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Analysis of C-type lectin receptor induced NF-kappaB signaling

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Tell me and I forget. Teach me and I remember. Involve me and I learn.

- Benjamin Franklin

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ZUSAMMENFASSUNG

Myeloide Zellen sind Wächter des angeborenen Immunsystems, die eindringende Pathogene, sterile Verletzungen des Gewebes und eine Vielzahl anderer Abweichungen vom Normalzustand erkennen. Mustererkennende Rezeptoren, sogenannte *pattern recognition receptors*, die zur Familie der C-Typ Lectin Rezeptoren (CLRs) gehören, ermöglichen diesen Wächterzellen die Erkennung essentieller, tragender Strukturen von Pilzen, Viren und anderen Keimen. β-glucan Kohlehydrate sind mit Pathogenen assoziierte molekulare Muster, sogenannte *pathogen associated molecular patterns*, die in den Zellwänden von Pilzen vorkommen und spezifisch aktivierend auf den Dectin-1 Rezeptor wirken. Zusammen mit den verwandten CLRs Dectin-2 und Mincle ist Dectin-1 entscheidend an der Signalvermittlung für die Entstehung von Entzündungsreaktionen und dem Selbstschutz des Wirtes gegen pathogene Pilze beteiligt. Diese drei Rezeptoren reagieren auf Erkennung ihrer Liganden, indem sie direkte oder indirekte Verbindungen mit der Kinase Syk eingehen und unter Einbeziehung des zytoplasmatischen Adapterproteins Card9 den Transkriptionsfaktor *nuclear factor* κB (NF-κB) aktivieren, um somit die Produktion entzündungsfördernder Zytokine auszulösen. Allerdings sind speziell die in unmittelbarer Nähe der Rezeptoren ablaufenden Prozesse dieser Signalkaskade, die ab der Aktivierung der Kinase Syk zur Einbindung des zentralen Card9 Moduls führen, nicht vollständig bekannt.

Für die hier beschriebenen Analysen solcher rezeptorproximalen Abläufe wurden die CLR Liganden Zymosan und Curdlan verwendet, um aus dem Knochenmark von Mäusen gewonnene dendritische Zellen, sogenannte *bone marrow-derived dendritic cells (BMDCs)* zu stimulieren. Von Dectin-1 ausgehende Signale hatten die Phosphorylierung von Tyrosin und damit die Aktivierung der Protein Kinase C-δ (PKCδ) in einer von Syk abhängigen Art und Weise zur Folge. In *Prkcd*$^{-/-}$ BMDCs war die Zytokinproduktion in Reaktion auf die Stimulation von Dectin-1, Dectin-2 oder Mincle reduziert, während PKCα-, PKCβ-, oder PKCθ-defiziente Zellen im Vergleich zum Wildtyp normal reagierten. Es konnte gezeigt werden, dass die Phagozytose von Zymosanpartikeln unabhängig von PKCδ stattfindet. Sowohl die Dectin-1 abhängige Induktion des klassischen, *canonical* NF-κB Signalweges, einschließlich der Gruppierung eines Card9 und dessen Effektor Bcl10 enthaltenden Komplexes, als auch die Aktivierung der Kinase TAK1 waren in *Prkcd*$^{-/-}$ BMDCs gestört. Zellen, die kein PKCδ exprimieren, zeigten in Folge von *Candida albicans* Infektionen eine deutlich eingeschränkten Produktion entzündungsfördernder Zytokine.

Insgesamt beschreiben die Daten in dieser Arbeit PKCδ als wesentliches Bindeglied für die Dectin-1 induzierte, Syk-vermittelte Signalweiterleitung über Card9 zur Aktivierung von NF-κB. PKCδ wird damit als essentielles Molekül dieses Signalwegs identifiziert, welches speziell für die von CLRs ausgelöste angeborene Immunantwort und die Verteidigung des Wirtes unabdingbar ist.

SUMMARY

Myeloid cells are sentinels of the innate immune system that detect invading pathogens, sterile tissue damage, and various other forms of deviation from normality. Pattern recognition receptors of the C-type lectin receptor (CLR) superfamily enable those sentinel cells to recognize essential scaffolding structures of fungi, viruses, and other microbes. β-glucan carbohydrates are pathogen associated molecular patterns of fungal cell walls and specific agonists of the CLR Dectin-1. Together with its cognate CLRs, Dectin-2 and Mincle, Dectin-1 is critical for the instruction of inflammation and host protection in response to pathogenic fungi. Upon ligand binding, these three receptors directly or indirectly couple to the spleen tyrosine kinase (Syk) and involve the cytoplasmic adaptor caspase recruitment domain-containing protein (Card)9 to induce nuclear factor κB (NF-κB) signaling, leading to the production of proinflammatory cytokines. However, particularly the CLR-proximal events in this signaling cascade, linking Syk activity to engagement of the central Card9 module, are incompletely understood.

Here, the CLR ligands zymosan and curdlan were used to stimulate mouse bone marrow-derived dendritic cells (BMDCs) for the analysis of such receptor-proximal events. Dectin-1 signaling caused tyrosine phosphorylation and activation of protein kinase C-δ (PKCδ) in a Syk-dependent manner. Cytokine production in response to Dectin-1, Dectin-2, or Mincle stimulation was found to be reduced in *Prkcd*$^{-/-}$ BMDCs, while PKCα-, PKCβ-, or PKCθ-deficient cells responded normally, when compared to the wild-type. Phagocytosis of zymosan particles was shown to be independent of loss of PKCδ. Dectin-1-dependent induction of canonical NF-κB signaling, including the assembly of a complex involving Card9 and its effector protein Bcl10, as well as TAK1 kinase activation were defective in *Prkcd*$^{-/-}$ BMDCs. Finally, cells lacking PKCδ were impaired in the production of inflammatory cytokines in response to an infection with *Candida albicans*. Together, these data suggest that PKCδ is an essential link in Syk-mediated signaling via Card9 to induce NF-κB activity and specifically required for CLR triggered innate immunity and host defense.

1. INTRODUCTION

In spite of her beauty, the world we live in is a hostile and dangerous environment. Human beings, together with animals, plants and all other living creatures are constantly exposed to physical, chemical, and microbial threats. An organism's ability to protect and defend itself is therefore a prerequisite for survival. The science of immunology describes the mechanisms that organisms employ to defend themselves against environmental threats in the form of infection and to maintain the state of tissue homeostasis with a particular focus on the cellular and molecular level (Murphy *et al.*, 2012).

1.1. The Immune System

Multicellular organisms are provided with a dynamic network of specific organs and tissues, highly specialized cells, molecular mediators, and a vasculature, collectively termed the immune system. Its components collaborate to protect the body from disease or foreign structures called antigens and to clear, where necessary, established infections by mounting a concerted reaction in the form of an immune response. Threatening microbial invaders that need to be detected and eliminated can be grouped roughly into four categories of pathogens. Those are viruses, bacteria, fungi, and unicellular or multicellular eukaryotic organisms also referred to as parasites (Kindt *et al.*, 2007; Murphy *et al.*, 2012). Also host cells that are altered and may lead to or already have developed cancerous traits will be, under certain conditions, recognized and eliminated. Correct and precise functioning of the immune system therefore depends on its ability to faithfully distinguish between foreign and the body's own cells and molecules, and to destroy only non-self or abnormal and damaged cells. Failure in this identification mechanism can lead to severe infections whereas deregulation of the system may cause allergies, asthma, and in the worst case the development of malignancy. Attack of the body's healthy cells, in turn, leads to serious inflammatory or autoreactive conditions as it is the case, for example, in multiple sclerosis, rheumatoid arthritis, psoriasis, inflammatory bowel disease, or lupus, to name but a few (Kindt *et al.*, 2007; Murphy *et al.*, 2012). In higher vertebrates, this very effective host defense system has evolved to yield the two fundamental mechanisms of innate and adaptive immune responses.

1.1.1. Innate Immunity

The first line of defense against invading pathogens is constituted by the innate immune system. It can be traced back far in evolution and in some form has been found in all multicellular plants and animals examined until now. Innate immunity is the dominant mechanism of host defense in most organisms. It is also referred to as the natural or the native immune system, highlighting its constant and immediate availability in healthy individuals. It provides an early and rapid protection against infections, with the detection of foreign or dangerous patterns leading to an immediate maximal response (Litman *et al.*, 2005; Kindt *et al.*, 2007). Innate immune responses usually last no longer than some hours or a few days and enable an organism to efficiently clear a broad spectrum of infectious microbes, ideally without ever triggering adaptive immunity. The innate immune system is well equipped to precisely discriminate between pathogens and self but its detection mechanisms are not specialized enough to distinguish subtle differences in foreign molecules. Its response is consequently generic and therefore considered the non-specific part of the defense machinery. Innate immune responses alone are also not designed to generate immunologic memory and hence cannot convey lasting immunity (Kindt *et al.*, 2007; Murphy *et al.*, 2012).

Mechanisms of innate immunity that protect an organism against microbial infection comprise physical barriers, defense molecules, and innate immune cells. Evident and preferred entry sites for pathogens are the body's surfaces most exposed to the environment. The skin, the mucosae of the respiratory tract, the lungs, the gastrointestinal and the genitourinary tract are therefore protected by continuous epithelial barriers, armed with specialized cells, for example γδ T cells, and special anti-microbial agents such as β-defensins (Agerberth and Gudmundsson, 2006). Tears, breast milk, and saliva further contain phospholipase A2 and lysozyme. Those enzymes also have anti-bacterial functions and build a chemical barrier against the entry of pathogens (Hankiewicz and Swierczek, 1974; Moreau *et al.*, 2001). Gastric acid together with proteases produced by the stomach effectively counter ingested microorganisms. Insect bites or injuries to the skin, in contrast, literally open gates for microbes to break through. Pathogens that cross this defense layer are attacked by phagocytic cells, such as macrophages (MPs), neutrophils, and dendritic cells (DCs), or by specialized lymphocytes known as natural killer (NK) cells. Moreover, a number of plasma proteins and proteins of the complement system help to clear invading pathogens. These tools of innate immunity are specifically tailored to recognize and to react against pathogenic structures but they do not respond to non-infectious foreign particles (Kindt *et al.*, 2007;

Abbas and Lichtman, 2009). The effector cells of innate immunity will be discussed in more detail in chapter 1.1.3.

Efficient immune responses require coordination of the individual components involved, which in turn depends on communication. Cells of the immune system interact through direct cell to cell contact but importantly also through the production and secretion of cytokines (Table 1). These are soluble extracellular messenger proteins mediating immune responses and influencing inflammatory reactions. By convention, many cytokines are called interleukins, since they are produced by leukocytes and also act on leukocytes. Pathogen detection leads to well dosed cytokine release at low concentrations, affecting either directly the cells that secrete them in an autocrine feedback mechanism or targeting neighboring cells in paracrine action (Abbas and Lichtman, 2009).

Several cytokines are of particular importance for innate immunity and MPs are the central cytokine producing innate immune cells (Table 1). Their secretion of tumor necrosis factor (TNF), interleukin (IL)-6, and IL-1, together with chemokines (chemotactic cytokines) recruits blood neutrophils and monocytes to sites of infection and orchestrates inflammatory responses. High levels of lipopolysaccharide (LPS), caused by severe disseminated infections with Gram-negative bacteria, can trigger the production of very high concentrations of TNF and may lead to the potentially lethal clinical syndrome of septic shock. Another major cytokine produced by MPs is IL-12. It activates NK cells which, in turn, secrete interferon (IFN)-γ thereby activating again more MPs. NK cells in innate immunity and T helper (T_H) cells in adaptive immunity, where cytokines play an important role in cell-mediated responses, are the main producers of IFN-γ. Viral infections induce MPs and other infected cells to secrete type I IFNs that inhibit viral replication and prevent the infection from spreading to other cells (hence the name interferon), with IFN-γ being a weak agent in comparison to the effects caused by the type I IFNs, IFN-α, IFN-β, and IFNω (Abbas and Lichtman, 2009). Other cytokines such as IL-10 are involved in the downregulation of immune responses after an infection has been eliminated and therefore have modulatory or even inhibitory effects on the cells that they target.

Inflammation is one of the early and most essential protective mechanisms triggered by the immune system in response to pathogen detection (Abbas and Lichtman, 2009). In addition to cytokines, insulted or infected cells release eicosanoids such as prostaglandins and leukotrienes. These substances bring about fever, elicit the dilation of blood vessels, leading

to increased vascular permeability, and direct leukocyte accumulation at sites of infection. Those effects, in turn, cause the typical symptoms of inflammation including redness, swelling, heat, pain, and loss of tissue function. Termination of inflammation is followed by rapid wound healing and tissue repair (Medzhitov, 2008).

Table 1: Essential Cytokines in Innate Immune Responses and Inflammation.

Adapted from Abbas and Lichtman (Abbas and Lichtman, 2009).

Cytokine	Main producer cell(s)	Main target cells and biological effects
Tumor necrosis factor (TNF)	Macrophages (MPs), T lymphocytes	Endothelial cells: activation (inflammation, coagulation) Neutrophils: activation Hypothalamus: fever Liver: synthesis of acute phase proteins Muscle, fat: catabolism Various cell types: apoptosis
Interleukin-1 (IL-1)	MPs, endothelial cells, distinct epithelial cells	Endothelial cells: activation (inflammation, coagulation) Hypothalamus: fever Liver: synthesis of acute phase proteins
Chemokines	MPs, endothelial cells, T cells, fibroblasts, platelets	Leukocytes: chemotaxis, activation
IL-12	MPs, dendritic cells (DCs)	NK cells and T cells: IFN-γ synthesis, increased cytolytic activity T lymphocytes: T_H1 differentiation
Interferon (IFN)-γ	NK cells, T cells	Activation of MPs Stimulation of certain antibody responses T lymphocytes: T_H1 differentiation
Type I IFNs (IFN-α, IFN-β)	IFN-α: MPs IFN-β: Fibroblasts	All cells: anti-viral setup, increased class I MHC expression NK cells: activation
IL-10	MPs, DCs, T_H2 cells	MPs: inhibition of IL-12 production, reduced expression of costimulators and class II MHC molecules
IL-6	MPs, endothelial cells, T cells	Liver: synthesis of acute phase proteins B cells: proliferation of antibody-producing cells T lymphocytes: T_H17 differentiation
IL-23	DCs, MPs	T lymphocytes: T_H17 differentiation
IL-15	MPs and several others	NK cells and T cells: proliferation
IL-18	MPs	NK cells and T cells: IFN-γ synthesis

Another protective mechanism, referred to as the acute phase response of the immune system, causes the levels of several plasma proteins in the circulation, other than cytokines, to

rapidly increase during infections. Such factors include plasma mannose-binding lectin (MBL) involved in the recognition of microbial carbohydrates, C-reactive protein (CRP) which binds to phosphorylcholine on the surface of pathogens, antibodies secreted from B lymphocytes, and importantly the diverse proteins belonging to the complement system. These enzymes with protease function cooperate in form of a catalytic cascade and eventually target the cell surface of pathogens, thus "complementing" the antibody-mediated killing of microbes (Rus *et al.*, 2005; Murphy *et al.*, 2012). Activated complement molecules either opsonize (coat) invaders, marking them for phagocytosis or destruction, or operate by disrupting their plasma membrane (Figure 1). Moreover, the complement system produces chemoattractants to recruit other immune cells in addition to cytotoxic agents and growth factors. These substances also increase vascular permeability and facilitate the healing of injured tissue following the eradication of pathogens (Martin, P. and Leibovich, 2005; Abbas and Lichtman, 2009).

An essential function of the innate immune system is further to stimulate adaptive immune responses (Figure 1). Innate antigen presentation activates adaptive immunity and innate cells secrete cytokines together with other messenger molecules to tailor the adaptive responses, rendering them appropriate and maximally efficient in fighting a particular pathogen, thereby directing the nature of the adaptive immune response to follow (Abbas and Lichtman, 2009).

1.1.2. Adaptive Immunity

Even though innate immune responses very efficiently resolve a large number of infections, pathogenic microbes have been and will be evolving rapidly to evade, circumvent, or resist these defense mechanisms. In cases where the innate immune system alone cannot defeat invading pathogens, its activities trigger adaptive immunity which, in turn, will mount a stronger and antigen-specific response (Pancer and Cooper, 2006; Abbas and Lichtman, 2009). In contrast to innate immunity, adaptive immunity is not present at birth. Instead it has to be developed through an individual's numerous encounters with many different pathogens. Adaptive immunity is hence also referred to as specific or acquired immunity and can only be found in higher vertebrates. As suggested by its name, this type of immune response adapts to a particular infection with one specific pathogen and very often will lead to immunological memory, providing the individual organism with lifelong protective immunity against reinfection with the same pathogen. The individual is then said to be immune to that particular

pathogen, whereas naive individuals have not previously encountered those specific antigens. This effect of acquired immunity provides the basis on which the principle of vaccination functions but is also the reason for the lag time between exposure to a pathogen and a maximal response (Abbas and Lichtman, 2009; Murphy *et al.*, 2012).

Lymphocytes are the key effector cells of adaptive immunity. Like all immune cells, they originate from bone marrow-resident hematopoietic stem cells. B cells develop and mature in the bone marrow, T cells in the thymus. The mature but naive lymphocytes then leave these primary and central, or generative lymphoid organs. B cells travel in the blood, T cells in the blood and in the lymph and recirculate between there and the peripheral lymphoid organs, such as lymph nodes, the spleen, and mucosal or cutaneous tissues, ready and waiting to encounter the antigen for which they express the adequate receptors (Abbas and Lichtman, 2009; Murphy *et al.*, 2012).

