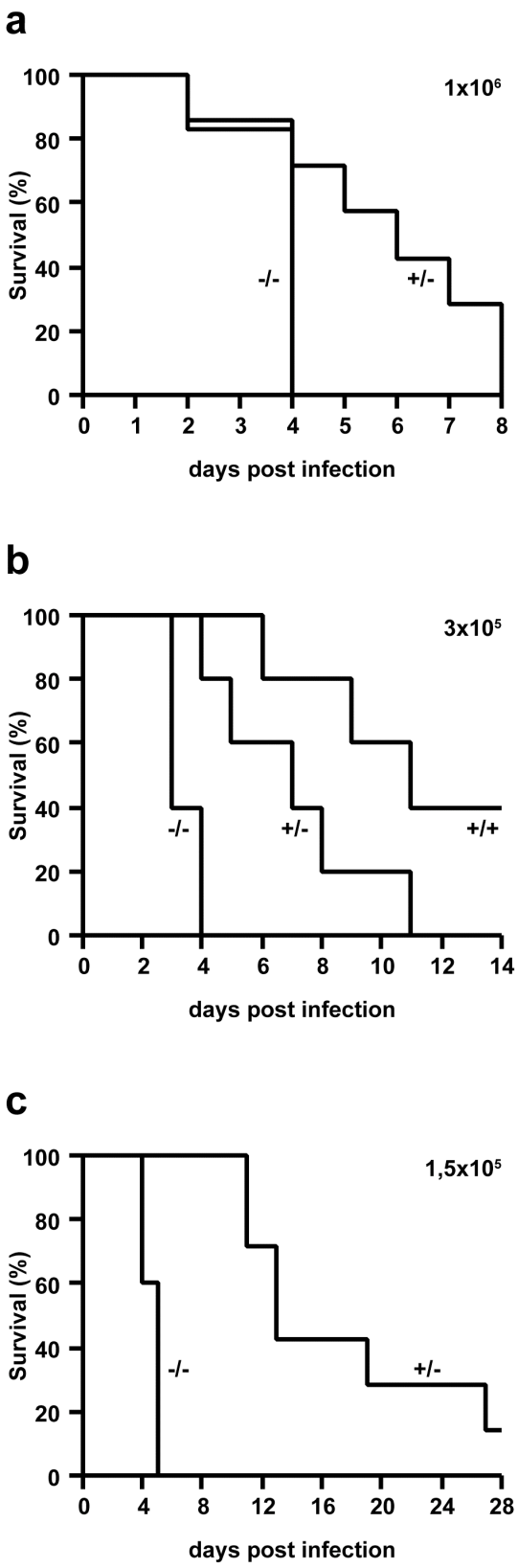

Figure 16: Strongly increased mortality of Card9 deficient mice after *Candida albicans* infection. Eight to ten week old Card9 deficient, wild type and/or heterozygous control female mice (five or seven per group, respectively, from two litters) were intravenously infected with *Candida albicans.* Injected doses were: in **a** $1x10^6$ CFU, in **b** $5x10^5$ CFU, in **c** $1,5x10^5$ CFU. Survival was checked on a 12h basis for 1 week and afterwards once a day.

Consistent with previous data the production of IL-12 (Rogers, Slack et al. 2005) and IL-6 is partly independent of Syk and also partly independent of Card9 function. Together, the results show that the cellular effects after inhibition of Syk or a Card9 deletion upon zymosan stimulation are comparable in terms of cytokine production. These findings further indicate an essential role of Card9 in Dectin-1/Syk signalling.

3.5 Card9 Relays Dectin-1/Syk Signalling to NF-κB

To identify the molecular function of Card9 in Dectin-1/Syk-mediated cell activation the surface expression of Dectin-1 and Dectin-1 triggered Syk phosphorylation was determined in Card9$^{-/-}$ BMDCs.

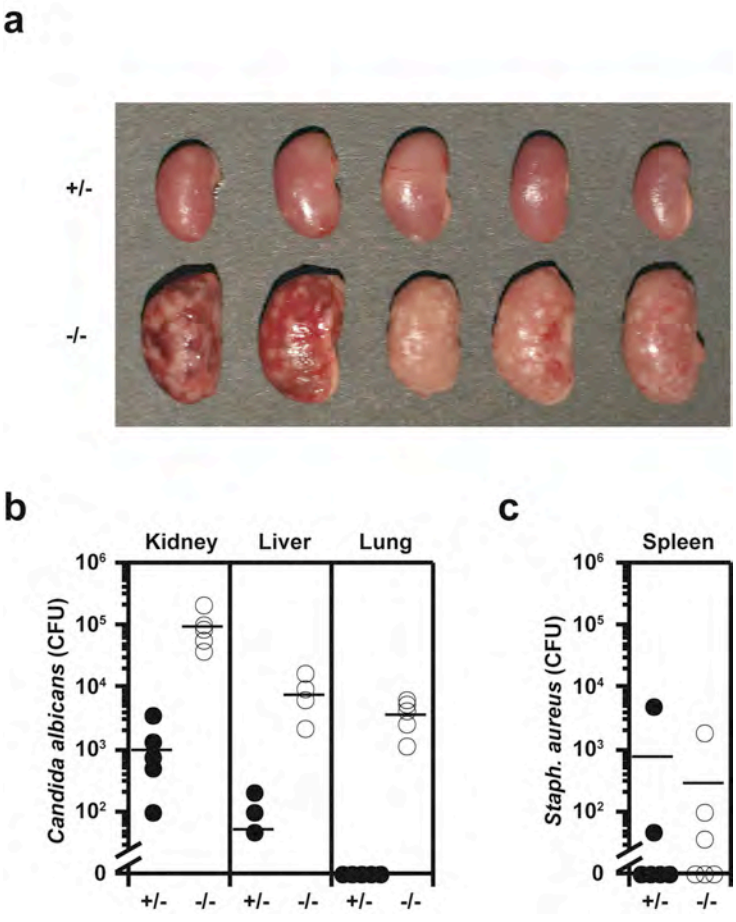

Figure 17: Determination of pathogen load in *Candida albicans* infected Card9 knock-out mice. Eight to ten week old Card9 deficient and heterozygous control female mice (five per group, out of two litters) were intravenously infected with 3x10^4 CFU *Candida albicans*. Four days after infection, Card9$^{-/-}$ mice showed the first signs of disease and were immediately sacrificed for examination. **a** Kidneys of Card9$^{-/-}$ mice were about double to trible the size of their control littermates and showed much higher amounts of white spots. **b** *C. albicans* titers were determined in the right kidney, lung and liver of these mice. **c** Card9$^{+/-}$ and Card9$^{-/-}$ mice were infected intravenously with 5x10^5 CFU *Staphylococcus aureus.* Five days bacterial titers were determined in the spleen.

The receptor is regularly expressed (Figure 20) and upon stimulation with zymosan normally activates Syk (Figure 21a). Syk activation in BMDCs not only controls cytokine production but also regulates zymosan phagocytosis (Rogers, Slack et al. 2005). To quantify Syk-dependent zymosan internalization, BMDCs were incubated with or without Syk inhibitor and fluorescently-labeled zymosan before flow cytometric analysis (Figure 21b). Regular Syk-dependent phagocytosis was observed in Card9$^{-/-}$ BMDCs demonstrating that certain Dectin-1/Syk controlled pathways are not dependent on Card9.Since overexpression studies have indicated that Card9 can induce NF-κB (Bertin, Guo et al. 2000), the activation of this transcription factor was probed by monitoring RelA nuclear translocation in zymosan- or LPS-stimulated Card9$^{-/-}$ BMDCs (Figure 22). Using confocal microscopy similar nuclear translocation of RelA in Card9$^{-/-}$ and control BMDCs was detected upon TLR4-activation with LPS. Microscopy also clearly demonstrated that Card9$^{-/-}$ BMDCs internalized zymosan in the normal manner. However, Card9$^{-/-}$ BMDCs exhibit a substantial impairment in zymosan-induced NF-κB activation although some NF-κB induction presumably via TLR2 could be observed. In addition, electromobility shift assays (EMSAs) for NF-κB DNA binding activity were performed using nuclear extracts from bone marrow derived macrophages BMDMs that had been stimulated with zymosan or the TLR ligands PGN, LPS or R848 (Figure 23). TLR ligation induced regular NF-κB activation whereas zymosan-induced NF-κB activation was diminished in the absence of Card9. Together, these results indicate a role for Card9 in transducing zymosan-activated Dectin-1 signals to NF-κB.

3.6 Card9 Signals via Bcl10 and Malt1

Since Card9 can interact with Bcl10 (Bertin, Guo et al. 2000) it has been studied whether the two proteins functionally cooperate (Figure 24). Consistent with previous results (Bertin, Guo et al. 2000) single overexpression of Card9 or Bcl10 weakly activates NF-κB. However, the co-overexpression of both proteins elicited more than additive NF-κB induction, indicating synergism (Figure 24a).