The specialized receptors on the surface of those cells mediate the antigen-specificity of adaptive immune responses. B lymphocytes are equipped with a B cell receptor (BCR) and in addition, secret soluble antigen receptors called antibodies or immunoglobulins whereas T lymphocytes express a T cell receptor (TCR). TCRs assemble from idiotypic αβ proteins, in combination with a set of immunoreceptor tyrosine-based activation motif (ITAM)-containing invariant cluster of differentiation (CD)3γδε chains and two ζ subunits (Bonefeld *et al.*, 2003). BCRs consist of one membrane-bound receptor immunoglobulin (mIg) molecule, which is non-covalently attached to one of each ITAM bearing immunoglobulin (Ig)α and Igβ proteins (Schamel and Reth, 2000). Those detectors are generated by random recombination events of receptor genes during B and T cell maturation, creating a great variety of structurally different receptors. Therefore, the specificity of the adaptive immune system is much more diverse and it detects significantly more chemically distinct structures than the innate immune system. The total lymphocyte population is able to recognize over a billion different antigens of both pathogenic and non-pathogenic nature. These antigens are not necessarily shared by certain classes of microorganisms and often vary among pathogens of the same type. Consequently, the adaptive immune system can also be evaded more easily than innate immunity, since the detected structures are usually not essential for pathogen survival and therefore may be mutated. Antigen receptors are beyond that clonally distributed, which means that each B and T cell clone expresses a different receptor, specific for one particular antigen (Abbas and Lichtman, 2009). Very importantly, this inherent specificity of adaptive immune receptors for pathogen structures also prevents the adaptive immune system from targeting the host's own

cells and molecules. In addition, mammalian cells express regulatory molecules to prevent immune reactions against self and a selection system already inactivates or kills lymphocytes which recognize self antigens during maturation (Abbas and Lichtman, 2009).

Adaptive immunity is divided into humoral and cell-mediated immune responses, further increasing its specificity in reactions against different types of infections. B cells mediate humoral immunity and provide defense against pathogens in extracellular fluids. Ligation of pathogen-derived antigens to the BCR induces the differentiation of naive B lymphocytes into antibody-generating effector cells, called plasma cells. They are the only cells in the body that can produce soluble antibodies and secrete them into mucosal fluids or into the circulation. Both antibodies and BCRs detect a great variety of shapes and conformations as well as soluble or cell surface-bound native macromolecules. Those include polysaccharides, lipids, proteins, and nucleic acids, in addition to small chemical groups or fractions of complex molecules. Each B cell clone and its progeny express a different antibody. Therefore, the collectivity of all B cell receptors represents the total set of antibodies that an organism is able to produce. The antibodies in the circulation block infections and prevent them from getting established, eliminate extracellular microbes but cannot function against pathogens that have already entered and infected a cell (Abbas and Lichtman, 2009; Murphy *et al.*, 2012).

Cell-mediated adaptive immunity on the other hand is carried out by T lymphocytes. Its purpose is to eliminate all forms of intracellular infections. T cells cannot detect native antigen molecules or even whole pathogens, a phenomenon referred to as T cell restriction. The TCR only recognizes peptide fragments of protein antigens which are bound to and displayed by major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs). This process is called antigen presentation and takes place in lymph nodes and in the spleen. The surface of interaction that builds between the membranes of an APC and a lymphocyte for this purpose is called the immunological synapse (IS) (Abbas and Lichtman, 2009).

There are three major subtypes of T lymphocytes. $CD4^+$ T_H cells activate phagocytes that have previously ingested pathogens to kill their prey and stimulate B cells for the production and release of antibodies. $CD4^+$ cells require the presentation of antigen peptide fragments on class II MHC molecules of APCs for their activation (Figure 2). They mediate effector functions through direct interaction with other cells but importantly also via the

secretion of proinflammatory cytokines. $CD8^+$ T killer cells are also known as cytotoxic or cytolytic T lymphocytes (CTLs) and detect peptides presented to them on class I MHC molecules. They recognize and kill host cells that have been infected and carry pathogens in their cytoplasm, an essential mechanism for the elimination of reservoirs of infection. Regulatory T (T_{reg}) cells are also important effectors of adaptive immunity. They prevent the immune system from overreacting and are critically involved in shutting-down immune responses after an infection is overcome (Abbas and Lichtman, 2009). T_{reg} cells work by direct cell to cell contact and, in addition, produce and secrete transforming growth factor (TGF)-β, IL-10, adenosine triphosphate (ATP), and cyclic adenosine monophosphate (cAMP), thereby suppressing the activity of other immune cells (Krammer and VanHook, 2011). γδ T cells in contrast are a minor subtype and express an alternative TCR. They are considered to form a link between innate and adaptive immunity, as they use TCR gene rearrangement to yield receptor diversity and are able to develop into memory cells. On the other hand, they respond to intact antigens, such as lipids and other pathogen-associated molecular patterns that are not presented on MHC molecules, a characteristic associated rather with innate pattern recognition receptor (PRR)-dependent pathogen detection (Holtmeier and Kabelitz, 2005).

$CD4^+$ lymphocytes are further distinguished into the T helper cell populations type 1 (T_H1), type 2 (T_H2), and the IL-17 secreting (T_H17) line. T_H1 lymphocytes support cellular immunity in the clearing of intracellular pathogens. Their signature cytokine, IFN-γ, primarily activates MPs. Importantly, T_H1 cells also produce the T cell growth factor IL-2, in addition to TNF, and to a lesser extent granulocyte macrophage colony-stimulating factor (GM-CSF) and the mast cell growth factor IL-3 (Mosmann *et al.*, 1986; Bettelli *et al.*, 2006). Gene expression in those cells is largely dependent on T box expressed in T cells (T-bet), the master transcription factor for T_H1 induction (Berenson *et al.*, 2004). T_H2 cells particularly foster humoral immunity for clearing extracellular pathogens (Bettelli *et al.*, 2006). They mainly secrete IL-4, which stimulates the production of IgE and makes T_H2 cell-mediated responses specifically effective against helminthic parasites, marking them for destruction. T_H2 cells further produce IL-5 to activate eosinophils but also secrete IL-13, IL-10, and IL-3 together with mast cell and T cell growth factors (Mosmann *et al.*, 1986). The T_H2-specific cytokine cocktail inhibits MP activation and suppresses T_H1 cell-mediated immunity. Likewise, the cytokines secreted especially from T_H1 cells oppose type 2 helper cell effects. T_H1 and T_H2 cell-mediated responses therefore balance each other, leading to a further enhancement of the

specific response mounted against a particular type of pathogen (Abbas and Lichtman, 2009). T_H17 cells, finally, are key drivers of inflammation but are also known to be responsible for autoimmune tissue injury (Bettelli *et al.*, 2006). T_H17 cells can be specified by their production of IL-17A, IL-17F, IL-21, IL-22, TNF, and GM-CSF (Littman and Rudensky, 2010). Their terminal differentiation *in vitro* requires the cytokines TGF-β, IL-6, IL-21, and IL-23, while IL-2 impedes this process. IL-4 and IFN-γ negatively influence the production of IL-17 during the T_H17 effector phase (Park *et al.*, 2005; Brustle *et al.*, 2012).

Any one T cell will be activated and respond only if it encounters an array of peptide-MHC complexes on an APC, since cross-linking of at least two or more TCRs is required for productive signaling to be initiated (Abbas and Lichtman, 2009). In order to become fully activated, naive T cells further require the APC to secrete cytokines and to provide other enhancing stimuli in addition to TCR engagement (Figure 1). The major receptor for such costimulatory signals is the cell surface molecule CD28 of T lymphocytes that interacts with its ligands CD80 and CD86 (B7-1 and B7-2) on activated APCs (Chambers and Allison, 1999). This requirement for additional signaling provided by innate immunity works as a safety mechanism ensuring that adaptive immune responses are directed against pathogens only and not against harmless, non-pathogenic substances. Very importantly, antigen binding to the TCR in the absence of such a CD28 signal is not simply insufficient for T lymphocyte activation. Instead, it will instruct the T cell to enter a stable state of unresponsiveness, referred to as anergy (Schwartz, 2003). Vice versa, also $CD4^+$ T cells express the costimulator CD40 ligand (CD40L, also known as CD154) after differentiating into effector cells. This molecule interacts with CD40 on APCs and together with the cytokines secreted by the T helper cell boosts the functions of MPs, DCs, and B cells in immune responses. Adaptive immune responses therefore often cooperate with the innate immune system employing its mechanisms to combat infections. To do so, the two arms of the immune system maintain constant bi-directional cross-talk (Abbas and Lichtman, 2009).

Successful TCR/CD28 stimulation activates the T cell and engages its intracellular signaling machinery. It involves molecules such as kinases, phosphatases, and small GTPases, leading to induction of the key transcription factors nuclear factor of activated T cells (NFAT), activator protein-1 (AP-1), and nuclear factor κB (NF-κB). The immediate-early genes subsequently expressed encode crucial cytokines promoting the expansion of antigen-specific T cell clones and their differentiation into a pool of effector and memory cells, ultimately resulting in the recruitment of other immune cells and leading to the development

of an effective immune response (Abbas and Lichtman, 2009; Baier and Wagner, 2009; Krammer and VanHook, 2011).

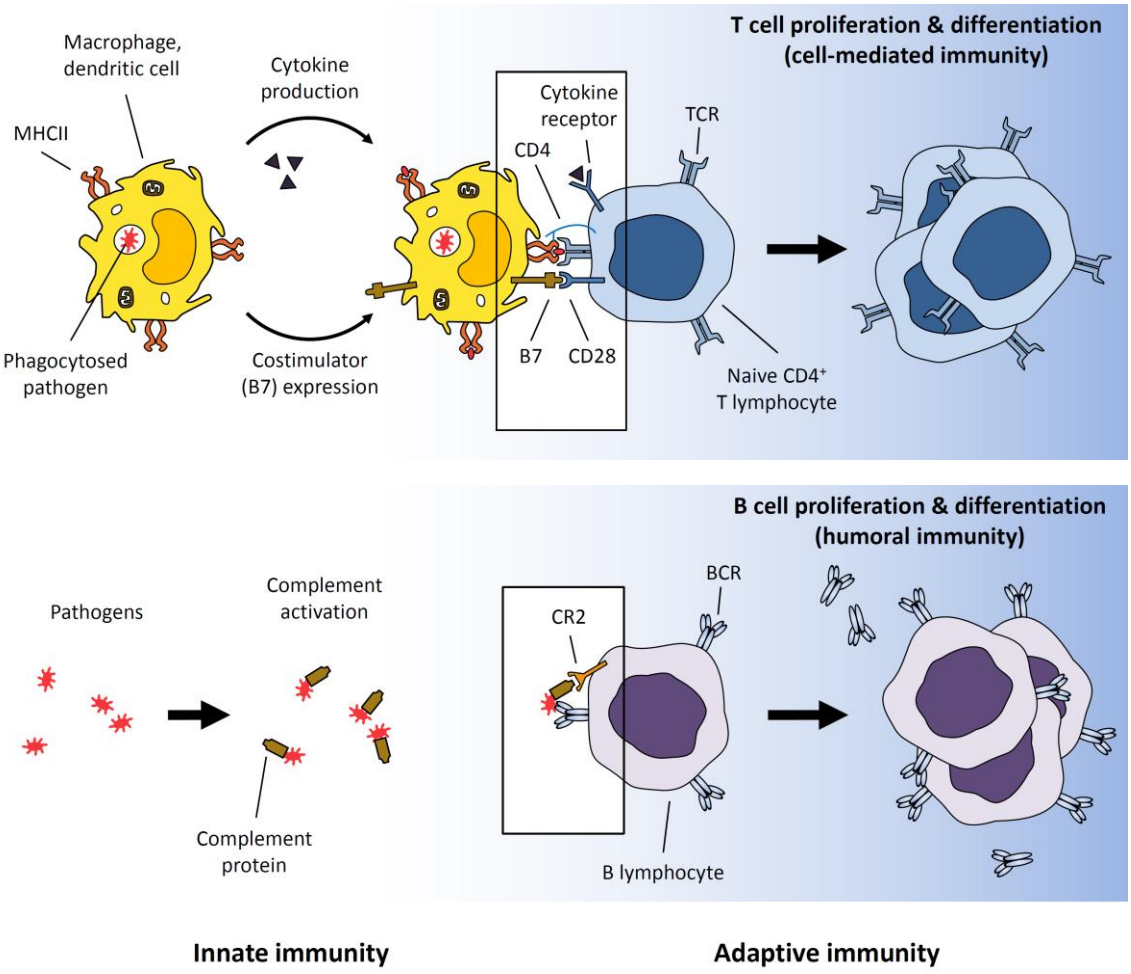

Figure 1: The Innate Immune System Stimulates Adaptive Immunity.

Top panel: Detection and uptake of pathogens activates phagocytes to produce cytokines, upregulate the expression of costimulatory molecules, and present MHCII-bound peptide fragments of antigens to naive $CD4^+$ T cells. In combination, these three signals induce T cell proliferation and differentiation.

Bottom panel: Invading pathogens trigger the complement system. B lymphocytes detect complement-tagged microbes with their antigen receptors and type 2 complement receptors (CR2), leading to B cell activation. Adapted from Kindt *et al.* and Abbas and Lichtman (Kindt *et al.*, 2007; Abbas and Lichtman, 2009)

In healthy individuals, adaptive immune responses are self-limited and will decline once an infection has been successfully cleared. Thereafter, subgroups of both antigen-specific B and T cells differentiate into so called memory cells, thus maintaining the ability of an organism to mount a tailored response against that particular pathogen. Those functionally inactive memory cells are long-lived. Reinfection with the same microbe will quickly reactivate them and lead to a stronger response with much faster and more efficient

elimination of the invader. The exact mechanisms of how immunologic memory is generated and maintained are so far not understood (Abbas and Lichtman, 2009).

1.1.3. Effector Cells of the Innate Immune System

Pathogens that manage to breach epithelia and evade detection by the complement system or enter an organism at any other site of the body will be confronted with a defense barrier of innate immune cells. Most significant amongst those first in line to attack invaders are phagocytic cells, such as monocytes/MPs, DCs, and neutrophils. Neutrophils, together with eosinophils and basophils also belong to the group of granulocytes or polymorphonuclear leukocytes. Other important innate effectors are NK cells and mast cells (Abbas and Lichtman, 2009; Murphy *et al.*, 2012).

As indicated by their name, phagocytes are specialized immune cells with the capacity to employ the essential innate defense mechanism of phagocytosis. Receptor-mediated detection of a microbe activates phagocytic cells to extend their plasma membrane around the pathogen, thereby internalizing the invader and encapsulating it into a phagosome. Fusion of this intracellular vesicle with lysosomes inside the phagocyte then leads to the formation of a phagolysosome. Triggered by the initial receptor binding, several enzymes contained in those compartments will produce a mixture of anti-microbial substances. Phagocyte NADPH oxidase converts molecular oxygen into superoxide anions (O_2^-), which spontaneously react with other molecules to generate free radicals, referred to as reactive oxygen intermediates (ROIs). Inducible nitric oxide synthase (iNOS) catalyzes the conversion of arginine to nitric oxide (NO). In addition to these reactions collectively termed the oxidative burst, lysosomal proteases break down microbial proteins into peptide fragments. Inside the phagolysosome, all of these substances are toxic to the ingested pathogen and contribute to an effective killing without damaging the phagocytic cell. Very strong immune responses to pathogens in the extracellular matrix, though, may trigger phagocytes to release these agents. The following inflammatory reaction is intended to protect the host but may also lead to tissue injury. Uptake and processing of soluble pathogen-derived molecules is mediated by a similar process, called pinocytosis (Abbas and Lichtman, 2009), whereas autophagy facilitates clearing and degradation of self-proteins and damaged organelles inside double-membraned vesicles known as autophagosomes (Lee, H.K. *et al.*, 2007).

Phagocytosis is a critical initial step in antigen capture. For the induction of an appropriate response, it needs to be followed by presentation of the pathogen-derived antigens to T lymphocytes (Figure 2). Since naive antigen-specific T cells are rare in the circulation, the APCs subsequently leave the site of infection to migrate and thereby transport the captured pathogens into regional lymph nodes that drain the infected organ or tissue. Pathogens or portions thereof that enter the body through lymphatic vessels or the blood stream are captured by APCs residing in lymph nodes or the spleen. Naive T cells recirculate through those peripheral lymphoid organs, whose anatomy and organization facilitates the concentration of pathogen-derived antigens, lymphocytes, and APCs. Inside those structures, the APCs process the ingested extracellular pathogens and present the resulting complexes of peptide fragments bound to MHCII molecules on their cell surface to naive $CD4^+$ T cells or to differentiated effector T cells. The peripheral lymphoid organs thereby provide an ideal environment for the initiation of an adaptive immune response. This mechanism is very efficient and a T cell response to antigens usually begins within 12 to 18 hours after a pathogen enters the body at any given site (Abbas and Lichtman, 2009).

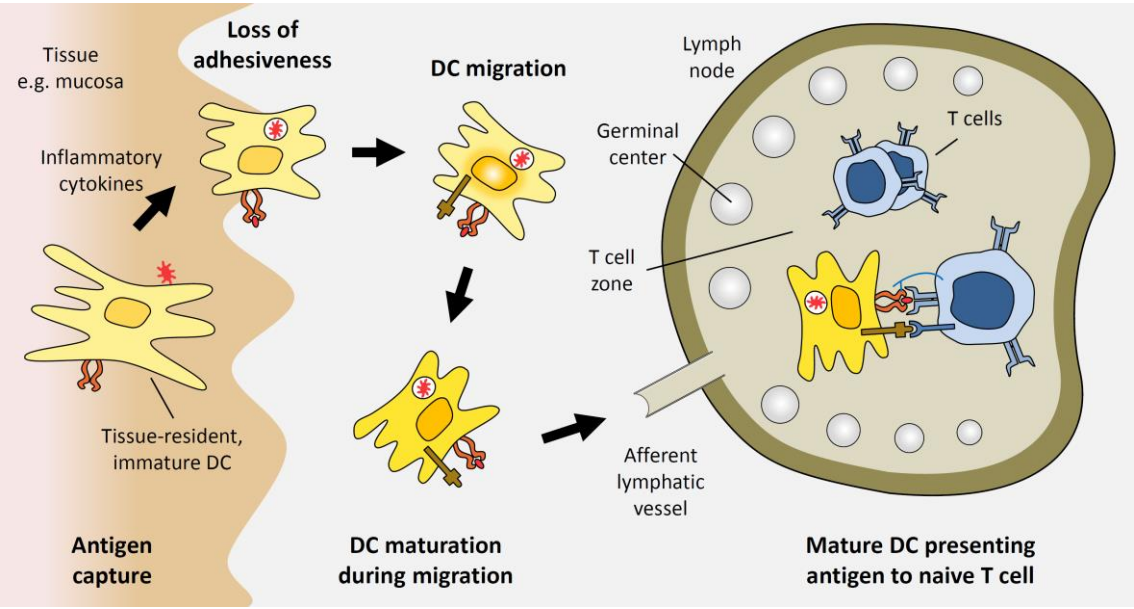

Figure 2: Antigen Capture and Display by DCs.