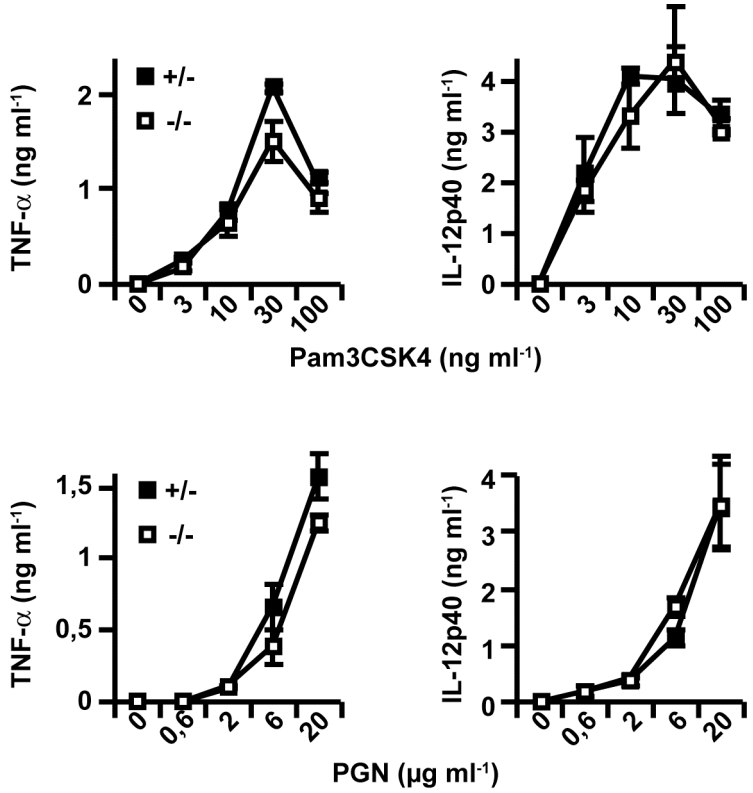

Figure 18: Normal TLR2 induced TNF-α production. BMDCs from Card9+/- (filled symbols) or -/- mice (open symbols) were stimulated for 24 h with the selective TLR2-ligands Pam3CSK4 or PGN and supernatants assayed for TNF-α concentration.

To directly investigate the role of a Card9/Bcl10 complex in Dectin-1 signalling, Dectin-1 and a combination of Card9 and Bcl10 either alone or together were introduced into 293 cells (that express low levels of Syk (Eliopoulos, Das et al. 2006)) followed by stimulation with zymosan (Figure 24b). Overexpression of Dectin-1 alone in 293 cells did not induce NF-κB luciferase activity. Upon zymosan stimulation, neither single Dectin-1 nor Card9 and Bcl10 overexpression increased the NF-κB luciferase signal. In contrast, coexpression of Dectin-1 together with Card9/Bcl10 conferred zymosan responsiveness, resulting in a substantial additional increase in NF-κB reporter activity upon zymosan treatment. To establish a hierarchy between Card9 and Bcl10, Card9 was introduced into wild type or Bcl10 deficient murine embryonic fibroblasts (Ruland, Duncan et al. 2001) (Figure 25). In parallel the known Bcl10 upstream activator Carma3 (McAllister-Lucas, Inohara et al. 2001) and the Bcl10 independent NF-κB inducer Nod1 were overexpressed.

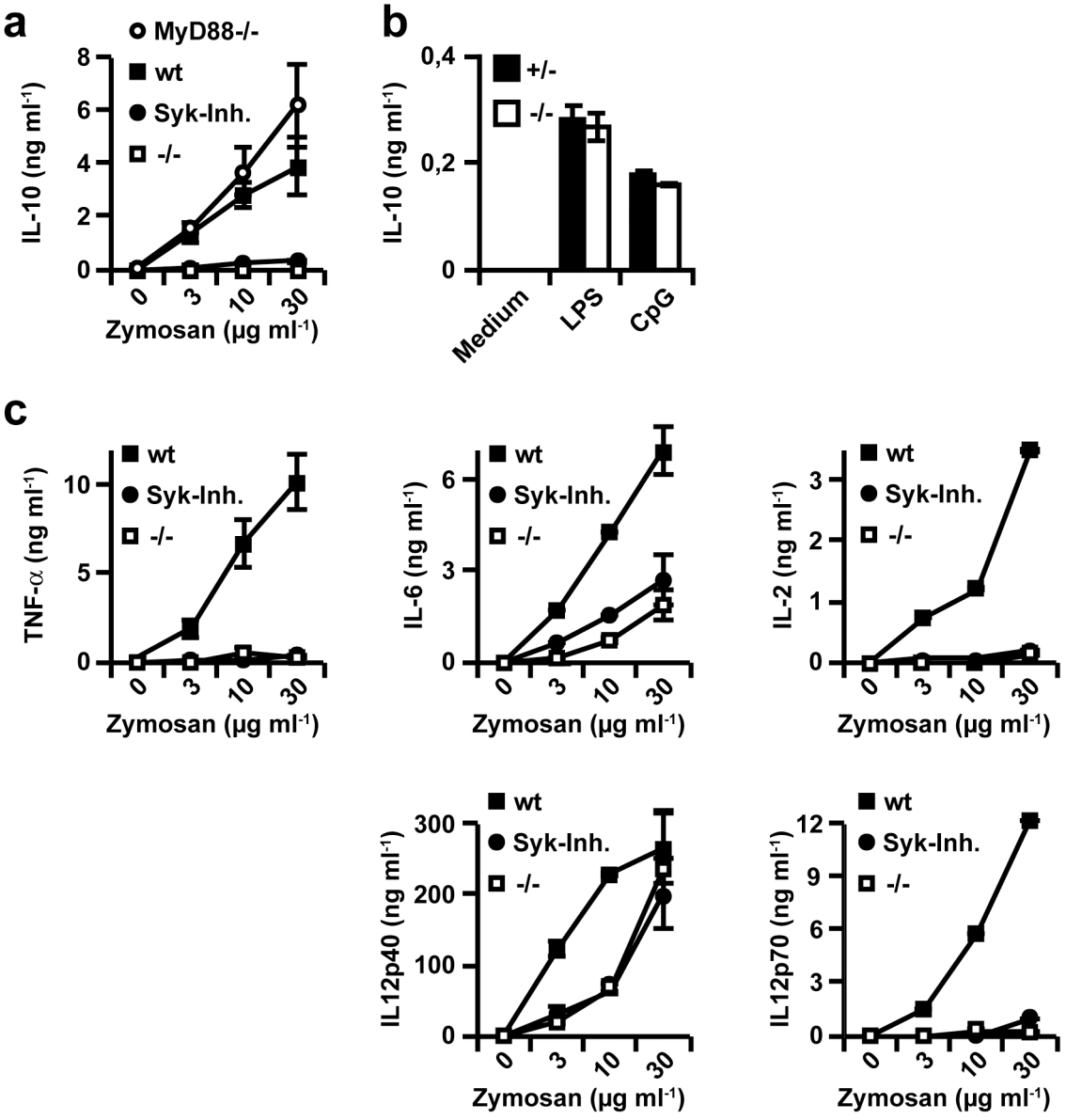

Figure 19: Comparative cytokine responses from Card9$^{-/-}$, MyD88$^{-/-}$ or Syk-inhibited DCs upon zymosan stimulation. **a** Defective Dectin-1/Syk dependent TLR/MyD88 independent IL-10 production in Card9$^{-/-}$ BMDCs. Wildtype BMDCs (wt, filled squares), Card9$^{-/-}$ BMDCs (-/-, open squares), wt BMDCs pretreated with 25µM Syk-inhibitor piceatannol (Syk-Inh., filled circles) or MyD88$^{-/-}$ BMDCs (MyD88$^{-/-}$, open circles) were stimulated with zymosan and 24 h later assayed for IL-10 production. **b** Normal IL-10 production in BMDCs from Card9$^{+/-}$ and -/- mice that were left unstimulated (Medium) or stimulated with TLR-ligands LPS (100 ng ml^{-1}) or CpG (1 µM) for 24h. ND, not detected. **c** Zymosan induced TNF-α, IL-6, IL-2, IL12p40 and IL12p70 production in wt BMDCs (wt, filled squares), wt BMDCs pretreated with Syk-inhibitor (Syk-Inh., filled circles) or Card9$^{-/-}$ BMDCs (-/-, open squares) stimulated for 24 h as in (a). Indicated values are means ±SD of triplicates and representative of three independent experiments.

As expected, Nod1-induced NF-κB in WT and Bcl10$^{-/-}$ cells was equal, whereas Carma3 only activated NF-κB in Bcl10$^{+/-}$ cells.

The requirement of Bcl10 and its downstream interaction partner Malt1 has also been investigated *in vivo* in mice deficient for these factors. These experiments were performed by Katrin Finger as part of her MD thesis work and are therefore not shown here. For this data, please refer to (Gross, Gewies et al. 2006). In brief, Bcl10 or Malt1 deficient BMDCs show the same defects in Dectin-1 signalling as Card9$^{-/-}$ dendritic cells do.

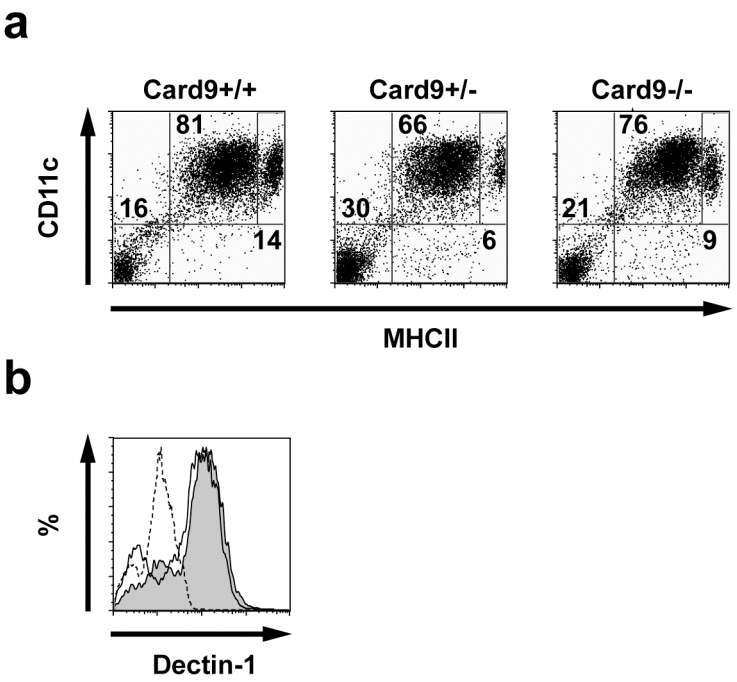

Figure 20: Differentiation and Dectin-1 expression on BMDCs. Prior to plating for stimulation on day eight after killing of the donor mice, BMDCs from wild type, heterozygous and Card9 deficient mice were FACS-analysed for surface marker expression. **a** Cells were stained with CD11c and MHCII to determine the extent of differentiation and spontaneous maturation. Gates indicate the population of total, CD11c^{+} dendritic cells and CD11c^{+} - MHCII^{high}, mature dendritic cells. Numbers represent percentages of total viable cells. Viability was assayed by propidium iodide (PI) stain. **b** Dectin-1 expression on CD11c^{+} BMDCs. Wild type versus Card9 deficient (grey area) and isotype stained (dashed line) cells.

3.7 Card9-Dependent Activation of T_H17 T Cell Responses

The *in vitro* investigation of Dectin-1 mediated effects has been hindered by the lack of a pure stimulus. Most recently curdlan was discovered to be such an agent (LeibundGut-Landmann, Gross et al. 2007). Curdlan is a β-glucan preparation from the agrobacterium *Alicaligenes faecalis*, an obligate aerobic gram-negative bacterium commonly found in the environment. It can selectively stimulate Dectin-1 without costimulating TLR. In order to test whether curdlan induces signalling via Card9, Card9$^{-/-}$ and wildtype control dendritic cells were stimulated with different doses of curdlan und cytokine production was measured. Card9 deficient dendritic cells produced virtually no TNF-α, IL-2, IL-10 and IL-12p70 while wildtype cells responded in a dose-dependent manner (Figure 26a). Remarkably, also the production of IL-12p40 and IL-6 was reduced to a higher extend as compared to zymosan stimulation. This more pronounced defect might be due to the costimulation of TLRs by zymosan. It has been shown that TLR stimulation induces higher amounts of IL-12 and IL-6 than Dectin-1, while Dectin-1 triggers more TNF-α, IL-10 and IL-2 than TLRs (Rogers, Slack et al. 2005; LeibundGut-Landmann, Gross et al. 2007).

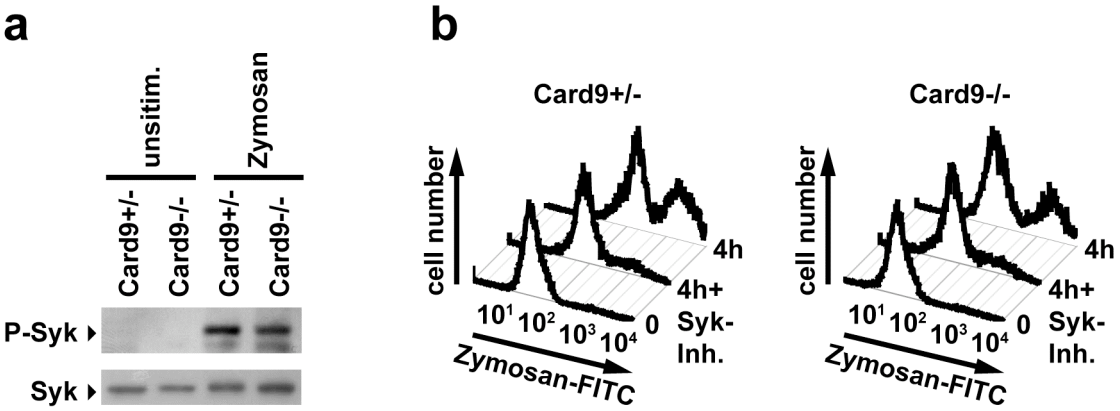

Figure 21: Regular Syk activation and phagocytosis in the absence of Card9

a Regular Syk activation in Card9-/- BMDCs. Cells were zymosan stimulated for 45 min and Syk activation was determined by immunoblot with anti-phospho-Syk antibodies. **b** Regular Syk dependent zymosan phagocytosis. Internalization of FITC labeled zymosan was measured after 4h in CD11c-gated control (+/-) and Card9-/- BMDCs that were preincubated with or without Syk inhibitor (Syk-Inh).

Activation of dendritic cells is a major step in the activation of adaptive immune responses. It has been indicated that fungal immunity preferentially involves the activation of the lately defined T_H17 T helper cell subpopulation (Huang, Na et al. 2004). DC derived IL-23 is, in addition to IL-6 and TGF-β, essential for the activation of T_H17 cells (Ivanov, McKenzie et al. 2006). Utilizing curdlan, it was investigated whether dendritic cells induce IL-23 in response to Dectin-1 stimulation in a Card9 dependent manner. Indeed, Curdlan induced IL-23 in a dose dependent manner in wildtype, but not Card9$^{-/-}$ cells (Figure 26b). In contrast IL-23 production in response to TLR stimulation was unaffected by the absence of Card9. In addition to certain proinflammatory cytokines, namely IL-12p70 for T_H1 and IL-23 for T_H17 responses, the upregulation of costimulatory factors on APCs is essential for the activation of sustained helper T cell responses.

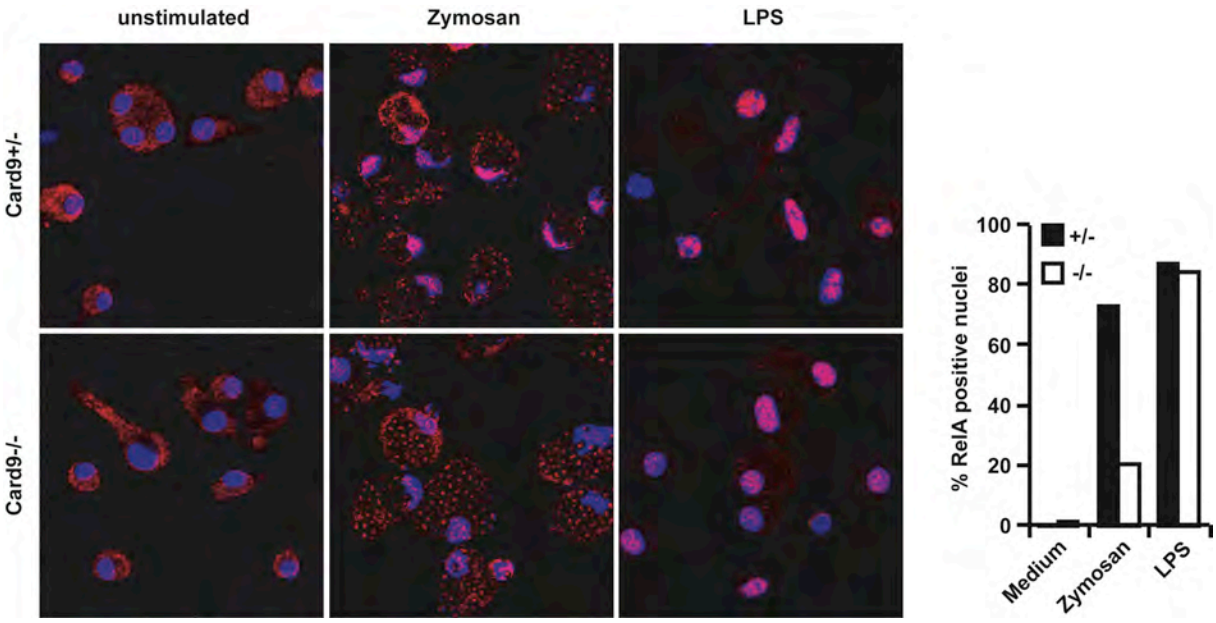

Figure 22: Specific defect in zymosan-induced NF-κB activation in Card9-/- cells. BMDCs from Card9+/- or -/- mice were stimulated for 1 h with 100 µg/ml zymosan or 200 ng/µl LPS. RelA (red) translocation into the nucleus (DAPI stained, blue; co-localization pink) was monitored by immunofluorescence and quantified by determining the frequency of RelA positive nuclei in at least 100 individual cells.

Stimulation of DC with curdlan strongly induced upregulation of the costimulatory molecules CD40, CD80 and CD86 on dendritic cells as demonstrated by FACS analysis (Figure 27). In the absence of Card9, this upregulation was clearly reduced. The defects appeared to be milder though, as compared to cytokine production. The lack of IL-12p70 and IL-23 production as well as the reduced upregulation of costimulatory factors strongly indicates that Card9 might play a role in the activation of adaptive anti-fungal T cell responses. In order to test this, it was finally determined whether T_H1 and/or T_H17 T cell responses were induced during fungal infection and to what extent this depends on the Dectin-1/Card9 signalling pathway. Large amounts of IFN-γ and IL-17 were produced by $CD4^+$ spleen cells from mice infected with *Candida albicans* after restimulation with heat-killed organisms (Fig. 28a). Card9 was indispensable for the T_H17 response and partially contributed to the T_H1 response (Fig. 28b). Therefore, adaptive immune responses to *Candida albicans* infection include the induction of T_H17 cells and this process is dependent on innate signalling via Card9.