Immature DCs guard many types of tissues. Phagocytosis of invading pathogens induces phenotypical changes, causing the DC to exit the site of infection and to migrate into a draining lymph node. The process of migration, together with the effects of pathogen encounter, result in DC maturation. Inside the lymph node, DCs and other professional APCs present peptide antigens of captured pathogens to T cells for the engagement of adaptive immune responses. Adapted from Abbas and Lichtman (Abbas and Lichtman, 2009).

In fact, all nucleated cells express class I MHC molecules and may thus function as non-professional APCs by presenting antigens from pathogens in their cytoplasm to $CD8^+$ CTLs. Class II MHC molecules, in contrast, are primarily expressed by professional APCs. Both MHC I and MHC II mechanisms of presentation involve distinct pathways, organelles, and molecules that function to sample any protein detected in intracellular or extracellular compartments, respectively, thereby permitting the recognition of antigens retrieved from different environments by individual classes of T lymphocytes. Consequently, antigen capture and presentation are mainly performed by professional APCs. These cells form a group of specialized sentinels that patrol the immune system and most importantly comprise monocytes/MPs, DCs, and B cells. To be called professional, these APCs have to provide all costimulatory signals necessary for full activation of naive T cells to proliferate and differentiate, in addition to antigen presentation (Abbas and Lichtman, 2009).

DCs are a heterogeneous population of antigen detecting and presenting cells. Several different subtypes with unique functions and regulatory requirements have been described in both lymphoid and non-lymphoid tissues (Ginhoux *et al.*, 2009). Their name is derived from their morphology, as most DCs are characterized by long arm-like extensions, comparable to the dendrites of nerve cells. Immature DCs originate from bone marrow precursors and travel with the blood stream to sites of infection. Alternatively, located in epithelia they guard the main potential entry sites for pathogens ready to engulf any invaders (Figure 2). DCs recognize and capture pathogens early during an infection via their invariant PRRs. This leads to DC activation and the production of proinflammatory cytokines, particularly TNF and IL-1. The combined effects of cytokines and PRR signaling induce the DCs to undergo phenotypical and functional changes. The expression of pathogen receptors, along with that of surface molecules enabling the DCs to adhere to epithelia, is downregulated. Instead, receptors specific for chemokines produced in T cell-rich zones of lymph nodes are now predominantly expressed. These alterations in the composition of surface molecules cause activated DCs to exit the epithelia and to migrate via lymphatic vessels into draining lymph nodes. Pathogen encounter and the process of migration induce DCs to mature. From cells devised to capture antigens they turn into APCs with increased and more stable expression of MHC II molecules for the display of antigens, together with all costimulators necessary for the efficient induction of T cell responses (Abbas and Lichtman, 2009; Murphy *et al.*, 2012). They are the most important professional APCs as they control the initiation of primary T cell-dependent immune responses, thereby linking innate and adaptive immunity (Steinman,

2007). As highly potent immunostimulators, DCs not only initiate immune responses but also influence the type of T_H cells that will differentiate out of naive $CD4^+$ lymphocytes in response to a certain type of pathogen, thereby directing the adaptive response that will follow (Abbas and Lichtman, 2009; Murphy *et al.*, 2012).

Other important professional APCs are those that belong to the monocyte/MP population. Monocytes are phagocytic cells that circulate with the bloodstream. Several receptors enable them to recognize pathogens, which they ingest and destroy intracellularly. Monocytes become recruited to sites of infection. Hence, they have the ability to leave the circulation where necessary and to enter extravascular tissue. There, they differentiate into MPs and survive for long periods. Monocytes and MPs belong to the same cell lineage, often also referred to as the mononuclear phagocyte system (Abbas and Lichtman, 2009).

Resident MPs, in contrast, phagocytize pathogens that manage to transverse epithelial barriers and can be found in connective tissue as well as in every organ of the body. They are called microglia in the CNS, Kupffer cells in the liver, alveolar MPs in the lung, and osteoclasts in the bone. MPs are typically among the first cells of the immune system to encounter pathogens, which triggers their activation. They kill the ingested microbes and respond by producing a variety of chemical substances including TNF, IL-1, IL-12, chemokines, and other signaling molecules such as prostaglandin E_2 (PGE_2) (Krammer and VanHook, 2011). Together with chemokines produced by epithelial cells at the site of infection, these messenger molecules attract more MPs and neutrophils. Activated MPs also secrete growth factors and enzymes such as fibroblast growth factor (FGF), angiogenic factors, and metalloproteinases that contribute to the repair of infected and injured tissue. MPs are also called scavenger cells, as they clear the organism of decrepit cells or debris. Also MPs are able to present antigen-loaded MHC molecules together with coactivators for the stimulation of T cells. In contrast to DCs, MPs are responsible for maintaining immune responses that already have been initiated by inducing the effector phase of cell-mediated adaptive immunity. The effector T cells, in turn, activate the MPs to kill the ingested pathogens by producing the most important MP activating cytokine, IFN-γ (Abbas and Lichtman, 2009; Murphy *et al.*, 2012).

Neutrophils, next to monocytes/MPs, are the second essential group of phagocytes in the blood. They develop rapidly from bone marrow precursors in response to infections and represent the most abundant leukocyte species in the circulation. Neutrophils function very

similarly to monocytes in the detection, uptake, and destruction of pathogens. They are also recruited to sites of infection by chemotaxis, but in contrast to monocytes die a few hours after leaving the circulation and entering the extravasculature. Neutrophils are usually among the first cell types to respond to infections, in particular to those caused by bacteria or fungi (Abbas and Lichtman, 2009).

B lymphocytes are also professional APCs. They present ingested protein antigens to T_H cells, thereby critically influencing the development of humoral immune responses. B cell activation, in contrast to antigen recognition by T cells, is much less regulated and may be triggered by various types of pathogen-derived cell wall components or soluble antigens. It occurs in peripheral lymphoid organs, such as the spleen and lymph nodes, where B lymphocytes reside. Not much is known about the requirement or existence of an antigen processing and display system for the activation of B cell responses (Abbas and Lichtman, 2009).

NK cells are lymphocytes which, unlike B or T cells, do not express clonally distributed antigen receptors and therefore belong to innate immunity. A primary NK cell function is the detection and killing of tumor cells or of host cells that were infected and damaged by intracellular pathogens. Yet, healthy cells of the organism need to be spared. To this end, NK cells express both activating and inhibitory receptors. Alterations on the surface of stressed host cells trigger receptors that activate NK cells and induce them to discharge perforating and apoptosis-inducing agents from their cytoplasmic granules. This kills infected host cells and critically contributes to the eradication of intracellular reservoirs of infection, particularly those of obligate intracellular pathogens such as viruses. The second essential function of NK cells in response to the detection of pathogens is the secretion of IFN-γ, which leads to the activation of MPs. IL-12 secreted from activated MPs, in turn, further stimulates NK cell activity, highlighting how those cell types cooperate in fighting intracellular pathogens. Normal, autologous, and nucleated cells, in contrast, express class I MHC receptors loaded with self peptides. Those molecules on the surface of host cells interact with inhibitory NK cell sensors such as killer cell Ig-like receptors (KIRs) and receptors consisting of CD94 and the lectin subunit NKG2. Both detector classes signal via immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on their intracellular domains, resulting in shutting-off of NK cells. Viruses often block the expression of MHC I molecules in the cells they infect in order to evade recognition and killing of the host cell by virus-specific $CD8^+$ CTLs. In such cases, the

inhibitory NK cell receptors are not triggered and the NK cell destroys the infected cell (Abbas and Lichtman, 2009).

Mast cells are large granule-rich cells, mainly found in mucous membranes and connective tissue. Their activation leads to the release of bioactive molecules such as the vasoactive amine histamine. Mast cells contribute to the regulation of inflammatory reactions and play important roles in the context of allergies and anaphylaxis (Murphy *et al.*, 2012).

1.1.4. Pattern Recognition in Innate Immunity

Neutrophils, monocytes/MPs, and very importantly DCs are myeloid sentinel cells of the innate immune system that constantly scan their environment for the presence of microbes and other danger signals. These cells, as well as many non-professional immune cells, are equipped with PRRs for the specific detection of typical structures conserved among various microbial species, so called pathogen associated molecular patterns (PAMPs). Bacteria, viruses, or fungi all have their own distinct structures and pathogens of the same type share the same PAMPs. Examples for bacterial patterns are LPS, terminal mannose residues on glycoproteins, and DNA containing unmethylated CpG motifs. Viral particles are usually detected due to virus-specific modifications of the nucleic acids that encode them and β-glucans are important PAMPs in the cell walls of yeast and pathogenic fungi. None of these patterns are found in mammals and homologous mammalian molecules differ in their composition. Importantly, PAMPs are structures critically required for the functionality and survival of the microbes that carry them. Pathogens, therefore, cannot simply evade innate immune recognition by alteration of these molecules or by expressing them in a non-functional form since this would render them unable to persist or to infect and colonize their host. Beyond that, most PRRs also detect endogenous molecules, collectively termed danger associated molecular patterns (DAMPs), which are sent out by injured, damaged or stressed cells (Abbas and Lichtman, 2009; Takeuchi and Akira, 2010; Murphy *et al.*, 2012).

Innate PRRs are, in contrast to antigen receptors of lymphocytes, not generated by somatic gene recombination events. PRRs are instead germline-encoded and non-clonally distributed, meaning that identical receptors are expressed on all cells of a particular population (*e.g.* all MPs express the same types of receptors). PRRs probably recognize less than a thousand different pathogen-associated structures and repeated exposure to the same PAMP does not enhance or accelerate the subsequently triggered immune responses (Abbas

and Lichtman, 2009). Four different subgroups of PRRs are known. Most prominent and best studied is the family of Toll-like receptors (TLRs) that recognize mainly bacterial structures. The recently discovered and ever more emerging family of C-type lectin receptors (CLRs) constitutes the second group of transmembrane detectors. Cytoplasmic PRRs include retinoic acid-inducible gene (RIG)-I-like helicases, responsible for the detection of viral RNA and nucleotide-oligomerization domain (Nod)-like receptors (NLRs) that chiefly engage the inflammasomes. PRR signaling induces activation and nuclear translocation of the transcription factors NF-κB, AP-1, IFN-regulatory factors (IRFs), and CCAAT/enhancer binding protein β (C/EBPβ). They respond to receptor ligation and concurrently regulate the transcription of their target genes. Different PRRs trigger individual sets of target genes and overactivation of this machinery may cause immunodeficiency, septic shock, or induce autoimmunity (Takeuchi and Akira, 2010).

Toll-like receptors are homologous to the *Drosophila* protein Toll, which is essential for host defense in these flies. There are 10 different family members in humans and 12 in mice that respond to pathogens outside of the cell or within intracellular endosomes and lysosomes. Moreover, TLRs also detect several self-components. The receptors typically assemble from amino (N)-terminal leucine-rich repeats (LRRs), a transmembrane region, and a cytoplasmic Toll/IL-1 receptor (IL-1R) homology (TIR) domain (Abbas and Lichtman, 2009; Takeuchi and Akira, 2010). TLR2 critically requires heterodimerization with TLR1 or TLR6 for ligand detection and recognizes lipoglycans from bacteria and mycoplasma, in addition to various fungal and viral components. TLR4 chiefly recognizes LPS, together with myeloid differentiation factor 2 (MD2) on the cell surface but on its own may also be activated by viral envelope proteins. TLR5 binds to flagellin of bacterial flagella. The group of receptors, comprising TLR1, TLR2, and TLR4 through TLR6, is present on the cell surface while TLR3, TLR7, and TLR9 are mainly expressed in association with the endoplasmic reticulum (ER) membrane. These intracellular TLRs detect nucleic acids from viruses and bacteria, in addition to endogenous nucleic acids in pathogenic contexts. TLR3 senses viral double-stranded (ds)RNA in the endolysosome and recognizes the synthetic dsRNA analog polyinosinic polycytidylic acid (poly(I:C)). TLR9 detects unmethylated DNA with CpG sequences from bacteria and viruses as well as malaria parasite components (Parroche *et al.*, 2007; Abbas and Lichtman, 2009; Takeuchi and Akira, 2010).

TLRs signal mainly via two different pathways. The decision on which cascade will be activated depends on the recruitment of certain pairs of adaptor molecules. Those signaling

mediators from the family of TIR domain-containing adaptors most importantly include myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor inducing IFN-β (TRIF), TIR domain-containing adaptor protein (TIRAP)/MyD88 adaptor-like (Mal), and TRIF-related adaptor molecule (TRAM). Particularly TIRAP/Mal and TRAM have sorting functions and direct TLRs to localize to specific regions of the cell in order to engage signal transduction. Once sorted, downstream signaling is facilitated either via the MyD88 or the TRIF-dependent cascade (Takeuchi and Akira, 2010). All TLRs except TLR3 depend on the MyD88 pathway, which further involves members of the IL-1R-associated kinase (IRAK) and TNF receptor (TNFR)-associated factor (TRAF) families, in addition to a complex that assembles from TGF-β-activated kinase (TAK)1 and TAK1-binding protein (TAB)1, together with TAB2/3. Subsequently, the transcription factors AP-1 and NF-κB are activated to translocate into the nucleus where they instruct the production of proinflammatory cytokines. After binding to their ligands, the two ER-associated receptors TLR7 and TLR9 additionally require translocation to the endolysosome as well as endosomal maturation in the form of protease and endopeptidase-mediated processing. Thereafter, they signal in a MyD88, IRAK, and TRAF-dependent manner to trigger NF-κB directed cytokine expression and IRF7-induced production of type I IFNs. TLR3 signaling is restricted to TRAM and the TRIF-regulated pathway but also LPS ligation to TLR4 has been found to activate this cascade. Downstream signaling further involves TRAF family members in combination with receptor-interacting protein (RIP)1, TANK-binding kinase (TBK)1, and similar to NAK-associated protein (NAP)1/TBK1 adaptor (SINTBAD). These events culminate in the dimerization of IRF3 with IRF7 to induce the expression of proinflammatory cytokines and type I IFNs (Takeuchi and Akira, 2010).

The family of RIG-I-like receptors (RLRs) comprises RIG-I, melanoma differentiation-associated gene 5 (Mda5), and laboratory of genetics and physiology 2 (LGP2) (Yoneyama and Fujita, 2008; Takeuchi and Akira, 2010). RIG-I and Mda5 contain two amino (N)-terminal caspase-associated recruitment domains (CARDs), a DEAD box helicase/ATPase domain in the central region, and a regulatory domain at their C-terminus, which mediates ligand binding. Located in the cytoplasm, they recognize dsRNA from various RNA viruses and trigger type I IFN production in response to those pathogens, which again upregulates RLR expression in a positive feedback manner. Mda5 is responsible for the detection of long dsRNAs (more than 2 kb), while RIG-I binds to short dsRNAs with 5’ triphosphate ends.

LGP2, in contrast, contains no CARD and mainly functions as a positive regulator upstream of RIG-I and Mda5 (Takeuchi and Akira, 2010).

RLR signaling is influenced by poly-ubiquitination of RIG-I. Its modification by the tripartite motif-containing 25 (TRIM25) E3 ubiquitin ligase leads to ubiquitin attachment at lysine (K)63 and induces the receptor. Ubiquitination on K48 by RING finger protein 125 (RNF125), in contrast, negatively influences RIG-I activity. Both RIG-I and Mda5 employ their CARDs to interact with IFN-β-promoter stimulator (IPS)-1 which, in turn, induces signaling via the phosphatase Eyes absent 4 (EYA4), in addition to TRAF3, SINTBAD, and TBK1, leading to IRF3/IRF7 dimerization and type I IFN expression. IPS-1 in parallel also activates NF-κB regulated cytokine production in a TNFR-associated death domain protein (TRADD), FAS-associated death domain-containing protein (FADD), and caspase-8/10-dependent manner (Takeuchi and Akira, 2010).

Sensing of viral, bacterial, or endogenous (ds)DNA in the cytoplasm leads to inflammasome activation depending on high-mobility group box 1 (HMGB1) protein and absent-in-melanoma 2 (AIM2) and causing the production of IL-1β (Schroder and Tschopp, 2010; Takeuchi and Akira, 2010). All members of the NLR family of cytoplasmic receptors contain carboxy (C)-terminal LRRs in combination with a central nucleotide-binding domain. Moreover, protein-binding motifs such as CARDs, pyrin domains, and baculovirus inhibitor of apoptosis protein repeat (BIR) domains in the N-terminal regions have been reported for most of these receptors. Pyrin or BIR domain-containing NLRs are inflammasome components that contribute to caspase-1 activation and do not promote the expression of proinflammatory mediators through regulation of gene transcription. The receptors Nod1 and Nod2, in contrast, detect structures of bacterial peptidoglycans. They contain CARDs, Nod, and LRR-domains and engage the adaptor RIP2/RICK for activation of NF-κB and the transcriptional upregulation of proinflammatory cytokine genes. As such, these NLRs collaborate with TLRs and synergistically mediate inflammatory responses (Takeuchi and Akira, 2010). The family of CLRs will be discussed in detail in section 1.3.

Sensing of pathogens by PRRs triggers a complex network of cellular mechanisms to induce pleiotropic outcomes and involves crosstalk among the various PRRs (Takeuchi and Akira, 2010). Central to the function of members from all of these receptor families is their ability to signal via the canonical NF-κB pathway to induce inflammatory responses and innate immunity.

1.2. The Nuclear Factor κB Pathway

Innate as well as adaptive immune responses critically depend on regulation by the essential and central NF-κB signaling cascade (Baltimore, 2011). Its key element, NF-κB, was originally described as a eukaryotic transcription factor which specifically interacts with a defined DNA sequence in the enhancer element of the Ig κ-light chain gene. Those initial studies characterized NF-κB to be active exclusively in mature B cells and plasma cells but not in early pre-B cells or T cells. Hence, the name NF-κB was chosen to highlight its properties (Sen and Baltimore, 1986a; Sen, 2011). It soon became evident though, that NF-κB activity is not solely limited to B lymphocytes. Rather, this ancient and evolutionarily conserved molecule plays a dominant role in regulating inducible gene transcription in almost every mammalian cell type examined so far (Sen and Baltimore, 1986b; Sen, 2011).