4 Discussion

Collectively, these results identify a novel signalling pathway in innate immunity with the potential to activate adaptive responses. Whereas Card9 is dispensable for antigen receptor signalling, the data presented here suggests a model in which Card9 operates upstream of Bcl10 to transduce, together with Bcl10 and Malt1, Dectin-1 signals for NF-κB activation, cytokine production and dendritic cell controled activation of T_H17 T cells responses *in vivo*. For a model, see Figure 29. Dectin-1 was recently discovered as the key non-TLR PRR for fungal β-glucan detection (Brown 2006). This receptor uses an intracellular YxxL ITAM motif to initiate signalling via Syk through novel MyD88-independent pathways (Rogers, Slack et al. 2005). The results presented here provide mechanistic insights into these pathways that can cooperate with TLR signalling for full innate immune cell activation and for the induction of pathogenspecific innate and adaptive responses (LeibundGut-Landmann, Gross et al. 2007). These findings also demonstrate that Bcl10–Malt1 signalling can be engaged by an ITAM-containing PRR through the adaptor Card9, presumably in an analogous manner to ITAM-containing lymphocyte antigen receptors that use Carma1 to couple to Bcl10 and Malt1. This hypothesis suggests an evolutionarily conserved mechanism for immune cell signalling and puts the concept forward that ITAM-receptors in innate and adaptive immune cells can differentially connect to the conserved Bcl10/Malt1 complex via distinct CARD-coiled-coil adaptor proteins.

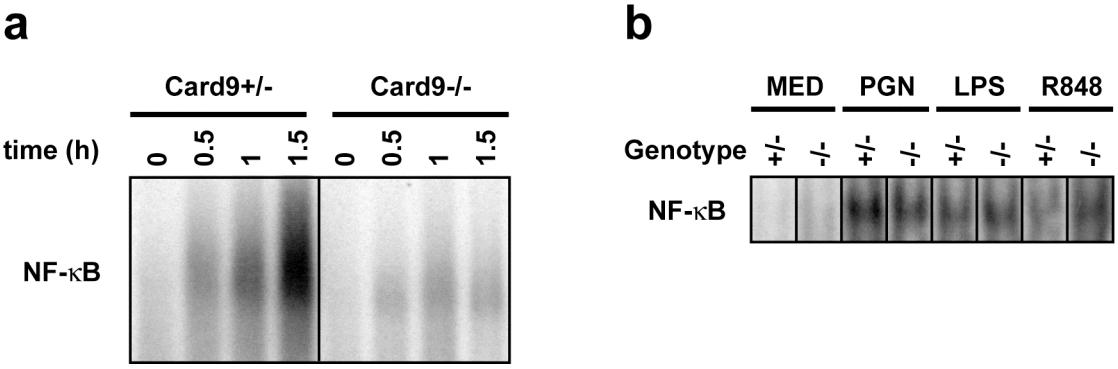

Figure 23: NF-κB activation was determined by EMSA in BMDMs stimulated for indicated time points with 100 µg/ml zymosan (**a**), or for 1h with 10 µg/ml PGN, 500 ng/ml LPS or 500 ng/ml R848 (**b**)

4.1 Card9 is Dispensable for Lymphocyte Activation

Prior to the findings presented here, it's been suggested that Card9 could potentially play a role in Bcl10-mediated antigen receptor signalling (Thome and Tschopp 2003). Bcl10-deficient mice show severe defects in virtually all NF-κB-related responses mediated by the B- or T-cell receptor (Ruland, Duncan et al. 2001; Xue, Morris et al. 2003). These mice show impaired lymphocyte development, manifested by defects like the accumulation of CD4/CD8 double negative, CD44-/CD25-thymocytes after the beta-checkpoint of thymocyte development and the total absence of marginal zone B-cells in the spleen as well as recirculating B-cells in the bone marrow. Consequently, humoral responses are severely reduced in the absence of Bcl10. In these mice, B- and T-cells don't proliferate after antigen-receptor stimulation and NF-κB does not translocate to the nucleus. All these Bcl10 dependent lymphocyte functions were unaffected by the absence of Card9. Therefore, Card9 is unlikely to directly interfere with Bcl10 regulated antigen-receptor signalling.

4.2 Card9 Controls Innate Immunity

Card9 conducts signals downstream of Dectin-1, the receptor for fungal β-glucans and is not involved in the reactions triggered by the well-defined Toll-like receptor family. In myeloid cells, Dectin-1 mediates its signals via Syk, which binds to the YxxL ITAM motif present in the cytoplasmic tail of Dectin-1 (Rogers, Slack et al. 2005). Dectin-1/Syk signalling activates multiple cellular outcomes in myeloid cells, including transcription-dependent and -independent reactions (Brown 2006). Even though independent ones, like phagocytosis, were found to be Card9-independent, certain transcription-dependent functions, such as the production of cytokines were clearly Card9 dependent. This defect was pinpointed to defective nuclear translocation and activation of the transcription factor NF-κB after Dectin-1 ligation. Nevertheless, additional transcription factors are also important and necessary for gene transcription to mount sustained immune responses. Some of these are the signal transducer and activator of transcription proteins (STATs), SMA- and MAD-related proteins (SMADs) (Shuai and Liu 2005), forkhead-box (FOX) transcription factors (Coffer and Burgering 2004), interferon (IFN)-regulatory factor (IRF) family members (Lohoff and Mak 2005),

nuclear factor of activated T-cells (NFAT) and also transcription factors regulated by mitogen-activated protein kinase (MAPK) family members like extracellular-signal-regulated kinases (Erk), Jun N-terminal kinases (Jnk) and p38 (Ashwell 2006), i.e. AP-1. Whether some of these transcription factors are also regulated via Card9 mediated signals will be a focus of future studies.

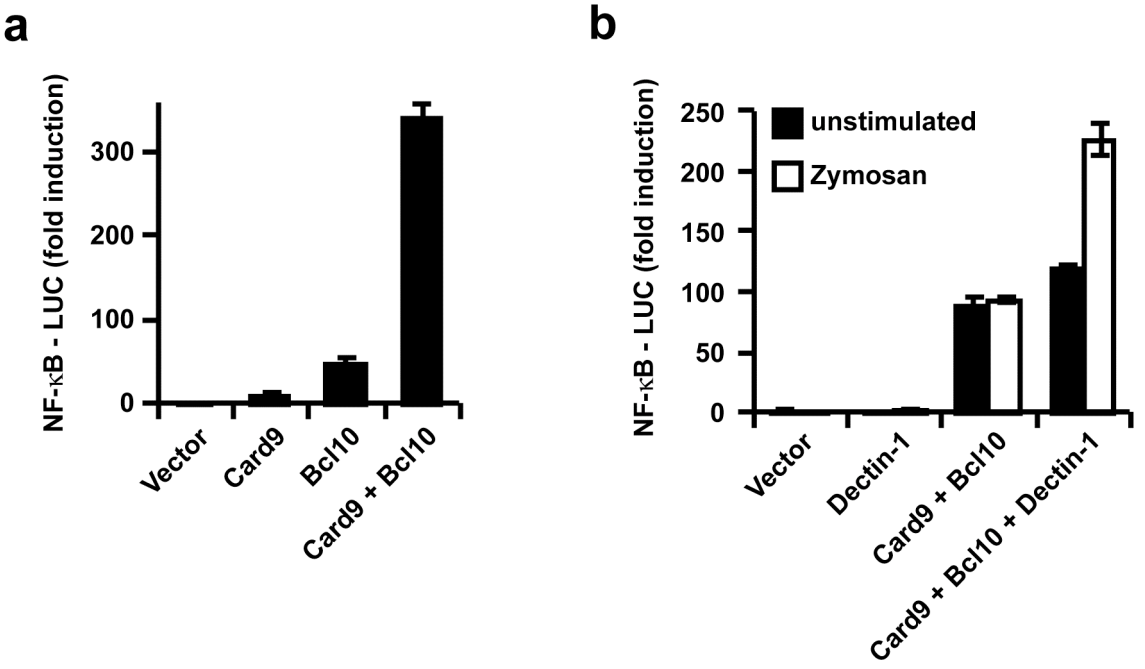

Figure 24: Card9 and Bcl10 synergize for NF-κB activation. **a** NF-κB luciferase assays in 293T cells upon lipid reagent transfection with Card9 and/or Bcl10 expression vectors (50 ng each / well in 96 wells). **b** Dectin-1 ligation with zymosan augments Card9/Bcl10 induced NF-κB induction. 293T cells were transfected with Dectin-1 (50 ng / well in 96 wells) and/or a combination of Card9 and Bcl10 expression vectors (5 ng each / well in 96 wells). NF-κB luciferase assays were performed with or without zymosan stimulation. The data shown is a representative result from four independent experiments.

Card9 can directly bind to Bcl10 (Bertin, Guo et al. 2000). Transient expression and reporter gene studies showed that Bcl10 is required for NF-κB activation by Card9 transient expression, indicating that Card9 signals upstream of Bcl10. This study also demonstrated that it is possible to reconstitute the Dectin-1 to NF-κB pathway by transient expression of combinations of Card9, Bcl10 and Dectin-1 in otherwise zymosan unresponsive cells. An additional study performed in our lab focused on Dectin-1 signalling in cells from Bcl10- and Malt1-deficient mice [Katrin Finger]. Malt1

binds to Bcl10 to transduce signals downstream of Bcl10 in lymphocytes. It became apparent that Bcl10- and Malt1-deficient cells show the same defects in cytokine production and NF-κB activation after Dectin-1 triggering as Card9 deficient cells. For example, when stimulated with zymosan or *Candida albi*cans cells, dendritic cells from Bcl10-deficient or Malt1-deficient mice have defects in production of TNF-α, IL-2, IL-10 and IL-6, while producing normal amounts of these cytokines in response to various TLR-ligands like PGN, LPS or CpG. In addition, dendritic cells deficient in either Bcl10 or Malt1 are also unable to induce nuclear translocation of NF-κB when exposed to zymosan. Thus, these results suggest a signalling pathway in which Card9 relays Dectin-1/Syk signals via Bcl10 and Malt1 for NF-κB activation.

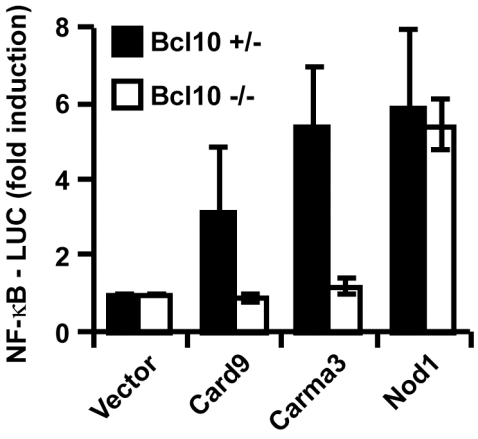

Figure 25: Bcl10 is required for Card9 induced NF-κB activation. Card9, Carma3 or Nod1 expression plasmids were introduced into Bcl10+/- or -/- MEFs by lipid reagent transfection and NF-κB luciferase assays performed.

Over the last years, signalling components downstream of Malt1 have been the focus of extensive work. In T cells, the signal is transuded by TRAF2, TRAF6 and TAK1 (Rawlings, Sommer et al. 2006). It is therefore conceivable that similar mechanisms could operate in myeloid cells, but are as yet not formally proven. The components upstream of Card9 that connect the signal to Syk remain much more ambiguous. The molecules relaying the analogue signals in lymphocytes include protein kinases C beta and theta (PKCβ, PKCθ) and the membrane-associated guanylate kinase (MAGUK) and linker-domain of Carma1. The MAGUK domain anchors Carma1 to the cell membrane

and mediates homophile interaction. The linker region between the MAGUK and coiled-coil domain is a flexible region by which the N-terminus of Carma1 folds back to the C-terminus, thereby presumably inhibiting interaction of the CARD with other CARD-carrying proteins. PKCθ serine-phoshorylates the linker region of Carma1 and thereby induces its activation (Sommer, Guo et al. 2005).

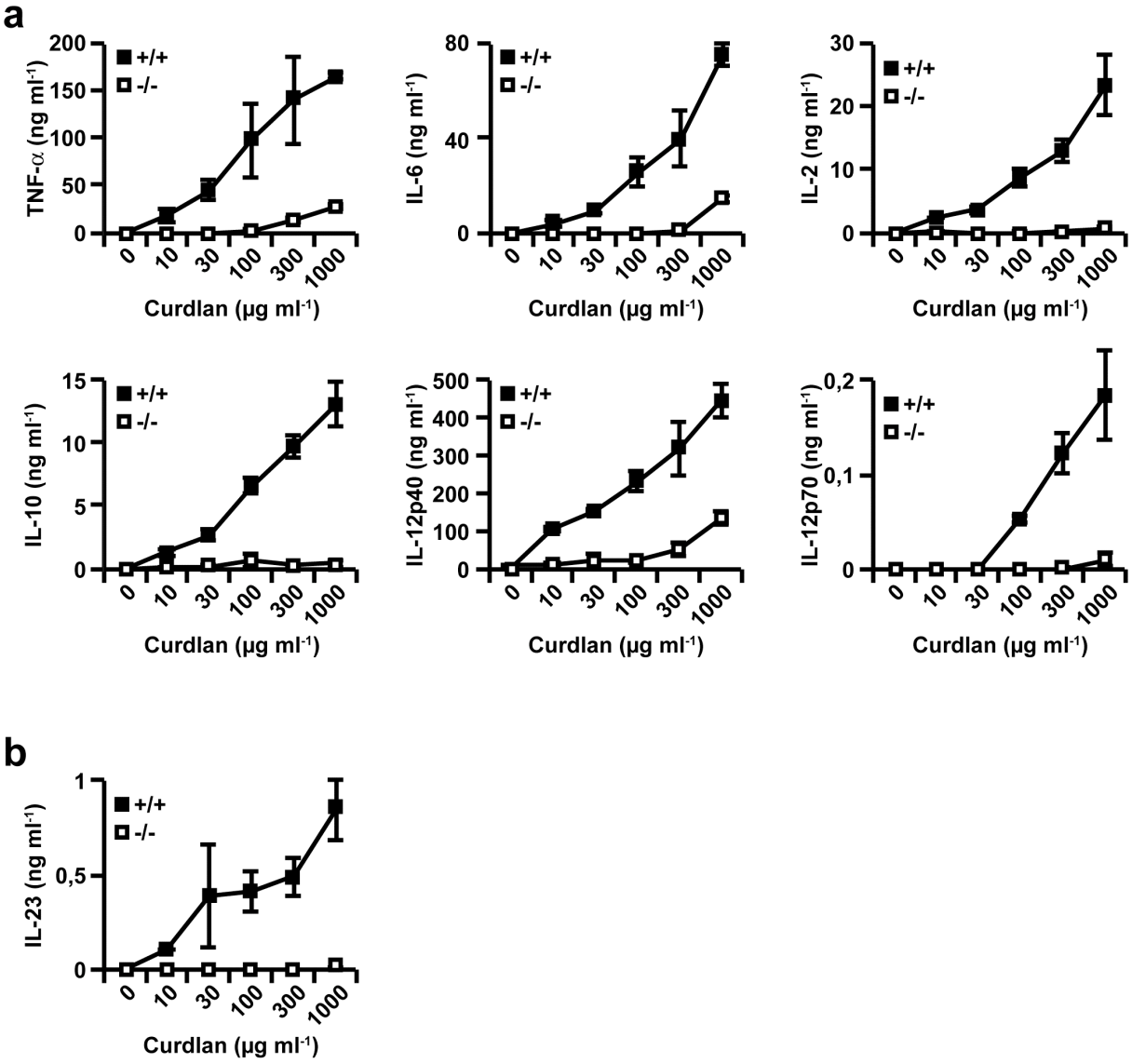

Figure 26: Curdlan is a pure β-Glucan that activates the Dectin-1/Card9 pathway. Production of TNF-α, IL-12p40, IL-12p70, IL-10, IL-2, IL-6 (**a**) and IL-23 (**b**) in response to curdlan depends on Card9. Cytokine production by BMDCs from Card9-/- or wild-type control mice after stimulation with curdlan was measured by ELISA. Data are the mean ± S.D. of triplicate stimulations and are representative of three independent experiments.

Card9 lacks the MAGUK domain and the linker region, suggesting that PKCs might not be involved in Card9 signalling. Consistent with this hypothesis, stimulation of dendritic cells with phorbol ester to directly activate PKCs does not induce cytokine production, further indicating that PKCs are not required for Dectin-1 signalling (data not shown). Future work will focus on the identification of novel proteins that act upstream of Card9. Another subject will be the identification of mechanisms involved in Card9 activation, like serine or tyrosine phosphorylation and other modifications and conformational changes, mechanisms reported for the activation of Carma1 and Bcl10.