1.2.1. NF-κB Engagement

NF-κB activity is involved in many important biological functions. A wide variety of external, internal, and environmental stimuli can induce NF-κB activation (Sen, 2011; Smale, 2011). The proinflammatory cytokines IL-1 and TNF were the first physiological inducers of NF-κB activity identified (Oeckinghaus *et al.*, 2011). It is now known that many bacterial PAMPs, like LPS or exotoxin B, next to numerous viral particles (HIV-1, HTLV-1, HBV, EBV, Herpes simplex) induce NF-κB signaling in DCs and MPs, as well as in B and T cells. Also DNA-damaging chemicals including ROIs or radiation (UV- or γ-irradiation), in addition to pro-apoptotic and necrotic stimuli trigger NF-κB signaling (Li, Q. and Verma, 2002; Oeckinghaus and Ghosh, 2009). Yet, the activation of NF-κB response genes is not only limited to factors which promote inflammation and apoptosis. Glutamate, for example, activates NF-κB in nerve cells, which triggers neuron survival and memory formation (Schölzke *et al.*, 2003).

Ligation of the different stimuli to their specific receptors on the cell surface or within the cell triggers signaling events tailored to both the agonist as well as to the responding cell type. The diversity of possible activators and signaling outcomes implicates the requirement for a multitude of individual pathways to be induced, all of which ultimately signal to NF-κB. The exact mechanisms leading to their activation are not completely understood. It is generally agreed upon, though, that not one of them is completely identical. For sure, they all require the assembly of multiprotein signaling complexes, the formation and attachment of

poly-ubiquitin chains, and the phosphorylating activity of specific kinases (Oeckinghaus *et al.*, 2011).

Consequently, NF-κB is known as a central orchestrator of inflammation and immune responses. Microbial invasion results in NF-κB activity in the nucleus, where it regulates the expression of many cytokines and acute-phase defense genes, but also allows for the production of several other inflammatory mediators including iNOS and cyclooxygenase 2 (COX-2) (Li, Q. and Verma, 2002). Even in the absence of danger, NF-κB plays a critical role in immune cell homeostasis by maintaining the expression of pro-survival genes and is further essential for cell differentiation, tissue development, and wound repair, as its activation triggers the production of growth factors, and other effector enzymes in response to stress (Ghosh *et al.*, 1998; Li, Q. and Verma, 2002; Bonizzi and Karin, 2004; Mémet, 2006).

1.2.2. Rel and IκB Protein Families

NF-κB is not one single protein. Rather, the abbreviation describes an entire group of structurally similar transcription factors. In mammals, the five members – p65 (RelA), c-Rel (Rel), RelB, p100/p52 (NF-κB2), and p105/p50 (NF-κB1) – have been identified so far (Li, Q. and Verma, 2002). These molecules are collectively called the NF-κB- or reticuloendotheliosis oncogene (Rel)-family of proteins (Figure 3). The latter name refers to a conserved N-terminal 300 amino acid sequence, known as the Rel homology domain (RHD) that all members have in common. p50 and p65 were the first two members of this group to be identified. Subsequently, the terms Rel-family of proteins and RHD were coined, referring to the high structural similarity between the N-termini of those molecules and the retroviral oncoprotein v-Rel, its cellular homologue c-Rel, as well as the *Drosophila* protein Dorsal (Steward, 1987; Ghosh *et al.*, 1990; Kieran *et al.*, 1990).

The RHD contains a region required for dimerization, a nuclear localization signal (NLS) near its C-terminal end, and a DNA binding domain within its N-terminus. Rel-family members use their RHD to assemble by forming homo- or heterodimers, which turns them into the actual NF-κB transcription factor molecules. This dimerization allows the active NF-κB/Rel proteins to translocate into the nucleus and induce gene expression by interacting with DNA. Rel proteins form various pairwise combinations. The p50-p65 heterodimer was the first to be identified and is commonly referred to as NF-κB (Baeuerle and Henkel, 1994; Ghosh *et al.*, 1998). With the exception of RelB, all Rel proteins contain a protein kinase A

(PKA) phosphorylation site that is located approximately 25 amino acids upstream of the NLS. PKA activity is not involved in NF-κB nuclear translocation or binding to DNA but has been shown to potently increase its subsequent *trans*-activation (May and Ghosh, 1997; Zhong *et al.*, 1997; Ghosh *et al.*, 1998).

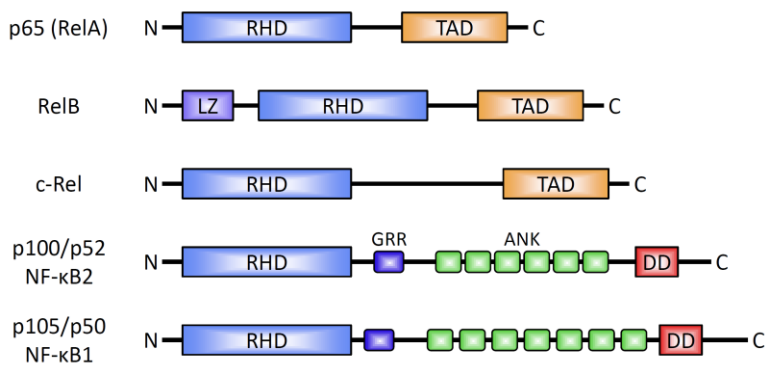

Figure 3: The NF-κB/Rel Family of Proteins.

All NF-κB proteins contain a Rel homology domain (RHD). NF-κB1 (p105/p50) and NF-κB2 (p100/p52) may, alternatively, be grouped into the IκB family of proteins. ANK, ankyrin repeats; DD, death domain; GRR, glycine-rich region; LZ, leucine-zipper; TAD, transcriptional activation domain. Adapted from Oeckinghaus and Gosh (Oeckinghaus and Ghosh, 2009)

Secondary to their similarities, proteins from the Rel-family further show significant differences that divide them into two subgroups. RelA, c-Rel, and RelB contain so called transcriptional activation domains (TADs), also referred to as transactivation domains, within their C-termini. TADs are necessary for the activation of target gene expression and consist of several serine residues combined with other mainly acidic and hydrophobic amino acids. Consequently, homo- and heterodimers of TAD containing Rel proteins, as well as their heterodimers formed with either p50 or p52, activate target gene transcription (Hayden and Ghosh, 2004; Oeckinghaus and Ghosh, 2009). RelA, c-Rel, and RelB proteins mainly consist of an RHD and a TAD. p50 and p52, in contrast, do not contain TADs (Figure 3). They form homo- and heterodimers with each other and compete with activating NF-κB dimers for DNA binding, thereby usually causing the repression of transcription (Baeuerle and Henkel, 1994; Zhong *et al.*, 2002). Beyond that, p50 and p52 differ from the other NF-κB subunits, since they are synthesized as larger precursors, p105 and p100, respectively, which belong to the inhibitor of κB (IκB) family of proteins (Figure 4).

NF-κB is not produced *de novo* in response to engagement of upstream pathway components. Instead, preformed NF-κB dimer complexes are always present, even in the cytosol of unstimulated cells. Silencing of NF-κB transcriptional activity until encounter with a proper stimulus is, therefore, essential and the reason why NF-κB is called a latent transcription factor. In most cell types, NF-κB dimers are sequestered in the cytoplasm through non-covalent interactions with inhibitory IκB proteins. These form a group of labile repressors which govern the DNA-binding activity of NF-κB (Baeuerle and Baltimore, 1988). To date, seven members of this family have been identified (Figure 4). Those include IκBα, IκBβ, B cell lymphoma (Bcl)3, IκBε, and IκBζ, in addition to the precursor proteins p105 and p100. All IκB proteins contain multiple copies of a 30 to 33 amino acid sequence known as the ankyrin repeat (ANK) module. These ANKs interact with a region in the RHD of NF-κB proteins, thereby masking their NLS and preventing translocation of NF-κB dimers into the nucleus. In p105 and p100, the ANKs are found on the C-terminal end, separated from the N-terminal RHD by a glycine-rich region (GRR). This GRR serves as a termination signal for the proteasome, which processes the precursors p100 and p105 upon activation to release p52 and p50, respectively (Oeckinghaus and Ghosh, 2009).

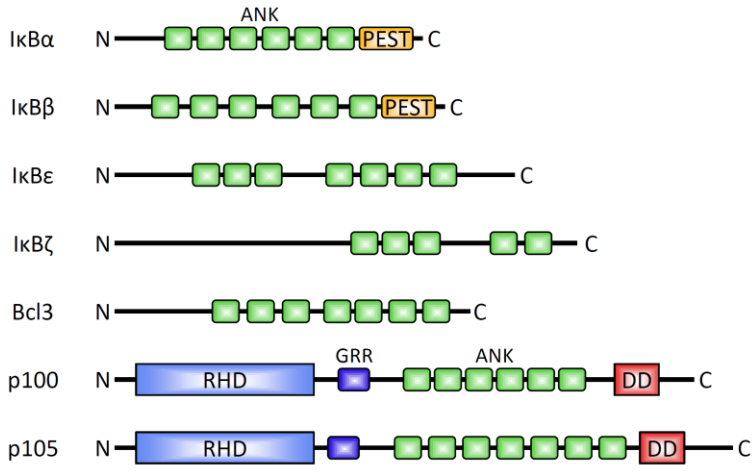

Figure 4: The IκB Family of Proteins.

Ankyrin repeats (ANK) are a typical feature of IκB proteins. The precursor proteins p100 and p105 further contain a Rel homology domain (RHD), which is characteristic for NF-κB/Rel family members. DD, death domain; GRR, glycine-rich region; PEST, proline-, glutamic acid-, serine-, and threonine-rich region. Adapted from Oeckinghaus and Gosh (Oeckinghaus and Ghosh, 2009)

1.2.3. Signal Transduction

Two different signaling pathways lead to the engagement of NF-κB. They are referred to as the canonical (or classical) and the non-canonical (or alternative) mechanisms of NF-κB activation (Chen, Z.J. *et al.*, 1996; Mercurio *et al.*, 1997; Woronicz *et al.*, 1997; Zandi *et al.*, 1997; Ghosh *et al.*, 1998; Senftleben *et al.*, 2001; Scheidereit, 2006). The common regulatory step in both signaling cascades is the activation of an IκB kinase (IKK) complex. This multiprotein structure consists either of IKKα-IKKβ heterodimers or of homodimeric IKKα catalytic kinase subunits, but in both cases includes the regulatory scaffold protein NF-κB essential modulator (NEMO), also referred to as IKKγ (Rothwarf *et al.*, 1998; Yamaoka *et al.*, 1998; Mercurio *et al.*, 1999). The two kinases, IKKα and IKKβ, carry out rapid phosphorylation of IκB proteins, leading to the release of active NF-κB dimers (DiDonato *et al.*, 1997; Mercurio *et al.*, 1997; Régnier *et al.*, 1997; Woronicz *et al.*, 1997; Zandi *et al.*, 1997). IKKα and IKKβ share significant sequence homology and have important structural domains in common including leucine-zippers which allow them to dimerize. Both IKKs play crucial but distinct roles in early development. IKKα was shown to be essential for the differentiation of skin epidermal cells (keratinocytes) as well as for general skeletal development, and cannot be replaced by IKKβ for these purposes. The cytokines IL-1 and TNF, on the other hand, activate NF-κB solely via IKKβ without the need for IKKα (Hu *et al.*, 1999; Takeda *et al.*, 1999) and IKKα cannot compensate for the loss of IKKβ in cytokine-induced NF-κB activation (Li, Q. *et al.*, 1999). IKKβ is critical for the transduction of NF-κB-mediated pro-survival signals in order to prevent cells, in particular hepatocytes, from undergoing TNF-induced apoptosis. Mice lacking IKKα die shortly after birth (Hu *et al.*, 1999; Takeda *et al.*, 1999), whereas IKKβ-deficient animals are not viable (Li, Q. *et al.*, 1999). NEMO preferentially associates with IKKβ, does not contain a catalytic kinase domain, and is critically required for the activation of IKKα-IKKβ heterodimers in response to proinflammatory cytokines (Rothwarf *et al.*, 1998; Yamaoka *et al.*, 1998). Another protein called IKAP has been shown to associate with both IKKs and may function as a scaffold for the formation of functional IKK complexes (Cohen *et al.*, 1998).

Various physiological stimuli such as proinflammatory cytokines, pathogen-derived peptides presented to antigen receptors, and PAMPs triggering PRRs, engage the canonical NF-κB cascade (Oeckinghaus and Ghosh, 2009). The individual receptors, in turn, employ multiple signaling adaptors upon ligation, thereby channeling activity to the IKK complex. Very important in this context is the family of TRAF adaptor molecules that have been

described to function downstream of various receptors. TNF receptor signaling in addition specifically involves TRADD and FADD adaptors which, together with TRAF2 and the kinase RIP1, recruit and activate TAK1. Other important adaptors belong to the mitogen-activated protein (MAP) kinase (MAPK)/extracellular signal-regulated kinase (Erk) kinase kinases (MEKKs), the IRAK, and the protein kinase C (PKC) families (Lee, F.S. *et al.*, 1997; Lee, F.S. *et al.*, 1998; Nakano *et al.*, 1998; Oeckinghaus *et al.*, 2011). This large variety of potential interaction and activation partners reflects the fact that many different signaling pathways converge at the IKK complex. The engagement of any of these adaptor proteins leads to the phosphorylation and induction of IKKβ within the IKK complex (Li, Q. and Verma, 2002; Guo *et al.*, 2004). Being a kinase itself, IKKβ subsequently phosphorylates the p50-p65-associated IκB proteins at specific serine (Ser) residues (Ser32 and Ser36 in IκBα, and Ser19 and Ser23 in IκBβ) within their N-terminal signal responsive regions (SRRs). This chain of events is referred to as the post-translational mechanism of NF-κB induction. It results in rapid IκB dissociation, releasing active NF-κB dimers from their inhibited state, which then translocate into the nucleus (Sen and Baltimore, 1986b; Smale, 2011). The phosphorylated IκBs, in turn, interact with β-TrCP which employs an ubiquitin-ligase complex to mediate IκB poly-ubiquitination at K48 and the subsequent degradation of IκB by the 26S proteasome (Maniatis, 1999; Baltimore, 2011). Poly-ubiquitination is not only essential for NF-κB signaling but has been described as an important regulatory mechanism for many different pathways (Emmerich *et al.*, 2011). Activation of the canonical NF-κB pathway is a rapid and transient process that depends exclusively on IκBα degradation (Vallabhapurapu and Karin, 2009; Shih *et al.*, 2011). In most cases, signaling via this cascade activates NF-κB dimers that consist of p65-p50 subunits, although combinations involving RelB or c-Rel are also possible. Once inside the nucleus, the active dimers induce κB site regulated target gene expression (Figure 5).

In contrast to classical NF-κB signaling, the non-canonical or alternative pathway is activated only by a small number of stimuli (Figure 5). Those include specific cytokines of the TNF family such as CD40L, the B cell activating factor (BAFF), and lymphotoxin-β (LT-β). The alternative NF-κB cascade is associated primarily with signaling in the context of lymphoid organ development and the generation of B and T cells. TRAF2 and TRAF3 are essential receptor proximal adaptor molecules in non-canonical NF-κB signaling (Oeckinghaus and Ghosh, 2009). TRAF2 facilitates K63-linked ubiquitination of cIAP1 and cIAP2, leading to TRAF3 ubiquitination at K48 and its subsequent proteasomal degradation.

This releases and activates NF-κB-inducing kinase (NIK), enabling the enzyme to accumulate in the cytosol as a consequence of both protein stabilization and *de novo* synthesis (Vallabhapurapu *et al.*, 2008; Zarnegar *et al.*, 2008). In unstimulated cells, this mechanism ensures a low level of NIK, thereby preventing faulty activation due to receptor-unrelated signals and causing the kinetics of response to be relatively slow (hours) (Shih *et al.*, 2011). The non-canonical IKK complex typically consists of two IKKα molecules and one NEMO subunit. NIK phosphorylates and activates IKKα which, in turn, phosphorylates the IκB domain of p100 molecules, leading to K48 ubiquitination of p100 and its partial proteolysis to p52 (Ling *et al.*, 1998; Giardino Torchia *et al.*, 2013). The resulting p52/RelB heterodimers then translocate into the nucleus, where they activate the transcription of κB site-dependent target genes. The NF-κB subunit p105, in contrast, is constitutively cleaved to yield p50 and thereby clearly discriminated from p100. Whether inducible processing of p105 also plays a role in NF-κB signaling awaits further clarification (Xiao *et al.*, 2001; Moynagh, 2005).

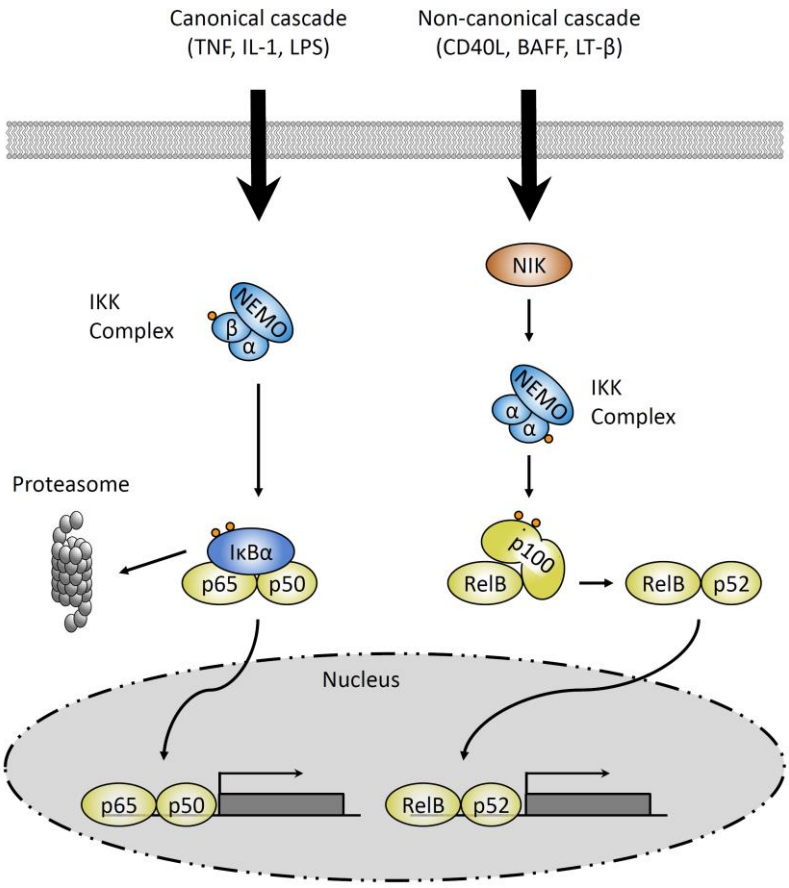

Figure 5: Canonical and Non-Canonical NF-κB Signaling.