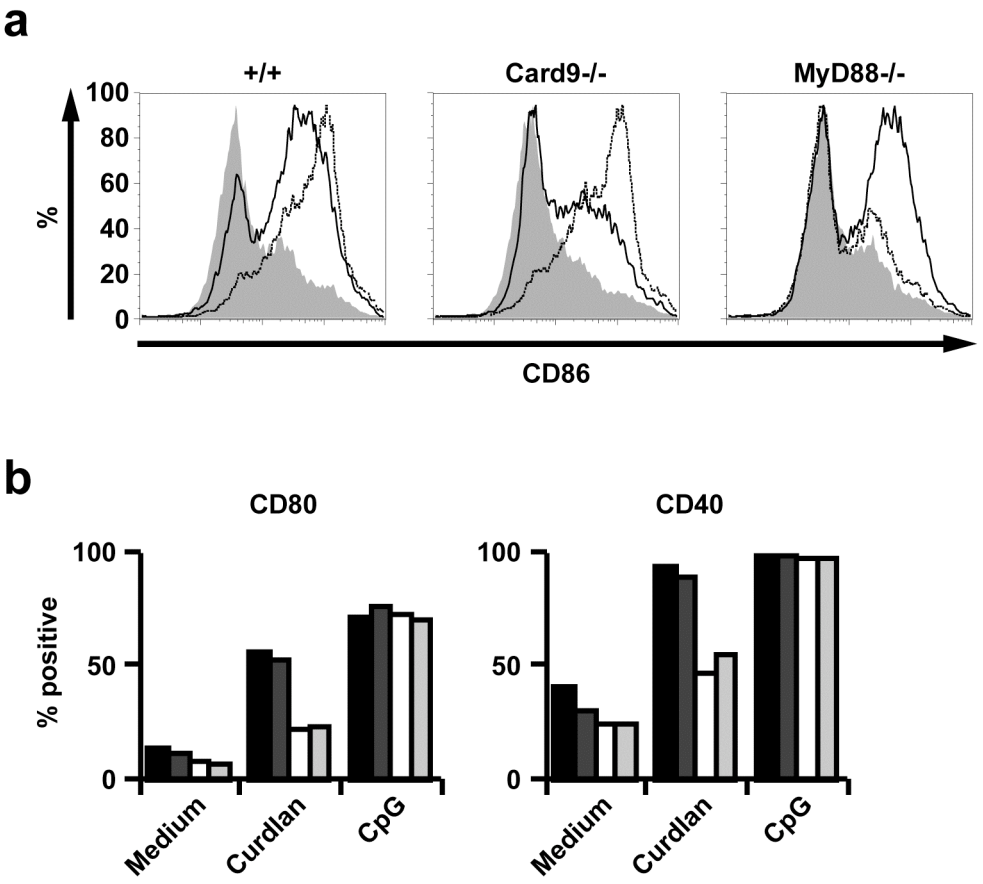

Figure 27: Selective Dectin-1 signalling induces Card9-dependent DC maturation. **a** Flow cytometry analysis for surface expression of CD86 on wt (C57BL/6), Card9-/- or MyD88-/- BMDCs after either no stimulation (solid area) or overnight stimulation with curdlan (5 µg/ml, solid line) or CpG (1 µM, dotted line). **b** FACS analysis for expression of CD40 and CD80 on the surface of BMDCs from two wild-type (black and dark-grey columns) and two Card9$^{-/-}$ mice (white and light-grey columns), either left unstimulated (Medium) or stimulated with curdlan or CpG as in a.

On the basis of similarities and differences in Bcl10/Malt1 activation in innate and adaptive immunity, a signalling model has been outlined (Figure 29). In adaptive immunity, ITAM-containing molecules like the B-cell receptor Igα/β chains and T-cell receptor CD3γ/ζ subunit utilize Carma1 to transduce signals to Bcl10/Malt1 and eventually NF-κB. On the other side, innate ITAM-containing PRRs like Dectin-1 use the Carma1 homologue Card9 to perform this action. Future research should address the question of whether the differential use of Card9 and Carma1 is a feature of different receptor families or different cell types and lineages.

After this data was published, two other groups also reported findings using independently generated Card9 deficient mice (Underhill and Shimada 2007). Both groups find similar expression patterns of Card9 as have been reported here and also identified roles for Card9 in innate immunity, but with a different focus. The group of Xin Lin reports that Card9 forwards signals from the intracellular PRR Nod2 for activation MAP Kinases (Hsu, Zhang et al. 2007). They stimulated thioglycolate-elicited macrophages with various PAMPs and found impaired IL-6 and TNF-α cytokine responses with muramyl dipeptide (MDP, a Nod2 ligand) and poly(I:C) (a TLR3 ligand), mild defects with peptidoglycan (PGN, a TLR2 ligand) and loxoribine (a TLR7 ligand) and normal responses with lipopolysaccharide (LPS, a TLR4 ligand) or diacylated lipopeptide FSL-1 (a TLR2/6 ligand). In terms of signal transduction, they reported impaired activation of MAPK p38 upon stimulation with MDP or poly(I:C), while Erk and Jnk as well as NF-κB activation were normal. Due to the fact, that poly(I:C), PGN and loxoribine have, in addition to their TLR stimulating capacity also, like MDP the potential to stimulate intracellular receptors, they conclude that Card9 participates in the perception of intracellular infections. To test this theory, they infected macrophages with the intracellular bacterium *Listeria monocytogenes* and vesicular stomatitis virus (VSV). They then found impaired cytokine production as well as reduced p38 and Jnk activation but normal Erk and NF-κB activation in response to both pathogens. In overexpression experiments and coimmunoprecipitations direct interaction of Nod2 and Card9 was demonstrated. Reporterassay experiments also showed a collaboration of Nod2 and Card9 for ATF-2 (a MAPK regulated transcription factor) activation. Interaction of native proteins was shown in cellular extracts from macrophages upon infection with *Listeria monocytogenes*. In infection experiments with the same

pathogen, Card9 mice were impaired in early cytokine production and unable to clear the infection (Hsu, Zhang et al. 2007).

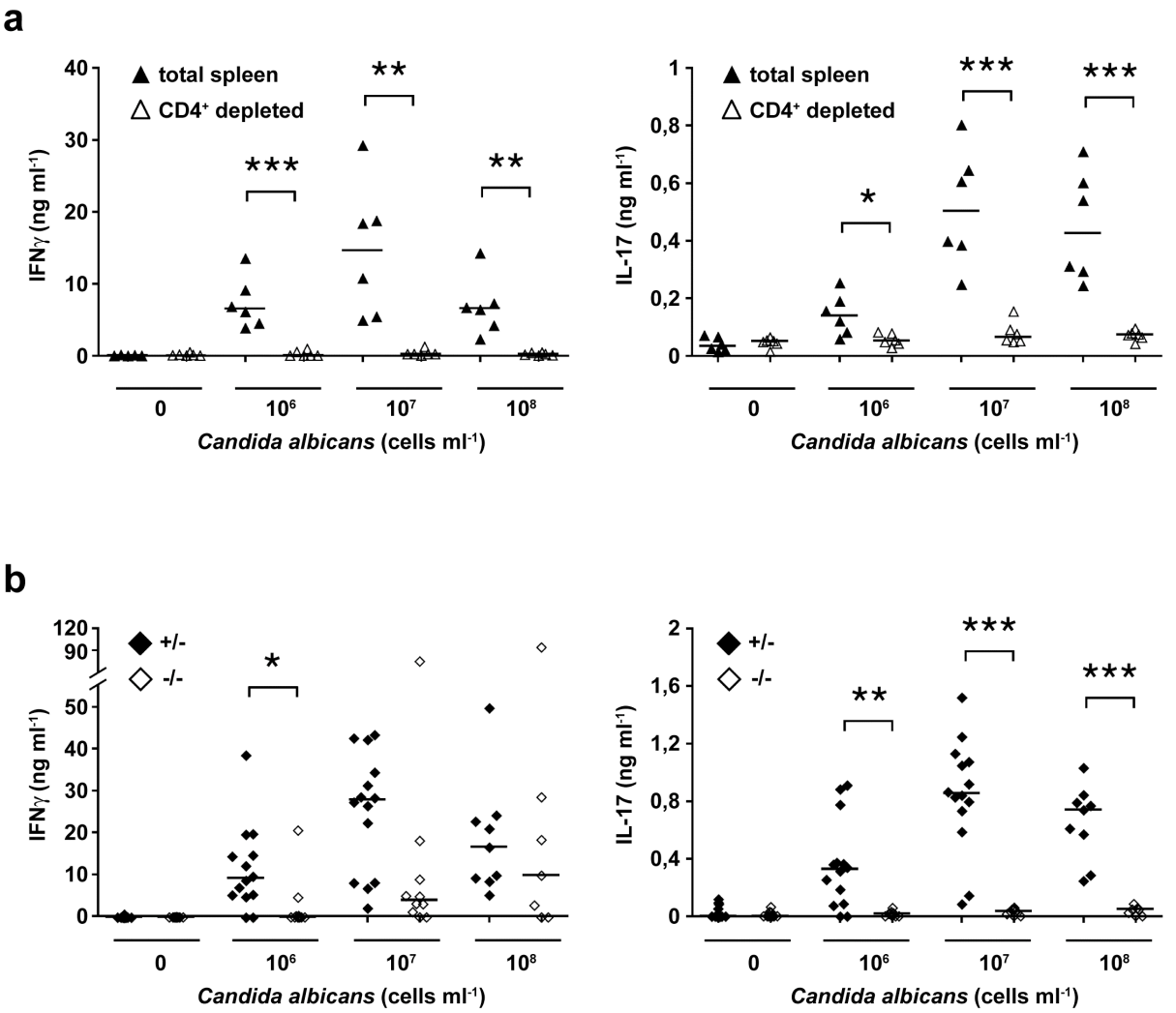

Figure 28: Infection with *Candida albicans* induces Card9-dependent T_H17 antifungal responses. **a** ELISA of IFN-γ and IL-17 production by total splenocytes (total spleen) or splenocyte samples depleted of $CD4^+$ cells ($CD4^+$ depleted); cells were obtained from wild-type mice infected with *Candida albicans* and were restimulated with heat-killed organisms. **b** ELISA of IFN-γ and IL-17 production by total splenocytes from Card9–/– or Card9+/– control mice infected with *Candida albicans* before restimulation of cells with heat-killed organisms. Each symbol represents one mouse (mean of triplicate restimulations); horizontal bars, median. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data is from six to fourteen mice per group pooled from two independent experiments.

The group of Saito also generated Card9-deficient mice (Hara, Ishihara et al. 2007). In addition to data on normal B and T cell function in the absence of Card9, this

group demonstrated Card9-dependent Dectin-1 activation. Moreover, they also tested other receptors that don't contain an ITAM themselves but signal via the ITAM containing adapter proteins DAP12 or FcRγ. FcR (CD16), triggering receptor expressed on monocytes 1 (TREM-1), osteoclast-associated receptor (OSCAR) and myeloid-associated Ig-like receptor (MAIR) also activated dendritic cells in a Card9-dependent manner. Upon TLR stimulation, Hara *et al.* additionally reported a partial defect in TNF-α and IL-6 production for several ligands, suggesting a potential role of Card9 in TLR signalling (Hara, Ishihara et al. 2007). Future work is therefore warranted to test whether Card9 is a direct element of TLR signal transduction or whether it cooperates with TLR pathways for full innate immune cell activation.