Several ligands such as TNF, IL-1, or LPS activate the canonical NF-κB pathway. Various adaptor molecules couple receptor engagement to activation of the IKK complex. IKKβ then phosphorylates serine residues in canonical IκBs, leading to the ubiquitination of IκB for proteasomal degradation and the release of NF-κB

dimers which translocate into the nucleus where they target specific κB sites. Stimulation of the non-canonical cascade causes NIK-mediated phosphorylation of IKKα. IKKα, in turn, phosphorylates p100 thereby inducing proteasomal degradation of p100 to p52 and releasing RelB-p52 heterodimers for the regulation of gene transcription. Adapted from Oeckinghaus and Gosh (Oeckinghaus and Ghosh, 2009).

The following sections will focus mainly on the mechanisms activated as a consequence of canonical NF-κB signaling.

1.2.4. Regulation of Target Gene Transcription

As mentioned above, signals from a plethora of receptors, channeled via many different routes will eventually result in the activation of NF-κB. In order to understand how such a multitude of pathways can converge and engage one single transcription factor, it is crucial to determine the molecular mechanisms that regulate its induction (May and Ghosh, 1999; Baltimore, 2011). Active NF-κB dimers in the nucleus recognize specific binding sequences that are collectively termed the family of κB sites (Leung et al., 2004; Baltimore, 2011). Those DNA segments, found within the promoters and enhancer regions of NF-κB-inducible genes, consist of nine or ten conserved nucleotides. Their consensus was originally described as the sequence g ggg ACT TTC C but it soon became clear that there is a certain degree of variation (Sen and Baltimore, 1986a). Meanwhile, the general consensus sequence ggg RNN YYC C, where 'R' is any purine, 'Y' is any pyrimidine, and 'N' is any nucleotide has been agreed on (Hayden and Ghosh, 2004). Moreover, the particular architecture of each site was shown to be important for the individual gene and, in at least one case, to dictate which coactivators are used by NF-κB (Leung *et al.*, 2004).

Until now, it has not been exactly defined how one particular stimulus can trigger the expression of a unique group of NF-κB-induced genes, whereas other stimuli activate different κB-site regulated subsets. It is generally accepted that the specificity of NF-κB is not simply a matter of dimer translocation into the nucleus and binding to DNA but most likely a question of context and induction. NF-κB transcriptional activity is known to involve transcriptional coactivators that can influence chromatin structure and other events required for transcriptional activation. In this regard, the accessibility of a particular gene is certainly an important aspect to consider (Baltimore, 2011; Smale, 2011). Prior to receiving an inductive stimulus, NF-κB responsive genes are in a latent state and show little or no expression. There are two types of NF-κB inducible genes; those that contain unmethylated CpG islands and potentially only need to bind NF-κB to be activated and others that are

blocked by nucleosomes and therefore require chromatin remodeling processes, in the form of histone modifications, before they can respond to NF-κB binding (Baltimore, 2011; Smale, 2011). Other studies examining the regulatory regions of NF-κB target genes and the transcription factors that associate with these motifs yielded an array of *cis*-recognition sites (Stein and Baldwin, 1993; Stein *et al.*, 1993; Apostolou and Thanos, 2008; Oeckinghaus *et al.*, 2011). Last but not least, secondary covalent modifications of NF-κB itself need to be considered since they appear to be of major significance in this context (Baltimore, 2011).

Most pathogens activate multiple signal-transduction mechanisms which usually lead to the engagement of several transcription factors, in addition to NF-κB (Oeckinghaus *et al.*, 2011). The degree of cooperation between these various regulators of target-gene expression is therefore another important aspect in the context of this chapter. After binding to a consensus site, Rel/NF-κB proteins have been shown to interact with other DNA-associated molecules including TBP, TFIIB, CBP/p300 and the general transcriptional apparatus. NF-κB further collaborates with transcription factors such as c-Jun or Sp1 in order to mediate efficient transcriptional activation. At the gene locus encoding IFN-β *(Ifnb1)*, a multi-molecular enhanceosome consisting of NF-κB together with the transcription factors IRF3, IRF7, and AP-1 is cooperatively formed to promote *Ifnb1* transcription (Ford and Thanos, 2010). In T cells, NF-κB and AP-1 activate the IL-2 gene by binding to the CD28 response element (CD28RE) of the corresponding gene promoter in a combinatorial manner (Hayashi and Altman, 2007). The results from these promoter studies suggest that a distinct combination of transcription factors, bound to specific promoter binding sites, is pivotal, leading to the conclusion that the constellation of factors other than NF-κB, induced in a particular cell at the time and for the duration of the signal, critically contributes to the selective regulation of gene expression (Baltimore, 2011; Oeckinghaus *et al.*, 2011; Smale, 2011). In spite of the multitude of mechanisms necessary for engaging NF-κB dependent gene expression, there is always some stochastic activation of NF-κB, even in the absence of a particular inducer. This often reported low transcriptional activity in the latent state is called the ‘basal level’ (Baltimore, 2011).

NF-κB regulates the expression of many different genes. These encode essential factors and mediators involved in various aspects of immune responses and include the major pro-inflammatory cytokines TNF, IL-6, IL-1α, and IL-1β. NF-κB further influences the expression of important leukocyte adhesion molecules like E-selectin, VCAM-1, and ICAM-1, as well as that of transporter of antigenic peptides (TAP)1 (involved in antigen

presentation to B and T cells) and the MHC molecules. Also, the response to and the induction of IL-2 secretion depend on NF-κB regulation. Beyond that, factors that control growth and/or transcription such as Ras, c-Myc, and p53 are induced by NF-κB, making it crucial for many aspects of cell growth, differentiation, and proliferation (May and Ghosh, 1997; Ghosh *et al.*, 1998). Importantly, NF-κB also activates genes which encode many of the subunits that assemble to build the individual NF-κB dimers. Hence, NF-κB becomes a complicated mixture of various subunits upon induction. Some of these dimers keep their activating potential even past the first wave of NF-κB production, especially those consisting of c-Rel-p50 and p65-p65 (Hoffmann *et al.*, 2002; Baltimore, 2011).

All genes encoding NF-κB subunits are being expressed, even in the absence of stimulation, during the latent phase. This effect causes the presence of abundant amounts of p65 and p50 in the cell. The molecules are bound by IκB inhibitory proteins and form the so called latent pool of p65-p50 dimers. p50 homodimers (p50-p50), in contrast, do not interact with IκB and accumulate mainly in the nucleus. Together with other regulators that keep genes silent, such as Bcl3, these p50-p50 dimers are thought to be important mediators of the off state. Furthermore, substantial amounts of c-Rel and RelB can be detected in unstimulated cells. Yet, their presence is less abundant than in stimulated cells, since the genes encoding these subunits depend on NF-κB for their expression. The p100 precursor of p52 is also present in cells prior to their activation (Ghosh *et al.*, 1998; Baltimore, 2011).

1.2.5. Resolving NF-κB Activity

Inflammation is a powerful weapon of the immune system when it comes to fighting intruders, but also known to be a double edged sword. An organisms turns to it only when its integrity is seriously challenged (Medzhitov, 2008). In particular, prolonged expression of inflammatory mediators can cause severe tissue damage. Therefore, termination of the inflammatory response is critical and needs to be tightly regulated. Shutting down NF-κB activity involves many different mechanisms since all processes that were activated during an inflammatory response now need to be switched off. After killing invading pathogens and the initiation of wound healing, the abundance of inflammatory cytokines will and has to decrease. Regulatory enzymes remove the secondary modifications that activate NF-κB proteins (Ruland, 2011). Moreover, many NF-κB target genes encode inhibitors of the signaling pathways through which they were induced, allowing the inflamed tissues to reset to normal function once danger has passed. The gene encoding IκBα is of special importance in

this context. It is regulated by a κB site and so engagement of the canonical pathway promotes rapid *de novo* synthesis of the inhibitor. Newly produced IκBα immediately travels into the nucleus, where it removes active p65-p50 dimers from the DNA and sequesters them into a complex. This terminates NF-κB transcriptional activity, rendering it a transient event unless the activation signal persists. The NF-κB subunits bound to IκBα subsequently return to the cytoplasm. Freshly activated NF-κB dimers in the cytoplasm, ready to enter the nucleus, will also be sequestered. (Le Bail *et al.*, 1993; Sun, S.C. *et al.*, 1993; Arenzana-Seisdedos *et al.*, 1995). This chain of events represents a fundamental mechanism of NF-κB biology, referred to as the inactivating feedback loop built into the system (Sen, 2011). Its effects can be measured starting approximately half an hour after the initial stimulation. Instances where the inductive signal continues, in contrast, favor degradation of the newly made IκBα, releasing sequestered NF-κB dimers to be activated once more and return to the nucleus. In theory, this process could continue infinitely. At the single-cell level it has been shown that oscillations are evident as long as data is collected (Hoffmann *et al.*, 2002; Nelson *et al.*, 2004).

Using once more the example of shutting down an inflammatory response, the oscillations of nuclear NF-κB will decrease and eventually cease, in parallel with a reduction of inflammatory cytokine abundance. Yet, due to the negative feedback mechanism, the first pulse of nuclear NF-κB will be much greater than any subsequent pulses, even if the cytokine concentrations are kept at a constant level. Moreover, not only is the shuttling of NF-κB to the nucleus oscillatory, but also its transcription, which tracks exactly the timing of NF-κB entry into the nucleus (Hao and Baltimore, 2009). Degradation of IκB and nuclear translocation of NF-κB trigger the continuous induction of κB-site regulated genes until IκB is resynthesized. Therefore, even a short pulse of induction will lead to an hour of induced synthesis. The phenomenon of periodic oscillations in NF-κB activity is best described in the cellular response to TNF (Hoffmann *et al.*, 2002).

Other relevant genes involved in the downregulation of NF-κB signaling are those that code for the deubiquitinase A20 or the cylindromatosis (Cyld) protein (Ruland, 2011; Roth and Ruland, 2013).

1.2.6. NF-κB Signaling in Disease

The high relevance as well as the large number of genes induced by NF-κB render this transcription factor very powerful and yet very dangerous. Its regulatory influence

significantly affects the homeostatic balance of an organism. Dysregulation of effector molecules involved in the NF-κB cascade may therefore have serious pathologic consequences, including the development of diverse chronic inflammatory or autoimmune disorders such as Crohn's disease, ulcerative colitis, and rheumatoid arthritis (Roman-Blas and Jimenez, 2006; Ben-Neriah and Karin, 2011; Roth and Ruland, 2013). All of these medical conditions are associated with a constant overproduction of inflammatory cytokines, chemokines, and other mediators of inflammation. Many of the corresponding genes contain one or more κB sites within their promoters, suggesting that upregulation of NF-κB activity might be involved in their onset. Increased cytokine production consequently induces a positive feedback loop, leading to further NF-κB activation and advancement of disease.

As mentioned above, NF-κB does not only mediate inflammatory responses. The transcription factor is also essential for the subsequent process of wound healing. Excessive activation of the innate immune response, however, may result from severe or chronic inflammation and can lead to an evasion from normal tissue growth-control mechanisms. NF-κB signaling is also known to prevent apoptosis in cells that would otherwise be lost. There is a reason for dangerous cells to be destroyed, however, and the NF-κB induced extension of their life span may allow cancerous cells to persist. Inflammation, together with anti-apoptotic functions, and mitogenic effects are the three main cancer promoting mechanisms that critically involve NF-κB signaling. Malignancies such as leukemia, lymphoma, colon cancer, and ovarian cancer have been reported to arise as a pathologic consequence and are, undoubtedly, the worst possible outcomes of NF-κB dysregulation (Rayet and Gelinas, 1999; Ben-Neriah and Karin, 2011). These three mechanisms function quite differently, though. Proliferation and anti-apoptotic processes that enable dangerous cells to stay alive and grow further are mediated in the cytoplasm. The inflammatory process, on the other hand, is maintained by supporting cells that surround an emerging tumor, not by the cancer cells themselves, and therefore takes place in the extracellular matrix. Interestingly, chronic inflammation has pro-oncogenic as well as anti-oncogenic potential, making it even more complicated to decipher the role of NF-κB in cancer (Ben-Neriah and Karin, 2011). Various intracellular alterations can promote the development of cancer by causing uncontrolled NF-κB activity. Those include mutations that lead to the inactivation of IκB proteins, cause the activation of upstream components of the pathway, and/or create amplifications or rearrangements of genes encoding certain NF-κB subunits. In human lymphomas, such mutations have been described for genes encoding critical regulators including MyD88,

CARD-containing membrane-associated guanylate kinase (MAGUK) protein (Carma1) and mucosa-associated lymphoid tissue lymphoma translocation protein (Malt)1, whereas in epithelial tumors the kinases TBK1 and IKKε are key drivers of constant NF-κB activity. The deletion of genes encoding proteins that act at any critical step in the NF-κB pathway, in contrast, leads to the death of such tumor cells. Yet, even inhibition of NF-κB itself has been described to lead to more severe disease in some settings (Ben-Neriah and Karin, 2011).

From all of the examples mentioned above, deregulation of upstream components activating the NF-κB signaling cascade is thought to have the strongest influence on cancer development. One such upstream molecule is the caspase recruitment domain-containing (Card)9 adaptor protein, a non-redundant mediator of CLR-triggered innate immune responses (Ben-Neriah and Karin, 2011; Roth and Ruland, 2013).

1.3. C-type Lectin Receptors in Innate Immunity

The superfamily of C-type lectin receptors (CLRs) is an important class of PRRs that critically depend on the NF-κB cascade for signal transduction. They enable myeloid cells to detect changes from normality in order to direct appropriate responses against invading pathogens or to repair damaged tissue. Myeloid CLRs recognize various forms of PAMPs and respond to danger signals from damaged cells, such as oxidized lipids and other indicators of abnormal self. Subsequent CLR signaling induces endocytosis and phagocytosis for the uptake of pathogens or defective self components and can trigger proinflammatory as well as anti-inflammatory responses. Activation of CLRs also leads to significant alterations in the transcriptome of phagocytes. This causes both, phenotype and function of the cells to be reprogrammed and enables, for instance, DCs to initiate adaptive immune responses. The variety of possible outcomes caused by ligand binding to CLRs is due to the multitude of downstream pathways that may be activated. CLR-induced signals have the capacity to potentiate, impair, or modify signaling from other (innate immune) receptors, including TLRs and NLRs, and hence significantly contribute to the fine-tuning of reactions against damage or pathogenic insult (Sancho and Reis e Sousa, 2012; Roth and Ruland, 2013).

CLRs are integral or transmembrane receptors with an extracellular calcium (Ca^{2+})-dependent (C)-type lectin-like domain (CTLD) that confers lectin (i.e. carbohydrate binding)

activity and is, as such, termed carbohydrate recognition domain (CRD). The CRD enables these receptors to detect specific microbial structures of viruses, bacteria, and fungi. Confusingly enough, not all CTLDs interact with Ca^{2+} or bind carbohydrates and show instead high specificity for the detection of lipids, proteins, and even inorganic ligands. CLRs are distinguished into structural categories according to their properties. Members of CLR group II are type II transmembrane proteins composed of a short cytoplasmic N-terminus, a transmembrane section, and stalk region on the cell surface, followed by a Ca^{2+}-dependent receptor domain in the form of a single CRD. The stalk region is involved in receptor oligomerization and may vary in length between individual receptors. Most importantly Dectin-2 and MP-inducible C-type lectin (Mincle), next to SIGNR1 and Langerin belong to this group II. Dectin-1, in contrast, is a member of CLR group V, also referred to as the NK cell receptor-like group. These CLRs are homologous in their basic structure to group II receptors and differ mainly in their receptor domains, as the CTLD of group V CLRs lacks the typical motifs required for Ca^{2+} and carbohydrate binding. Most receptors that belong to group V are encoded in the natural killer gene complex, located on chromosome 12p13 and 6F3 in humans and mice, respectively. Other important group V members are the CLRs MICL (*Clec12a*), Clec-2, DNGR-1 (*Clec9a*), and LOX-1. CLRs belonging to group VI are type I transmembrane receptors with a ricin-like and fibronectin type 2 domain, in addition to eight to ten CTLDs on their extracellular N-terminus. A transmembrane region links their multiple CRDs on the cell surface to a short cytoplasmic tail. Mannose receptor (MR) and DEC-205 are the group VI CLRs expressed by myeloid cells (Sancho and Reis e Sousa, 2012).

In addition to mediating defense against infections, CLRs function also importantly by enabling myeloid cells to maintain homeostasis in the steady state. Most CLRs recognize self ligands, in addition to pathogens, and facilitate cell adhesion and migration, as well as cell-cell communication. Other CLRs detect damaged cells undergoing apoptosis or necrosis, or the debris that they release including oxidized lipids, heat shock proteins, and ribonucleo-proteins. Myeloid CLRs further participate in tissue repair and recognize alterations which mark cells as abnormal or transformed, thereby possibly contributing to a putative tumor immune surveillance by myeloid cells. Yet, such interactions between CLRs and cancerous tissue do not stringently protect the host, as they may be exploited by the tumor to persist (Sancho and Reis e Sousa, 2012).

1.3.1. Dectin-1 and Archetypal CLR Signaling

The most studied and best characterized receptor within the family of CLRs is dendritic cell-associated C-type lectin (Dectin)-1, also known as β-glucan receptor and CLECSF12. In mice, it is encoded by the gene *Clec7a* and was originally described to be a DC specific CLR. Further studies reported murine Dectin-1 to be expressed additionally in monocytes/MPs, neutrophils, and in a certain subset of γδ T cells (Taylor *et al.*, 2002; Martin, B. *et al.*, 2009). As a member of group V CLRs, it does not contain a typical CRD and binds its ligands in a Ca^{2+}-independent manner, mediated via several *N*-linked glycosylations. Dectin-1 recognizes PAMPs in the form of β-(1,3) or β-(1,6)-linked glucans, so called β-glucans, that are critical components in the cell walls of (pathogenic) fungi including *Candida albicans* (*C. albicans*), but have also been found in several bacteria and plants (Brown, 2006; Palma *et al.*, 2006). Humans as well as mice deficient for Dectin-1 are significantly more susceptible to various fungal infections (Roth and Ruland, 2013). Moreover, the receptor detects a self ligand, which is expressed in T cells but remains to be identified (Ariizumi *et al.*, 2000). Another article that was published very recently suggests the intermediate filament protein vimentin as an endogenous ligand (Thiagarajan *et al.*, 2013). Activation of Dectin-1 facilitates ligand uptake as the receptor shows late endosome-lysosome endocytic activity and induces downstream signaling that critically promotes innate and adaptive immune responses (Sancho and Reis e Sousa, 2012).

The Dectin-1-induced pathway serves as a paradigm for CLR signaling. Ligand binding to Dectin-1 leads to the recruitment of Src family protein tyrosine kinases (SFKs) that phosphorylate an ITAM-like motif within the cytoplasmic tail of the receptor. Conventional ITAMs are tandem repeats of the consensus YxxL/I (with x representing any amino acid). The intracellular domain of Dectin-1 is structured differently though, as it possesses only one tyrosine contained in a single YxxL sequence, which is hence referred to as a hemITAM (Figure 6). Other CLRs that have been described to contain a hemITAM include Clec-2, DNGR-1, and SIGN-R3 (Suzuki-Inoue *et al.*, 2006; Huysamen *et al.*, 2008; Tanne *et al.*, 2009). In the cytoplasmic domains of Dectin-1 and Clec-2 the YxxL motif is additionally preceded by the conserved amino acid sequence DEDG which has been speculated to serve as a triacidic lysosomal targeting signal (Sancho and Reis e Sousa, 2012). Dectin-1 was the first spleen tyrosine kinase (Syk)-coupled CLR described to be essential for mammalian immunity and is able to directly recruit and activate the kinase. The underlying mechanism critically depends on the above described SFK-mediated phosphorylation of the single tyrosine residue

within the hemITAM. This distinguishes Dectin-1 from other CLRs that have been reported to autophosphorylate their hemITAMs (Rogers *et al.*, 2005; Underhill *et al.*, 2005; Mocsai *et al.*, 2010).

Syk contains a tandem Src homology (SH)2 domain, enabling it to interact with bis-phosphorylated ITAMs and mutation of either SH2 was shown to block Dectin-1-induced signaling (Fuller *et al.*, 2007). This implicates a requirement for both Syk SH2 domains to be engaged for successful signaling, and raises the question as to how a hemITAM, containing only one tyrosine, may provide a docking site for the kinase. It is conceivable that one Syk SH2 domain could interact with the hemITAM, while the other SH2 binds to an unknown partner. A different and more recognized hypothesis suggests that dimerization of hemITAM-containing CLRs brings two YxxL/I sequences in close proximity to each other, thereby forming a pseudo-ITAM in *trans* (Sancho and Reis e Sousa, 2012; Roth and Ruland, 2013). Association of Syk with the phosphorylated docking sites leads to a conformational change of the kinase and mediates its autophosphorylation and activation (Mocsai *et al.*, 2010).

Signals are transduced as the interaction of activated Dectin-1 with Syk facilitates Card9 recruitment to the cell membrane or to phagosomes containing fungal particles, via a direct or an indirect mechanism (Rosas *et al.*, 2008; Goodridge *et al.*, 2009; Hernanz-Falcon *et al.*, 2009). This leads to the assembly of a myeloid Card9-Bcl10-Malt1 (CBM) signalosome which, in turn, engages the IKK complex that initiates canonical NF-κB signaling and gene expression (Gross *et al.*, 2006). Card9 and Bcl10 appear to be critical for the Dectin-1-induced activation of all canonical NF-κB subunits, whereas Malt1 has been reported to relay signals selectively to c-Rel (Figure 6), thereby directing the specific production of IL-1β and IL-23p19 (Gringhuis *et al.*, 2011). Dectin-1 is further able to activate non-canonical RelB in a manner that requires NIK but does not involve Card9 (Gringhuis *et al.*, 2009).

Myeloid cells critically depend on Dectin-1-stimulated Syk-coupled NF-κB signaling for the activation of their proinflammatory program. In the case of DCs, this induces cell maturation and triggers the production of several cytokines including IL-2, IL-10, IL-6, TNF, and IL-12/23p40 (Sancho and Reis e Sousa, 2012). The DCs are thus rendered competent to instruct the polarization of naive $CD4^+$ T helper cells into T_H1 and T_H17 subsets, to promote the priming of $CD8^+$ CTLs, and to initiate antibody-mediated immune responses (LeibundGut-Landmann *et al.*, 2007; LeibundGut-Landmann *et al.*, 2008; Osorio *et al.*, 2008).

In addition to the central NF-κB pathway, Dectin-1 activity further engages signaling cascades that involve the MAPKs p38, Erk, and c-Jun N-terminal kinase (JNK) together with the transcription factor NFAT (Goodridge *et al.*, 2007; LeibundGut-Landmann *et al.*, 2007; Slack *et al.*, 2007). This is similar to the outcome of antigen-receptor stimulation in lymphoid cells and contrary to TLR signaling which does not mobilize Ca^{2+} or activate NFAT. The combined activation of all these Syk-dependent pathways defines the typical proinflammatory cytokine pattern which is generated by myeloid cells in response to CLR stimulation (Sancho and Reis e Sousa, 2012; Roth and Ruland, 2013). In DCs, it was further shown that phospholipase C γ (PLCγ)2 activity, which causes Ca^{2+} influx and engages the Erk and JNK pathways, is essential for the Dectin-1-mediated production of cytokines (Tassi *et al.*, 2009; Xu *et al.*, 2009b). The activation of Syk-dependent signaling in myeloid cells not only allows for responses on the transcriptional level but also influences cell migration, engages the phagocytic machinery, and induces killing of ingested pathogens. Production of microbicidal reactive oxygen species (ROS) in MP phagosomes is regulated via Syk and activates the NLR family, pyrin domain containing protein (Nlrp)3 inflammasome. This involves the recruitment of pro-caspase-1, which upon induction processes pro-IL-1β and culminates in the secretion of mature IL-1β (Figure 6). It has been reported that IL-1β is critical for anti-fungal responses and that Nlrp3-deficient mice rapidly succumb to challenges with *C. albicans* (Gross *et al.*, 2009; Roth and Ruland, 2013).

Dectin-1-triggered activation of the rapidly accelerated fibrosarcoma (Raf)-1 serine-threonine kinase, in contrast, functions independently of Syk. Yet, both pathways converge at the level of NF-κB induction (Figure 6). Raf-1-directed phosphorylation selectively targets the NF-κB subunit p65 on Ser276 and allows for its subsequent acetylation, mediated by the histone acetyl-transferases CREB-binding protein and p300. Acetylated p65 has a significantly elevated affinity for DNA and may partner with p50 to produce a transcriptional output that instructs human DCs to secrete IL-12p70 together with other cytokines (Gringhuis *et al.*, 2007; Gringhuis *et al.*, 2009). Raf-1 activity downstream of Dectin-1 is thus able to augment the transcription of Syk-dependent cytokine genes in human DCs. Alternatively, acetylated p65 can also form inactive dimers together with Syk-induced RelB, thereby negatively affecting the secretion of RelB-dependent cytokines such as IL-23p19. In sum, these effects have been reported to favor the development of T_H1 responses (Gringhuis *et al.*, 2009; Sancho and Reis e Sousa, 2012).

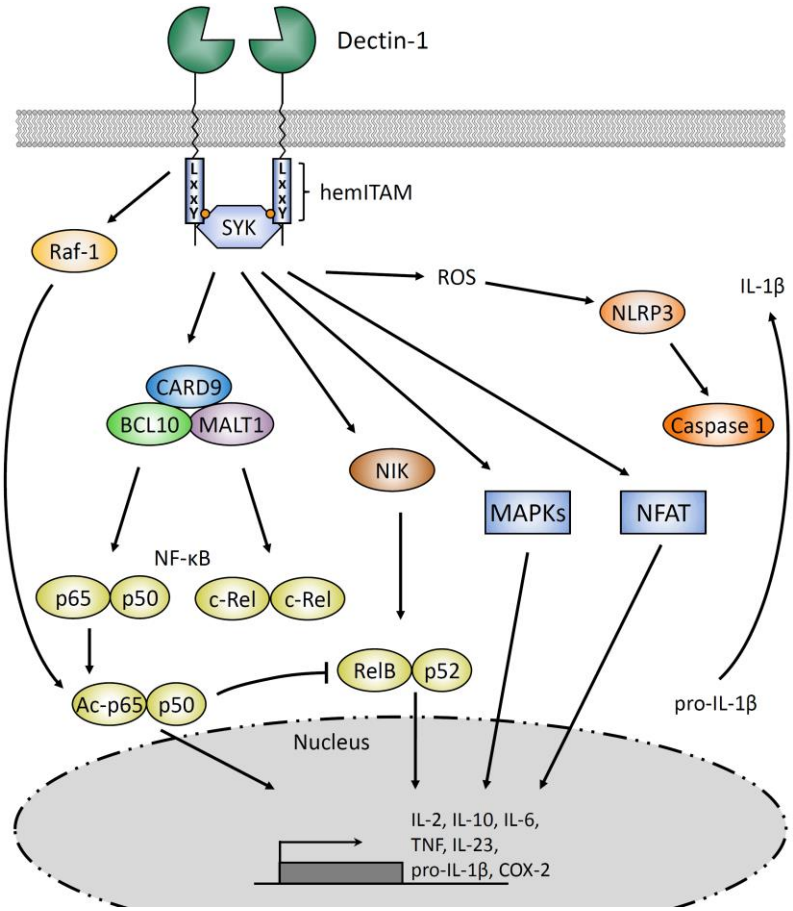

Figure 6: Dectin-1 Signaling.

Ligand-induced Dectin-1 engagement triggers multiple signaling cascades. Phosphorylation of a tyrosine residue located within the intracellular hemITAM of Dectin-1 leads to recruitment and activation of the kinase Syk. The subsequent assembly of Card9 and Bcl10 activates canonical p65-p50 NF-κB heterodimers, while Malt1 specifically induces c-Rel homodimers in humans DCs. The non-canonical NF-κB cascade downstream of Syk functions independently of Card9 but requires NIK. Dectin-1-Syk signaling triggers the production of anti-microbial ROS, which further induces the Nlrp3 inflammasome and the secretion of IL-1β. Raf-1 activation in response to Dectin-1 engagement is independent of Syk and causes p65-p50 to be acetylated, leading to RelB inhibition and the alteration of NF-κB activity. Also the MAPKs p38, Erk, and JNK, in addition to the transcription factor NFAT, respond to Syk activity and regulate gene transcription together with NF-κB. Adapted from Sancho and Reis e Sousa (Sancho and Reis e Sousa, 2012).

Dectin-1 activation and the subsequent induction of proinflammatory cytokine production are significantly influenced by the nature of the detected ligand. Successful signaling depends on the formation of a synapse-like structure that involves intact lipid rafts and requires exclusion of the inhibitory phosphatases CD45 and CD148 (Xu *et al.*, 2009a; Goodridge *et al.*, 2011). Such microdomains may assemble when Dectin-1 binds to large particulate β-glucans that lead to extended Syk-signaling and trigger NF-κB activity. Solid

ligands, which are too large to be engulfed result in the phenomenon of frustrated phagocytosis and cause an even enhanced inflammatory reaction. Interaction with smaller particles and their endocytosis, in contrast, lead to a subsequent decrease in Dectin-1 signaling (Rosas *et al.*, 2008; Hernanz-Falcon *et al.*, 2009). Soluble ligands are unable to trigger the formation of a synapse-like domain and function as Dectin-1 inhibitors. These effects are thought to constitute a safety mechanism which ensures that myeloid cells develop anti-microbial activity only upon contact with pathogens and not in response to microbial debris (Goodridge *et al.*, 2011; Sancho and Reis e Sousa, 2012).

1.3.2. The Card9-Bcl10-Malt1 Signalosome

CLR signaling in myeloid cells facilitates the assembly of Card9, the adaptor protein Bcl10, and the paracaspase Malt1 (CBM) into a multimeric protein complex, referred to as the innate or myeloid CBM signalosome (Figure 7). This CBM complex is critical for and central to CLR-induced signal transduction via the canonical NF-κB cascade for the initiation of immune responses against fungal, bacterial, and viral threats (Roth and Ruland, 2013).

Card9 was initially discovered *in silico* as the result of a database search for novel proteins containing a CARD module and described to associate with Bcl10 for the activation of NF-κB (Bertin *et al.*, 2000). Card9 consists of an N-terminal CARD, that allows for interaction with other CARD-possessing proteins, and a C-terminal coiled-coil region which facilitates the formation of oligomers. It has been found in various lymphoid and non-lymphoid organs, with particularly high expression levels being reported for myeloid immune cells such as MPs and DCs (Ruland, 2008; Roth and Ruland, 2013). Card9 was characterized to function as a central and non-redundant adaptor molecule that channels signals from Syk-coupled ITAM receptors to canonical NF-κB activation for inflammation and host defense. Murine DCs lacking Card9 are unable to induce NF-κB signaling in response to Dectin-1 stimulation and show a significantly impaired production of cytokines when challenged with *C. albicans* or zymosan (Gross *et al.*, 2006; Hara *et al.*, 2007; Goodridge *et al.*, 2009). Additionally, Card9 is involved in NLR signaling and selectively controls RLR-activated cascades (Roth and Ruland, 2013).

In contrast to myeloid cells, lymphocytes express the Card9 homolog Carma1, also known as Card11 and Bcl10-interacting MAGUK protein (Bimp)3 (Colonna, 2007; Roth and Ruland, 2013). The family of Carma proteins, which also contains Carma2 (otherwise known

as Card14 and Bimp2) and Carma3 (alternatively called Card10 and Bimp1) was originally identified based on the CARD-mediated interaction of its members with Bcl10 for activation of the IKK complex (Gaide *et al.*, 2001; McAllister-Lucas *et al.*, 2001). Lymphoid CBM signalosomes that assemble in response to antigen receptor engagement thus contain Carma1 instead of Card9 (Figure 7) and Carma1 is critical for the subsequent activation of NF-κB and JNK in B and T cells (Guo *et al.*, 2004; Thome *et al.*, 2010). Carma molecules consist of an N-terminal CARD, followed by a coiled-coil domain, a linker region, and a C-terminal MAGUK module. This module is characteristic for Carma proteins and contains a PDZ-SH3-GUK tripartite structure that facilitates membrane association. Card9 lacks such a MAGUK motif (Roth and Ruland, 2013).

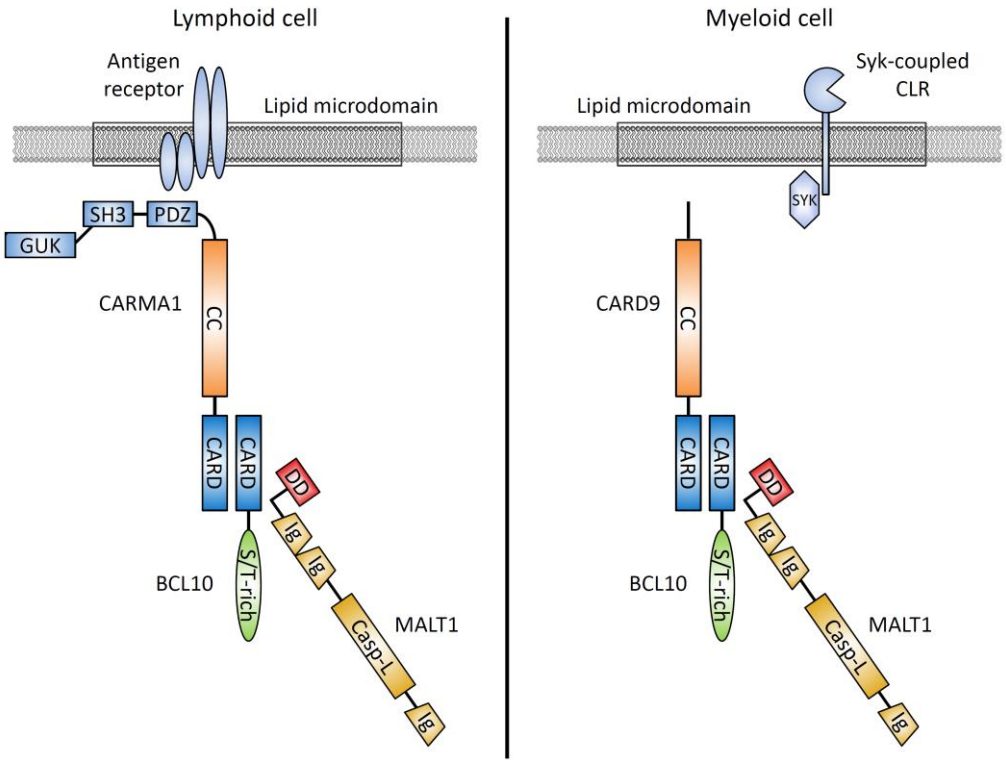

Figure 7: The CBM Complex in Lymphoid and Myeloid Cells.

Differential CBM complex assembly in different cell types. Carma1 is specifically expressed in lymphocytes and contains a PDZ-SH3-GUK (MAGUK) domain that mediates membrane association. The lymphoid CBM signalosome in B and T cells, hence, consists of Carma1, Bcl10, and Malt1. Myeloid cells, in contrast, predominantly express the Card9 adaptor protein, which does not have a MAGUK motif. The myeloid CBM complex contains Card9, Bcl10, and Malt1. Casp-L, caspase-like domain; CC, coiled-coil; DD, death domain; Ig, immunoglobulin-like domain; S/T-rich; serine and threonine-rich region; Adapted from Guo *et al.* and Roth and Ruland (Guo *et al.*, 2004; Roth and Ruland, 2013).

The signaling adaptor Bcl10 was initially described for its role in B cell activation and neurodevelopment (Ruland *et al.*, 2003). An N-terminal CARD enables the molecule to directly interact with the CARDs of Card9 or Carma1 for signal transduction (Figure 7), whereas the C-terminal serine and threonine-rich region in Bcl10 mediates inhibitory effects (Roth and Ruland, 2013). Various pathogen-derived and self ligands stimulate myeloid cells to respond with Bcl10-dependent activation of NF-κB and engagement of the MAPKs p38 and JNK (Ruland, 2008; Hara and Saito, 2009; Mocsai *et al.*, 2010).