4.3 Card9 in Anti-Fungal Immunity.

In line with the finding that Card9 mediates fungal recognition in myeloid cells, severe defects in fungal infections of Card9-deficient mice were observed. Intra-venous infections lead to rapid lethality of Card9$^{-/-}$ animals after 4 to 9 days of infection and the organs of Card9 knockout mice showed up to 100-fold higher loads of infiltrating *Candida albicans* cells compared to wildtype littermates. How the defect in cytokine production by dendritic cells and the increased susceptibility to fungi *in vivo* are mechanistically connected is not clear as to now. One important role of tissue-resident dendritic cells is the onset of inflammation after pathogen recognition by proinflammatory cytokines like TNF-α and IL-6 (Romani 2004). Defective cytokine production in the absence of Card9 during fungal infection would lead to reduced inflammatory responses. Thus, it could be predicted that there would be a severe reduction in the recruitment of lymphocytes or any other effector cells from the bloodstream to the site of infection, which is key in controlling a pathogen. However, the observed defects in cytokine production by dendritic cells are insufficient in explaining the severity of infection. Full activation of adaptive immunity takes about one week, and defects in adaptive immunity can therefore be excluded as a reason for the early fatality in Card9 deficiency. Thus, other cell types, like neutrophils and macrophages that also carry the Dectin-1 receptor are probably involved. Dectin-1-deficient mice have already been studied in detail for defects in these cell types. Two

independent studies have addressed the role of Dectin-1 in innate immunity by generating Dectin-1-deficient mice. The group of Gordon D. Brown has focused on the role of Dectin-1 in macrophages and neutrophils (Taylor, Tsoni et al. 2007). The main effector functions of these cells are phagocytosis of pathogens and killing by release of reactive oxygen species (ROS), nitric oxide (NO) and lysosomal proteases. Dectin-1-deficient macrophages and neutrophils showed a severely reduced ability to bind and take up zymosan particles. The respiratory burst reaction was only reduced in neutrophils from Dectin-1 knockouts, while it was found to be normal in macrophages. In line with this, Dectin-$1^{-/-}$ neutrophils were significantly impaired in their ability to kill live fungal cells *in vitro*, while macrophages showed no difference. It should be tested whether that oxidative burst like phagocytosis is independent of Card9.

Dectin-$1^{-/-}$ mice show a defect in the recruitment of neutrophils, eosinophils and macrophages into the peritoneal cavity upon infection with *Candida albicans*. This phenomenon was linked to defective production of IL-6, the chemokines CCL2 and CCL3 as well as granulocyte- and granulocyte-macrophage colony-stimulating factors. Sources of these cytokines are resident macrophages of the peritoneal cavity that were present in regular frequencies in Dectin-1 knockout mice. This finding provides further evidence for the hypothesis that defective onset of inflammation in Card9 deficient mice, due to defective cytokine as well as chemokine production in both dendritic cells and tissue-resident macrophages, causes increased susceptibility to fungal infections.

Dectin-1 knockout mice showed increased susceptibility to disseminated candidiasis. However, after infection with a dose of 10^4 cfu *Candida albicans*, some Dectin-1 deficient mice survived long term and only succumbed to the infection after the tenth day. In contrast, in a Card9 deficient background, no mouse survived more than 9 days even with low doses of only 1000 cfu. In addition, IL-10 and IL-12 production from Dectin-1 deficient bone marrow derived dendritic cells in response to zymosan stimulation was normal (Taylor, Tsoni et al. 2007), although these cells show impaired cytokine production in response to curdlan stimulation (LeibundGut-Landmann, Gross et al. 2007).

In a second report on independently generated Dectin-1 deficient mice, the group of Tarakhovsky reports no defect in *Candida albicans* defence and only mild defects in response to *Pneumocystis carinii* infection (Saijo, Fujikado et al. 2007). In this

study, they demonstrated defects in cytokine production using pure β-Glucans like oxidized zymosan. In contrast, they show normal responses of Dectin-1-knockout dendritic cells to zymosan or fungal cells in terms of cytokine production.

4.4 Activation of T_H17 Responses by Card9

It was additionally demonstrated that Card9 signalling in dendritic cells mediates the activation of T_H17 responses *in vivo* and *in vitro* (Palm and Medzhitov 2007). Production of the T_H17 related cytokines IL-23 and IL-6, as well as the upregulation of T cell co-stimulatory molecules on dendritic cells has been shown to be Card9 dependent. Consequently, Card9-deficient mice don't induce anti-fungal T_H17 T cells upon infection. Thus, Card9-deficient mice might be a good model to specifically study T_H17 dependent responses. Emerging data suggests that T_H17 T cell mediated immunity plays a role in those infectious diseases that were shown to be largely independent of TLR recognition. Card9 dependent C-type lectin recognition might be more important for the recognition of these pathogens, which has to be tested, as outlined below in more detail. T_H17 T cells have also been connected to autoimmune diseases that were previously thought to be T_H1-related, like multiple sclerosis in the model of experimental autoimmune encephalomyelitis (EAE). In addition, T_H17 T cells were found to be abundant in the lamina propria of the gut, although their function in this tissue remains elusive. It's been speculated that these cells develop in the interaction of immune cells with the gut flora, but their development appears to be MyD88 independent (Ivanov, McKenzie et al. 2006). In correlation, IL-23 and T_H17 T cells are now implicated in the development of inflammatory bowel diseases such as Crohns disease (Strober, Murray et al. 2006). Future work will address the question whether Card9-dependent signalling contributes to the development of T_H17 T cells in the gut and the onset of inflammatory bowel diseases and other autoimmune diseases, possibly by accelerating IL-23 and T_H17 responses.

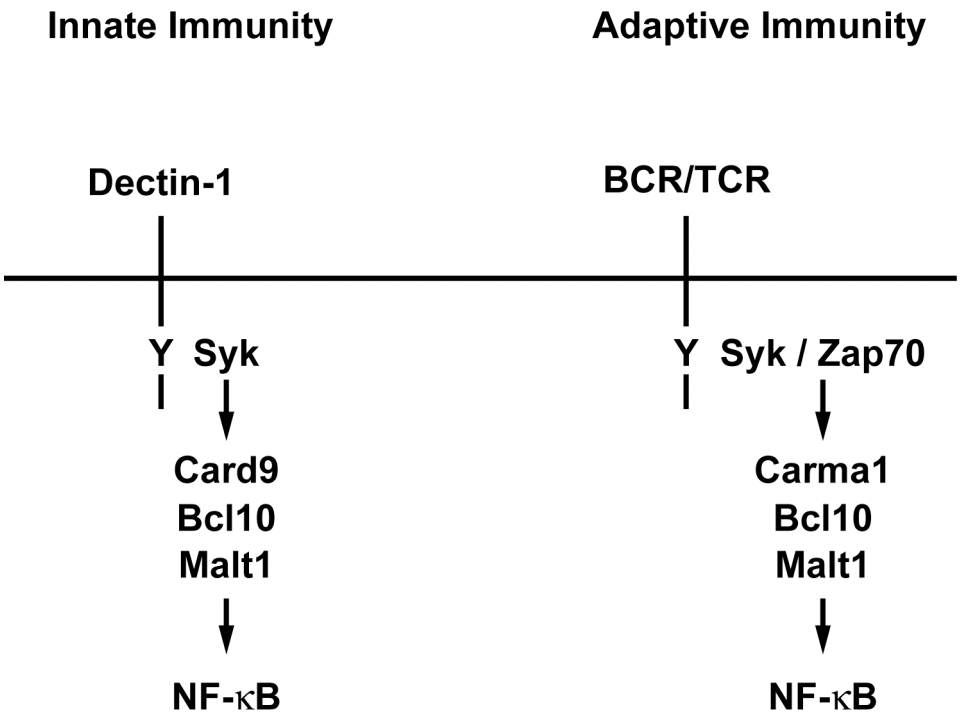

Figure 29: Simplified model for the differential control of Bcl10/Malt1 signalling in innate and adaptive immunity. Y, ITAM. For further explanation see page 70 of discussion.

4.5 Potential Role of Card9 in the Signal Transduction of other ITAM- Receptors

Card9 has been shown here and by others to be important in innate immune cell activation through ITAM bearing receptors or receptor proximal ITAM bearing adapters (Colonna 2007). Beyond those shown, many other receptors of the C-type lectin family are expressed on these cells and utilize ITAMs for signalling, opening the potential that Card9 might be related in their signalling pathways, too. Some of these receptors are summarized in Table 2.

The two studies on Dectin-1-deficient mice report a complete signalling defect only when pure β-Glucans like oxidised zymosan are used. With untreated zymosan, fungal cells or in fungal infection, they report a milder phenotype as compared to the data presented here for Card9$^{-/-}$ mice. Therefore, there might be other receptors that recognize fungal agents contained within zymosan. Some other C-type lectins were also demonstrated to bind to *Candida albicans* or other fungi, i.e. Mannose receptor, DC-SIGN, SIGN-R1 and Dectin-2 (Table 2). Dectin-2 was shown to forward signals via

the ITAM bearing adapter protein FcRγ. It selectively recognises the hyphal morphology of *Candida albicans*, whereas Dectin-1 recognises the yeast form (Gantner, Simmons et al. 2005). Candida hyphae "hide" their β-Glucans from Dectin-1 by covering it with other cell wall components, mostly mannans and mannoproteins. These mannoproteins are subject to oxidization of zymosan that destroys Dectin-1 independent but potentially Card9 dependent components of zymosan. To which component of *Candida albicans* cells Dectin-2 binds is still unclear, thou. Dectin-2 might thereby be a Card9-engaging anti-fungal proinflammatory receptor. Knockout mice deficient for Dectin-2 would provide more data to this question but are not yet available.