Malt1 consists of an N-terminal death domain (DD), two Ig-like motifs next to a central caspase-like (Casp-L) region, and a third Ig-like domain at its C-terminus (Thome, 2008). The Ig-like modules, together with the DD, enable Malt1 to associate with other proteins (Figure 7). The binding of Malt1 to Bcl10 is essential, as it facilitates recruitment of the IKK complex. The underlying mechanism, that leads to cell activation, depends on TRAF2, TRAF6-mediated K63 poly-ubiquitination of Bcl10 and Malt1, and the cooperation of other factors. In addition to its scaffolding function, Malt1 is a paracaspase that specifically targets cleavage sites preceded by a positively charged arginine residue. The proteolytic activity of its Casp-L domain is, therefore, distinct from that of classical caspases which process their substrates downstream of negatively charged aspartate (Staal *et al.*, 2011; Roth and Ruland, 2013). Formation of the CBM complex induces Malt1-mediated cleavage and inactivation of substrates such as A20 and Cyld (Thome, 2008; Staal *et al.*, 2011). Both molecules have been described to negatively regulate NF-κB signaling and activity of the MAPKs p38 and JNK (Roth and Ruland, 2013). Recent studies further report cleavage of non-canonical RelB by Malt1, thereby inactivating RelB and preventing it from localizing constitutively to the nucleus where it inhibits canonical NF-κB activity (Brustle *et al.*, 2012). In contrast to Bcl10, Malt1 is essential for normal B cell development but only has a minor role in B cell activation and is not required for neurodevelopment (Ruefli-Brasse *et al.*, 2003; Ruland *et al.*, 2003).

In spite of its essential role in CLR-stimulated signaling to NF-κB, the exact molecular mechanisms for activation of the CBM signalosome and those that relay information to downstream pathways remain to be determined (Roth and Ruland, 2013).

1.3.3. CLR-Mediated Detection of Fungal Invaders

In addition to Dectin-1, several other myeloid CLRs are required for successful defense against infections with pathogenic fungi such as *C. albicans* (Figure 8). Those include

Dectin-2, Mincle, MR, DC-SIGN, and SIGNR1, in addition to TLRs, NLRs, and others that orchestrate their signaling capacities to provide host protection in a unique and non-redundant manner (Osorio and Reis e Sousa, 2011; Sancho and Reis e Sousa, 2012). In spite of this multitude of receptors involved, Card9-deficient (*Card9*$^{-/-}$) mice are unable to mount innate immune responses or to induce the polarization of T_H17 cells following an experimental challenge with *C. albicans*, while T_H1 responses appear to be unaffected (Gross *et al.*, 2006; LeibundGut-Landmann *et al.*, 2007). T_H17-mediated immunity is essential for anti-fungal protection and T_H17-deficiencies are strongly associated with opportunistic infections such as chronic candidiasis in mice and humans. Severe cases have been described in pedigrees that carry mutations causing the expression of either non-functional Card9 or Dectin-1 (Ferwerda *et al.*, 2009; Glocker *et al.*, 2009). These findings once more underscore the central role of Card9 in CLR signaling for inflammation and host protection (Sancho and Reis e Sousa, 2012; Roth and Ruland, 2013). The only other Syk-coupled receptors within this group of critical CLRs involved in anti-fungal immunity are Dectin-2 and Mincle (Figure 8). In contrast to Dectin-1, their intracellular tails do not possess an ITAM-like structure. Dectin-2 and Mincle, therefore, associate with the Fc-receptor γ (FcRγ) chain for signaling, which contains multiple ITAM repeats (Sato *et al.*, 2006; Yamasaki *et al.*, 2008).

Dectin-2 is a group II CLR encoded by the *Clec4n* gene in mice and expressed primarily in monocytes/MPs and DCs (Sancho and Reis e Sousa, 2012). The receptor is mainly activated by α-mannans and high-mannose structures that are found in fungal cell walls but also detects other microbial molecules and a self ligand from $CD4^+$ $CD25^+$ T cells (Aragane *et al.*, 2003; McGreal *et al.*, 2006; Saijo, S. *et al.*, 2010). Association of Dectin-2 with the FcRγ chain depends on an arginine residue adjacent to its transmembrane region (Sancho and Reis e Sousa, 2012). The ITAM-bearing adaptor is required for stable cell-surface expression of the receptor and is critical for Syk recruitment and activation upon agonistic ligand binding. This induces Card9-dependent signal transduction to NF-κB and engages the Erk, JNK, and p38 MAPK pathways (Robinson *et al.*, 2009). Dectin-2 stimulation triggers the secretion of cytokines, including IL-1β and IL-23, that favor T_H17 cell differentiation and further instructs the uptake machinery to clear fungal pathogens (Sato *et al.*, 2006; Gringhuis *et al.*, 2011). Exposing myeloid cells to extracts from *Schistosoma mansoni* eggs, in contrast, elicits Dectin-2-mediated ROS production and potassium efflux, which promotes Nlrp3-dependent processing of pro-IL-1β and resembles the Dectin-1 response to fungi (Gross *et al.*, 2009; Ritter *et al.*, 2010). Moreover, a role for Dectin-2 in allergic reactions has been reported, as it

may detect the mold *Aspergillus fumigatus* and house dust mite derived mannose-bearing glycans. Subsequent signaling induces the production of proinflammatory lipids (cysteinyl leukotrienes) that contribute to allergic inflammation and promote T_H2 responses (Barrett *et al.*, 2009; Barrett *et al.*, 2011). This potentially mixed T_H2/T_H17 outcome of Dectin-2 signaling is thought to be shaped in one direction or the other by the nature of the ligand or via the interaction of Dectin-2 with other innate receptors (Sancho and Reis e Sousa, 2012).

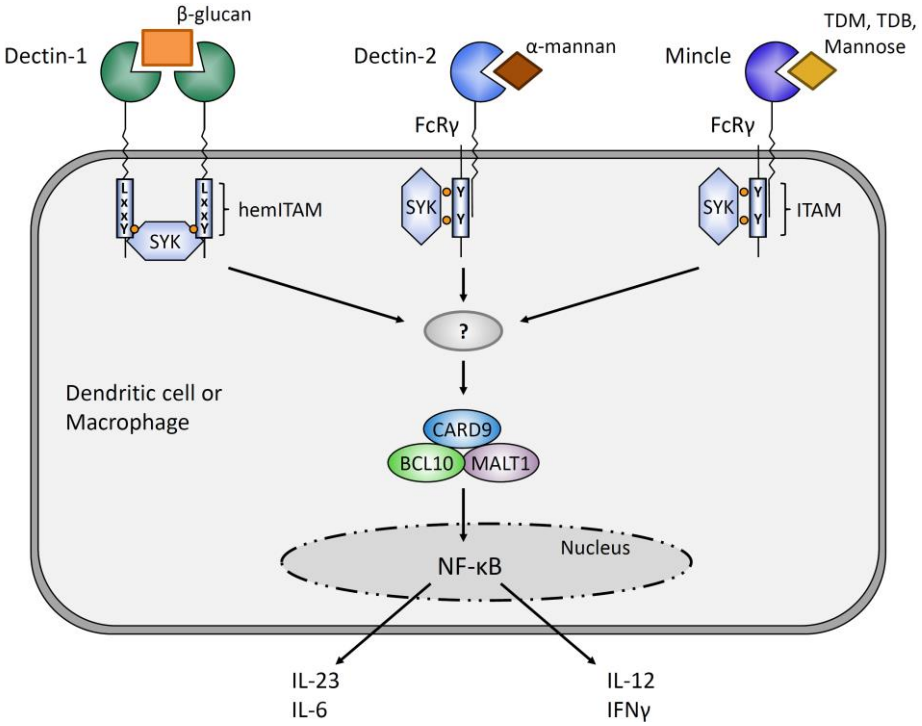

Figure 8: CLRs in Anti-fungal Immunity.

Dectin-1, Dectin-2, and Mincle are critical receptors for the detection of diverse fungal structures. Dectin-1 contains a hemITAM in its intracellular tail, whereas Dectin-2 and Mincle associate with the ITAM bearing FcRγ chain for signal transduction. Ligand binding causes tyrosine (Y) phosphorylation within those motifs and leads to Syk recruitment. The pathways downstream of all three receptors run via one or more unknown intermediates to the central Card9-Bcl10-Malt1 complex and engage cytokine production regulated by NF-κB and other transcription factors. Adapted from Drummond *et al.* and Roth and Ruland (Drummond *et al.*, 2011; Roth and Ruland, 2013).

Mincle is encoded by the murine *Clec4e* gene and belongs to the group II CLRs. It is expressed at low levels in MPs and neutrophils prior to pathogen encounter. The cells strongly upregulate its presence on the cell surface in response to proinflammatory cytokines or TLR stimulation (Yamasaki *et al.*, 2008). Mincle recognizes α-mannose-containing ligands from mycobacteria or the pathogenic fungal species *Malassezia* and *Candida* in a Ca^{2+}-dependent

manner (Bugarcic *et al.*, 2008; Wells *et al.*, 2008; Yamasaki *et al.*, 2009). Furthermore, the receptor is an essential detector of trehalose 6,6'-dimycolate (TDM) and its synthetic analog trehalose 6,6'-dibehenate (TDB). Both substances are *Mycobacterium tuberculosis (M. tuberculosis)*-derived glycolipids that function as potent activators of innate immune responses and as adjuvants (Ishikawa *et al.*, 2009; Schoenen *et al.*, 2010). The self ribonucleoprotein SAP130, released from damaged or necrotic cells, is bound by Mincle on MPs through a mechanism that involves distinct binding sites on the receptor in a Ca^{2+}-independent manner. An arginine residue within the transmembrane domain of Mincle facilitates its interaction with the FcRγ adaptor (Yamasaki *et al.*, 2008). Signal transduction in response to receptor engagement is mediated via the Syk- and Card9-dependent cascade, and leads to NF-κB activation, triggering the secretion of inflammatory cytokines and chemokines such as TNF, IL-6, C-X-C motif ligand (CXCL)2, and CXCL1, as well as nitric oxide production (Ishikawa *et al.*, 2009; Schoenen *et al.*, 2010). This cocktail of messenger molecules specifically attracts neutrophils to sites of tissue injury and instructs the repair of damage in a process called sterile inflammation (Yamasaki *et al.*, 2008). Yet, it does not favor immunity. As this outcome appears to contradict the requirement of Mincle signaling for the development of T_H1/T_H17-based immunity and anti-microbial defense, it has been proposed that ligand origin as well as the involvement of additional receptors might calibrate the outcome of Mincle activation (Sancho and Reis e Sousa, 2012).

The activities of Dectin-1, Dectin-2, and Mincle in anti-fungal immunity are supported by MR, DC-SIGN, and SIGNR1. The latter belong to a class of CLRs that do not contain distinct ITAM or ITIM modules and signal independently of Syk or phosphatase activities. Individual and isolated stimulation of these receptors does not lead to a detectable activation of myeloid cells. Yet, their engagement has been described to trigger the uptake machinery for pathogen clearing and antigen presentation to lymphocytes and to modulate the activatory potential of other CLRs (Geijtenbeek and Gringhuis, 2009). The exact mechanisms and pathways involved remain to be defined (Sancho and Reis e Sousa, 2012).

The opportunistic pathogen *C. albicans* belongs to the normal microbial flora of various mammalian species and can be found mainly on the skin as well as in the gastrointestinal and respiratory tracts. The innate immune system of healthy individuals usually restricts the spread of the fungus without further difficulties, whereas patients that are immuno-compromised due to age or disease often suffer from severe and chronic candidiasis (Peters-Golden and VanHook, 2012). *C. albicans* is clinically one of the most relevant pathogens and

systemic infections by it contribute significantly to worldwide morbidity and mortality (Gladiator *et al.*, 2013). The fungus is able to transform its morphology and can exist as a coccoid yeast, or in a hyphal form. It has been suggested that the Dectin-1-activating β-glucans are specifically produced during the yeast stage, while mannose and α-mannan, which trigger Mincle and Dectin-2, respectively, are predominant features of *Candida* hyphae (Brown *et al.*, 2002; Brown *et al.*, 2003; Sato *et al.*, 2006; Saijo, S. *et al.*, 2010).

1.4. The Family of Protein Kinase C Molecules

Protein kinase C (PKC) enzymes belong to the class of serine/threonine kinases. They were originally identified by Nishizuka and colleagues in 1977 and described as cyclic nucleotide-independent protein kinases that phosphorylate histone or protamine in bovine cerebellum (Takai *et al.*, 1977; Yamamoto *et al.*, 1977). In mammals, PKCs are encoded by a family of nine independent gene loci distributed across the entire genome. According to their biochemical properties, sequence homologies, and structural similarities, PKCs are clustered into three categories (Nishizuka, 1988). The isoforms PKCα, PKCβI/II (spliced variants), and PKCγ belong to the subgroup of classical or conventional PKCs (cPKCs) that require phospholipids, Ca^{2+} ions, and diacylglycerol (DAG) for catalytical activity. The novel PKC (nPKC) faction, which contains the isotypes PKCδ, PKCε, PKCη, and PKCθ, depends on DAG but not on Ca^{2+} or phospholipids for their engagement. The atypical isoforms (aPKCs) PKCλ/ι (PKCλ represents the murine ortholog of human PKCι) and PKCζ require none of the cofactors (Ca^{2+}, DAG, or phospholipids) for their activation (Newton, 1995; Mellor and Parker, 1998; Tan and Parker, 2003; Spitaler and Cantrell, 2004; Leitges, 2007).

All tissues and cells express at least two or more PKC isoforms. These are involved in the regulation of a multitude of cellular processes and act as critical signal transducers in a wide spectrum of pathways. PKCs direct metabolic processes and have been described to influence cell growth and differentiation as well as apoptosis, transformation, and tumor development. They contribute to cytoskeletal alterations and facilitate gene expression in response to a plethora of environmental cues (Tan and Parker, 2003; Rosse *et al.*, 2010). Individual PKC isoforms have been noted to show a high degree of similarity in their catalytic domains, paired with rather broad and overlapping substrate specificity. This explains why different PKC family members have been found to function in a redundant manner *in vitro*

(Tan and Parker, 2003; Spitaler and Cantrell, 2004). The aPKC isoform PKCι/λ, for example, is highly homologous to PKCζ and has been reported to compensate for PKCζ function (Leitges, 2007).

PKC *in vivo* functions, however, are very clear and distinct and may even vary for a single isoform in diverse cell types. The specificity of different PKC isotypes and their involvement in the network of signaling cascades therefore need to be well defined and organized. This is achieved for individual PKCs through specific and unique expression patterns and their differential subcellular localization (Tan and Parker, 2003; Leitges, 2007). Other regulatory mechanisms control lipid interaction, protein partners, and phosphorylation, thereby determining latent activity, location and agonist responsiveness. These mechanisms that collaborate to define the isoenzyme-, cell-, and tissue-selective functions of PKCs become particularly evident in PKC-mediated immune-cell signaling (Tan and Parker, 2003).

The involvement of PKCs in host defense is ancient and not exclusive to mammals. The DAG-PKC signaling cascade evolved in primitive organisms and appears to be conserved throughout evolution from yeast to man. In fact, even the nematode worm *Caenorhabditis elegans* expresses several isoforms including both classical and novel PKCs (Mellor and Parker, 1998; Spitaler and Cantrell, 2004) and plants also have been described to employ PKC-like serine-threonine kinases in their defense against fungal infections (Xing *et al.*, 1996).

Several PKC isoforms have been attributed non-redundant roles in individual cell types of the immune system and PKC-regulated signaling cascades are central to many of its functions. PKCs facilitate the development, differentiation, activation, and survival of lymphocytes, are involved in the induction of T cell proliferation and regulate the reactivation of effector CTLs, in addition to other aspects of cellular immune responses. They also mediate the activation of MPs (Truneh *et al.*, 1985; Tan and Parker, 2003; Isakov and Altman, 2012). In this manner, PKCs contribute significantly to the fine-tuning of immune response signaling thresholds by, on the one hand, preventing severe autoimmune diseases due to over-reactions to self-antigens and, on the other hand, by avoiding inadequate responses to foreign antigens that may increase the risk of infection or tumor development (Tan and Parker, 2003).

1.4.1. Structural Characteristics of the PKC Family

All PKCs assemble from an N-terminal regulatory and C-terminal catalytic moiety. Within these superior structures, PKCs contain the highly conserved regions C1 through C4, shared by most isoforms, in addition to variable (V) segments that differ between isotypes (Leitges *et al.*, 1996; Spitaler and Cantrell, 2004). The presence or absence of these motifs influences which cofactors are required for optimal catalytic activity (Tan and Parker, 2003).

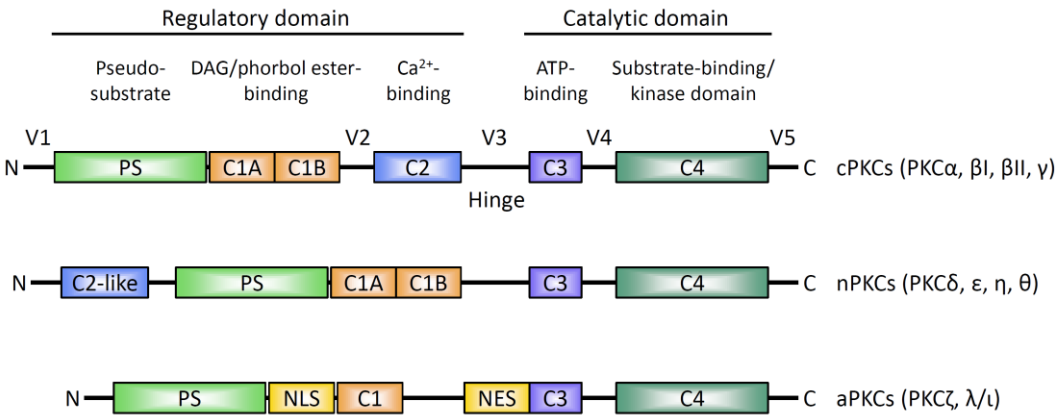

Figure 9: Structural Characteristics and Classification of PKC Isoforms.
All PKC isoforms contain a highly conserved catalytic domain at their C-terminus that consists of the ATP- and substrate-binding lobes C3 and C4, respectively, forming the kinase core. The N-terminal regulatory domains, in contrast, vary between the PKC categories. cPKCs share all regulatory modules, including the autoinhibitory pseudosubstrate motif, a cysteine-rich DAG and phorbol ester-binding tandem C1 domain, and a Ca^{2+}-binding C2 domain. The C2-like motif of nPKCs differs from the classical C2-domain in key molecules and hence cannot bind Ca^{2+}. aPKCs contain a single, modified C1 domain and function independently of DAG and Ca^{2+}. Instead, this subgroup is regulated mainly by subcellular localization, mediated by association with regulatory proteins, a nuclear localization signal (NLS), and nuclear export signal (NES) modules. V1 through V5 are variable regions. The amino acid sequence of the V3 hinge region is unique to individual isoforms and allows for conformational changes in response to activatory signals. Adapted from Tan and Parker, and Spitaler and Cantrell (Tan and Parker, 2003; Spitaler and Cantrell, 2004).