Dectin-1 is the first member of a family of seven NK-cell receptor-like-C-type lectins for which defined signalling capacities have been described (Brown, Herre et al. 2003; Cambi and Figdor 2003; Rogers, Slack et al. 2005). A group of six very similar receptor-encoding genes is located in the vicinity of the Dectin-1 gene. They show high sequence homology and might go back to a common ancestor gene that could have multiplied at this locus, possibly by viral or retroviral action (Brown 2006). Four of these genes, Clec2, Clec9a, Clec12a and Clec12b encode proteins possessing an YxxL ITAM motif in their cytoplasmic tail. Although it is possible that Dectin-1 might be an exception, these receptors might function similar to Dectin-1, possibly as PRRs, and engage analogous pathways (Brown, Herre et al. 2003; Rogers, Slack et al. 2005). Future work should therefore investigate the role of Card9/Bcl10/Malt1 signalling in these, as of yet, undefined pathways.

FcγR possibly also engages Bcl10 for downstream signalling. The closely related FcεR on mast cells was shown to signal via Bcl10 and Malt1 for NF-κB activation, cytokine production and late phase hyperresponsiveness (Klemm, Gutermuth et al. 2006). FcγR and FcεR use the same Fc receptor γ subchain and some of the cell types carrying one or the other are most closely related. Future work will investigate whether FcγR indeed signals via Bcl10 like FcRε and if they engage Card9 or Carma1 to do this.

4.6 Comparing Card9 and MyD88 Dependent Effects

An interesting feature of Card9 deficient mice is, that they don't develop spontaneous fungal infection. MyD88-deficient mice frequently suffer from bacterial

infections like *Staphylococcus aureus* infections of the middle ear. MyD88 knockout often die a few weeks after birth and seldom reach the age of one year. Compared to those, Card9 mice live more than two years and eventually pass away from causes other than infection (personal observation). Specific pathogen free (SPF) mouse facilities are not free of opportunistic fungi like *Candida* or *Aspergillus* species (Harlan Inc., personal communication). The immune system of Card9 deficient mice therefore seems to be able to deal with low-doses of fungal pathogens, probably by epithelial defence, while MyD88 knockouts are often unable to do the same for some bacteria. However, in fungal sepsis, very low doses of *Candida* already lead to the death of Card9 knockout mice. Card9 therefore seems to take action when an infection becomes serious, i.e. reaches the blood stream. C-type lectin domain receptors are relatively new in evolution, first appearing in gnathostomata (Kono, Sakai et al. 2003; Robinson, Sancho et al. 2006). Toll like receptors in contrast are much older and are conserved for example in insects and nemaotodes (Hoffmann and Reichhart 2002). Even in plants, LRR carrying PRRs are found. The abilities of C-type lectins in innate immunity might correspond to the special selective pressures on higher vertebrates. Not much is known about pathogen-host specificity in invertebrates. In vertebrates, pathogens-host specificity often correlates with the severity of the disease (Wolfe, Dunavan et al. 2007). Opportunistic pathogens like *Staphylococci* or *Candida* species are usually non-specific and only infect immunocompromised individuals. Many highly infectious diseases like human immunodeficiency virus (HIV) and other viruses or certain helmiths or protozoic pathogens like *Plasmodium falciparum* are highly host specific. Interestingly, these are mostly non-bacterial pathogens. Many of the C-type lectin receptors discussed above bind to such pathogens and some also bind to Mycobacteria (Table 2). If further research would verify these findings in *in vivo* models, it might turn out that C-type lectin receptors are more important in the recognition of eukaryotic or viral, highly host-specific pathogens, while the main role of TLRs might be the recognition of opportunistic prokaryotes and viruses. This would fit into place with the observation that, while TLRs turned out to be of extraordinary importance for the defence against bacteria, i.e. TLR2 for *Staphylococcus aureus*, the results for highly infectious pathogens didn't meet expectations (Bellocchio, Montagnoli et al. 2004; Malmgaard, Melchjorsen et al. 2004; Villamon, Gozalbo et al. 2004; Power, Marshall

et al. 2006; Ryffel, Jacobs et al. 2006; Fremond, Togbe et al. 2007; Lepenies, Cramer et al. 2007). It would also fit the fact that C-type lectin expression often varies greatly between individuals or species, as outlined for DC-SIGN between men and mice and for NK1.1 between different mice strains (Colucci, Caligiuri et al. 2003), possibly reflecting the needs of pathogen-host specificity. If indeed some of these receptors signal via Syk to Card9/Bcl10/Malt, this pathway might turn out to be of significant importance for many infectious diseases.

A novel study on the contribution of MyD88/TRIF pathways for vaccination has shown that TLR signalling also does not contribute to activation of adaptive responses by the most common clinical adjuvants, Freunds adjuvant and aluminium hydroxid (alum) (Gavin, Hoebe et al. 2006). However, it has been shown that whole recombinant yeast cells expressing foreign antigen work as a potent adjuvant for immunisation (Stubbs, Martin et al. 2001). Whether MyD88/TRIF and Card9 pathways work synergistically together for activation of adaptive responses by any of theses substances or whether both are dispensable for immunisation will allow further insights on the mechanisms of adaptive immune activation by the commonly used but poorly understood adjuvants.

4.7 Possible Involvement of Card9 in Cancer Development

Proteins of the Bcl10 pathway are recurrently involved in lymphomagenesis (Farinha and Gascoyne 2005). There are two major mechanisms participating in lymphoma development in this pathway. One is chromosomal translocation, bringing genes under the control of a strong promoter like the immunoglobulin heavy chain enhancer on chromosome 14 in humans. Bcl10 is an example for this mechanism being translocated t(1;14)(p22;q32) (Willis, Jadayel et al. 1999). Chromosomal translocation can also result in chimeric proteins like in the case of IAP2-Malt1 with the translocation t(11;18)(q21;p21) (Morgan, Yin et al. 1999). The underlying mechanism of all these pathogenic changes seems to be an increased and persistent oligomerisation of proteins of this pathway, either due to fusion to proteins containing homo-oligomerisation domains like IAP2, or by increasing the cellular concentration of proteins with domains capable of homo-oligomerisation, like CARD domains and thus

eventually inducing NF-κB (Thome 2004). Card9 was found be upregulated in lymphomas due to genomic gains of the Card9 locus. The Card9 gene is also reported to be involved in lymphoma development through genomic gains. It is amplified in translocation negative MALT-lymphoma and overexpressed in gastric B-cell lymphoma (Kono, Sakai et al. 2003) (Nakamura, Nakamura et al. 2005) (Zhou, Ye et al. 2006). Nevertheless, how Card9 actually contributes to lymphoma development is still unclear. This work demonstrated that Card9 is an important activation mediator in distinct cell types. Like experimental overexpression of Card9 can activate NF-κB in cell lines, upregulation of Card9 due to genomic gains could lead to constitutive NF-κB activation, thereby contributing to lymphomagenesis. Therefore, Card9 is, after Malt1 and Bcl10 the third member of Bcl10 pathways that can potentially promote cancer development if genetically altered.

4.8 Conclusion and outlook

In contrast to immunity directed against viruses and bacteria, far less is known about innate responses to fungal infections (Romani 2004). Yet, the incidence of these infections is rapidly growing worldwide with the number of immunocompromised patients, suffering from AIDS or undergoing immunosuppressive therapies for example during organ transplantations. In order to potentially manipulate anti-fungal immunity, it is necessary to understand the pathways controlling these responses. To this point, molecular insights into fungal infections have mostly been derived from studies of the receptor Dectin-1 (Brown 2006). The results presented here provide mechanistic insights into these pathways. This might prove to be important for the development of new anti-fungal immune therapies. This knowledge might also help to develop therapeutics that interfere with Card9/Bcl10/Malt signalling in disease.

In recent years, numerous important infectious diseases, as well as common adjuvants, were demonstrated to be TLR/MyD88 dependent to a minor degree (Bellocchio, Montagnoli et al. 2004) (Villamon, Gozalbo et al. 2004). In turn, various CLRs as well as other receptors and receptor families have been demonstrated to recognize human pathogens. Many CLRs use ITAM motifs to forward signalling, potentially via Card9. Future studies will test this hypothesis. Card9 might therefore be

a master regulator of innate immunity forwarding signals of many C-type lectins, functioning in an analogous way to MyD88 for TLRs. T_H17 T cell responses are emerging to be of major importance for many infectious diseases as well as various autoimmune diseases (Amadi-Obi, Yu et al. 2007) (Veldhoen, Hocking et al. 2006). Like TLRs activate T_H1 responses to many different pathogens via MyD88, Card9 might also activate T_H17 T cells for defence against pathogens other than fungi. In addition, T_H17 dependent autoimmunity might be related to Card9 dependent signals derived either from pathogens or from host-derived stimuli. Future work will have to investigate the potential involvement of Card9 in innate and adaptive immunity to various pathogens as well as in T_H17-mediated autoimmune models.

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