Mainly the N-terminal portion varies between the three PKC categories even though it contains the key regulatory motifs (Figure 9). cPKCs are characterized by the presence of all typical regulatory elements. The autoinhibitory pseudosubstrate (PS) motif is contained by all isoforms and binds to the catalytic domain, thereby rendering the enzyme inactive in the absence of activators. The two C1 domains (C1A and C1B) contain zinc-finger motifs for binding to DAG or phorbol ester while the C2 domain mediates binding to Ca^{2+} and

phospholipids. The cysteine-rich elements within the C1 portion are highly conserved among all PKC classes. nPKCs compensate for the lack of a Ca^{2+}-binding C2 motif by receiving regulatory signals and binding to DAG via an extended N-terminal region (C2-like domain). Regulation of the catalytic activity of Ca^{2+}- and DAG-insensitive aPKCs appears to be determined largely by their intracellular localization, which depends on the interaction with regulatory proteins, NLS, and nuclear export signals (NES) within the aPKC regulatory domain. aPKCs have a PS and a single modified C1 domain (Mellor and Parker, 1998; Newton, 2003; Tan and Parker, 2003; Guo *et al.*, 2004; Spitaler and Cantrell, 2004).

The catalytic domain, in contrast, is highly homologous in all PKC isoforms (Figure 9). It contains the ATP-binding domain C3 and the substrate binding lobe C4, which together form the kinase core (Tan and Parker, 2003). Most protein kinase inhibitors target the ATP binding site which is well conserved even among distantly related protein kinases (Baier and Wagner, 2009).

1.4.2. PKC Function

Following protein synthesis, classical and novel PKCs are initially immature in the sense that the presence of cofactors alone is not sufficient for their activation. Immediately thereafter, PKCs become constitutively transphosphorylated by DAG-engaged phosphoinositide-dependent kinase 1 (PDK1) within the activation loop of their catalytic domain. This priming event enables the PKCs to autophosphorylate their own C-termini, rendering them catalytically competent. Ca^{2+}, in the case of classical isoforms and other second messengers, such as DAG, are subsequently needed for full activation depending on the type of isoform (Newton, 2003; Spitaler and Cantrell, 2004).

DAG and inositol 1,4,5-trisphosphate (IP_3) are produced by PLCγ, which hydrolyzes the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) in response to receptor engagement. DAG is a hydrophobic molecule that remains attached to the plasma membrane where it is required for the activation of PKCs and influences other effector molecules like RasGRP. Hydrophilic IP_3, in contrast, diffuses through the cytosol and binds to IP_3 receptors. These ER-associated sensors function as ligand-gated calcium channels and facilitate the release of free Ca^{2+} ions into the cytoplasm (Spitaler and Cantrell, 2004; Isakov and Altman, 2012).

In resting cells or in the absence of lipid hydrolysis most PKCs are located mainly in the cytosol. cPKCs and nPKCs contain tandem repeats of C1, a highly conserved and cysteine-rich domain that functions as a minimal DAG-binding site. DAG directly acts on those isoforms and is critical for their recruitment to the plasma membrane, events that are essential but not sufficient for PKC activation (Tan and Parker, 2003; Leitges, 2007). The PS sequence sterically blocks substrate access to the catalytic center, thereby keeping the kinase in an inactive state. It closely resembles the ideal phosphorylation site recognized by the substrate-binding cavity in the catalytic domain, yet with an exchange of serine to alanine at the predicted serine-threonine phosphorylation site (Newton, 2003; Tan and Parker, 2003; Spitaler and Cantrell, 2004). DAG binding increases the affinity of cPKCs for membrane phospholipids (phosphatidylserine) and shifts their affinity for Ca^{2+} into the physiologic range. This leads to conformational changes in the enzyme that displace the PS domain from the active site, thereby increasing its catalytic activity and making the active site available for signaling effectors (Newton, 2003; Tan and Parker, 2003). Even though they are insensitive to Ca^{2+}, nPKCs do depend on DAG for activation. Moreover, they interact with phospholipids and regulatory proteins that influence PKC activation and translocation via a C2-like structure near the N-terminus (Mellor and Parker, 1998; Spitaler and Cantrell, 2004). In contrast to cPKCs and nPKCs, the general mechanisms for aPKCs activation are very different and do not necessarily require the activation of PLCs, leading to the generation of DAG and IP_3. aPKCs contain only a single C1 motif and are therefore unable to bind to DAG or phorbol esters. One example how aPKCs can be activated instead, is their induction in response to activation of the phosphoinositide 3-kinase (PI3K) pathway (Spitaler and Cantrell, 2004; Leitges, 2007).

In immune cells only very few PKC substrates have been identified so far. Yet, phosphorylation consensus sites have been described for most PKC isoforms to require positively charged amino acid residues directly upstream of the serine-threonine residues to be phosphorylated. Many PKCs have been described to autophosphorylate on several residues, thereby positively regulating their own activity and localization (Hayashi and Altman, 2007). Members of the protein kinase D (PKD) family of serine-threonine kinases are evolutionarily conserved direct substrates for nPKCs and cPKCs, and critically require PKC phosphorylation for their activation. PKC-mediated phosphorylation of PKD, important for the initiation of T cell precursor differentiation and proliferation (Spitaler and Cantrell, 2004), has been reported in antigen receptor stimulated B cells, T cells, and in mast cells and is

therefore likely facilitated by several different PKC isoforms. Other PKC substrates are involved in the rearrangement of the actin cytoskeleton (Hayashi and Altman, 2007).

1.4.3. PKCs in Lymphoid Signaling

Key signaling cascades for the initiation and homeostasis of immune reactions are regulated by PKCs, either in a positive or in a negative manner. Multiple PKC isoforms are activated differently by DAG and Ca^{2+}, mainly as a result of antigen receptor cross-linking in B cells, T cells, and mast cells. Together with other DAG-binding proteins, they interact in a network of central signaling pathways to control the biology of lymphocytes. The DAG-PKC axis, which directs many different gene transcriptional programs, is essential for controlling the activation of lymphocytes and influences their morphology, motility, and chemotaxis as well as their differentiation and proliferation (Leitges *et al.*, 1996; Spitaler and Cantrell, 2004).

1.4.3.1. T cell specific isoforms

T cells express the PKC isoforms α, βI, ε, η, θ, and ζ in varying amounts (Hayashi and Altman, 2007; Isakov and Altman, 2012; Stahelin *et al.*, 2012). Individual isoforms are involved in different aspects of T cell activation and effector functions including cytokine expression or regulation of the cells' adhesive capacity (Baier and Wagner, 2009; Isakov and Altman, 2012). PKCs function as amplifying kinases in T cell signaling and modulate the strength of upstream signals, thereby contributing to the elimination of negative regulators. The sustained activation of essential transcription factors leads to an amplification of cytokine signaling, which then mediates entry into the S-phase of the cell cycle and leads to cell survival and significant proliferation of clonotypic T cells. The sum of these essential cellular and molecular interactions determines whether downstream signaling in antigen-specific lymphocytes is successful or not (Baier and Wagner, 2009).

PKCθ is predominantly expressed in T cells and appears to be the key isoform, as it is reported to have important and non-redundant functions in the control of several fundamental processes of T lymphocyte biology (Figure 10). Results from biochemical and genetic studies describe PKCθ to be essential for a productive activation of mature T cells, their proliferation and survival. A unique property of PKCθ is its ability to translocate from the cytosol to lipid rafts and subsequently to the center of the IS in TCR/CD28 stimulated T cells. This event is essential for proper PKCθ function. The mechanism works indirectly, requiring the Lck protein tyrosine kinase as an intermediate, and the unique V3 (hinge) region of PKCθ, which

eventually anchors it to the cytoplasmic domain of CD28. Moreover, phosphorylation of PKCθ through the germinal center kinase (GSK)-like kinase (GLK), which also translocates to the IS of TCR-engaged T cells, is required. This involvement of adaptor molecules provides an explanation for a selective recruitment of DAG effector proteins. The formation of this PKCθ-Lck-CD28 tripartite complex is critical for the activation of downstream signaling events (Isakov and Altman, 2012). Consequently, PKCθ-deficient (*Prkcq*$^{-/-}$) T cells are impaired in their antigen receptor-induced activation of the transcription factors AP-1, NF-κB, and NFAT, resulting in incomplete T cell activation and in abnormal expression of pro-apoptotic proteins such as Bcl2-associated death promoter (BAD) and Bcl2-interacting mediator (Bim), in parallel to reduced expression of survival promoting genes like Bcl2 and Bcl-extra large (Bcl-x_L), causing reduced T cell survival (Hayashi and Altman, 2007). Moreover, the differentiation and the effector function of T helper subsets is compromised in *Prkcq*$^{-/-}$ mice, in particular the T_H2 and T_H17 subsets. The effect of PKCθ on T_H1 cell development, in contrast, appears to be only moderate. Several studies report PKCθ to be dispensable for T_H1-dependent and CTL-mediated anti-viral defense and suggest a possible compensatory innate immune mechanism (Marsland *et al.*, 2004; Salek-Ardakani *et al.*, 2004). Other activatory conditions may cause PKCθ to translocate into the nucleus instead, where its direct binding to chromatin regulates microRNAs and induces a gene expression program specific for T lymphocytes (Sutcliffe *et al.*, 2011). It is therefore clear that PKCθ critically regulates multiple aspects of T cell function (Spitaler and Cantrell, 2004; Hayashi and Altman, 2007; Isakov and Altman, 2012).

PKCθ integrates TCR and CD28 signals, leading to the activation of several transcription factors, including NF-κB (Figure 10). In this context, Carma1 is one of the most important substrates for PKCθ. Carma1 is selectively expressed in lymphocytes and associates constitutively with lipid rafts (Gaide *et al.*, 2002). It is required for TCR, but not for TNF or IL-1-induced NF-κB activation in Jurkat cells (Pomerantz *et al.*, 2002; Wang *et al.*, 2002). TCR/CD28 stimulation of mature T cells leads to PKCθ-dependent phosphorylation of Carma1 and promotes its association with Bcl10 and Malt1 (Matsumoto *et al.*, 2005; Sommer *et al.*, 2005). Together they form the lymphoid Carma1-Bcl10-Malt1 (CBM)-complex, which is subsequently recruited to the IS (McAllister-Lucas *et al.*, 2001; Hara *et al.*, 2003; Che *et al.*, 2004). This, in turn, induces the PKCθ-dependent phosphorylation and activation of Bcl10, which leads to activation of the IKK complex and NF-κB induction (Gaide *et al.*, 2002). All three proteins of the CBM complex (*i.e.* Carma1,

Bcl10, and Malt1) have been shown to be required for maximal activation of NF-κB in TCR/CD28 stimulated T cells (McAllister-Lucas *et al.*, 2001; Ruland *et al.*, 2001). Similar defects in antigen receptor-mediated NF-κB activation and subsequent cell proliferation are reported for mice deficient in PKCθ and Bcl10 (Ruland *et al.*, 2001). IκBα degradation is not detectable in *Prkcq*$^{-/-}$ T cells in response to TCR stimulation, supporting the model whereby PKCθ regulates NF-κB activity through effects on IKK-IκBα (Sun, Z. *et al.*, 2000).

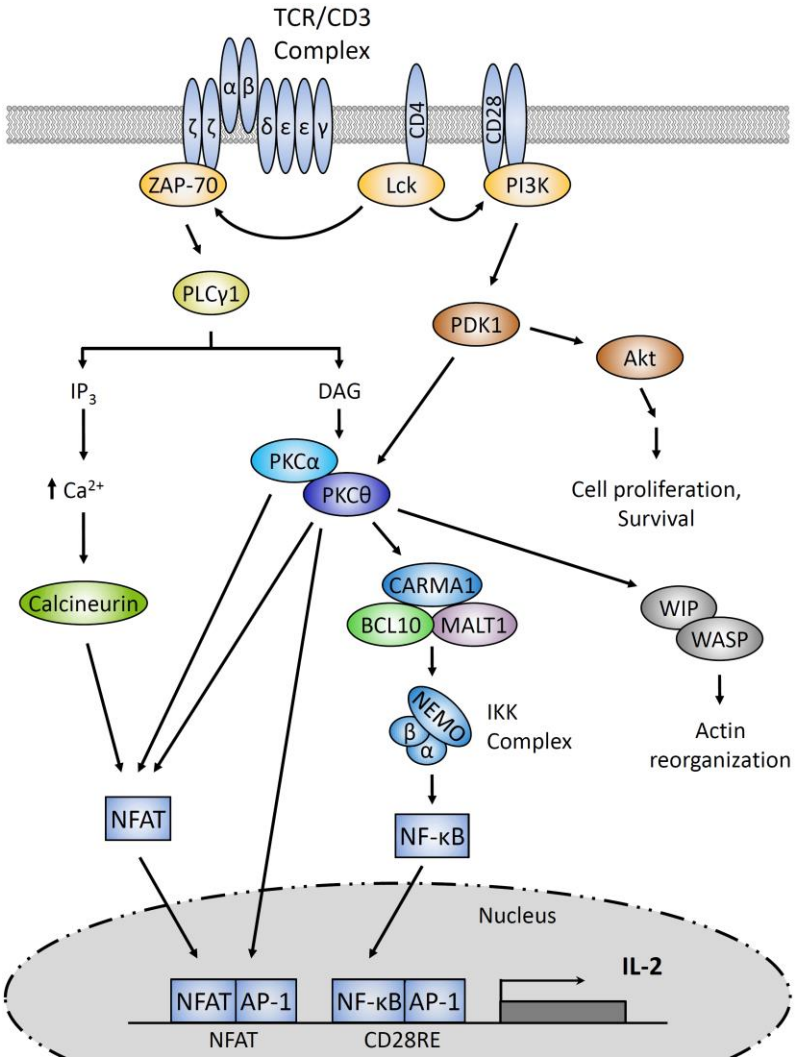

Figure 10: PKC Signaling in T cells.

PKCθ and PKCα, the two most important PKC isoforms in T lymphocytes, have essential and physiological functions in mediating TCR signaling for T cell activation and proliferation. CD28RE, CD28 response element. Adapted from Tan and Parker and Baier and Wagner (Tan and Parker, 2003; Baier and Wagner, 2009).

Signaling to the transcription factor AP-1 is also regulated by PKCθ and requires the PKCθ-mediated phosphorylation of the STE20-SPS1-related proline-alanine-rich protein

kinase (SPAK), a mitogen-activated protein kinase. Induction of NFAT activity, in contrast, requires cooperation between PKCθ and the Ca^{2+}-dependent serine-threonine phosphatase calcineurin (Figure 10). Binding of the three PKCθ-regulated transcription factors NF-κB, NFAT, and AP-1 to their consensus within the IL-2 gene promoter is required for an optimal IL-2 response and hence for T cell proliferation (Hayashi and Altman, 2007; Isakov and Altman, 2012).

Several other proteins have been reported to interact with PKCθ, such as moesin, Wiskott-Aldrich syndrome protein (WASP)-interacting protein (WIP), 14-3-3τ, Cbl, the tyrosine kinases Fyn and Lck, the serine-threonine kinase Akt, insulin receptor substrate (IRS)1, and the HIV nef protein. Lck phosphorylates PKCθ, thereby affecting its activity and subcellular localization. Other interaction partners, like Cbl, 14-3-3τ, WIP, and moesin are substrates for PKCθ and are involved in the regulation of functions including cytoskeletal reorganization (Tan and Parker, 2003; Spitaler and Cantrell, 2004; Hayashi and Altman, 2007; Isakov and Altman, 2012).

Other PKCs with important physiological functions in T cells are the isoforms PKCα and PKCβ, as they are central regulators of T cell fate and significantly influence the character of lymphocyte-specific effector responses *in vivo* (Baier and Wagner, 2009). A recently published study shows that both, PKCα and PKCβ cooperate in primary murine T cells in a PKCθ-independent manner to regulate IL-2 gene expression in response to anti-CD3 stimulation of the cells (Lutz-Nicoladoni *et al.*, 2013). For Jurkat cells it has been reported that PKCα but not PKCβ is required for TCR/CD28-triggered signaling and subsequent NF-κB activation. PKCα is believed to function upstream of PKCθ for induction of NF-κB after CD3/CD28 activation but this needs to validated *in vivo*. PKCα is thought to be involved in thymocyte development and probably plays a role in allelic exclusion and differentiation during thymocyte development. PKCα-deficient mice have been reported to be defective in their T_H1 response and show a significant reduction of IFN-γ production (Pfeifhofer *et al.*, 2006). PKCβ-deficient mice show normal T cell signaling but are reported to have defects in lymphocyte function-associated antigen-1 (LFA-1) dependent outside-in signaling, which facilitates T cell motility and T cell migration across vascular walls to sites of infection (Volkov *et al.*, 2001; Tan and Parker, 2003; Thuille *et al.*, 2004; Baier and Wagner, 2009). PKCδ, in contrast, is reported to be involved in a signaling pathway required for T cell attenuation (Gruber *et al.*, 2005).

1.4.3.2. B cell specific isoforms

B cells and mast cells mainly express the PKC isoforms β, δ, λ, and ζ. Single knockouts of these enzymes have been reported to affect B cells and mast cells but not T cells. Only minimal expression of PKCθ is reported in B cells but mast cells express both PKCβ and PKCθ (Sun, Z. *et al.*, 2000; Su *et al.*, 2002). B cells clearly require the coordinated interaction of multiple PKCs for the induction of productive BCR signaling and for the fine-tuning of signals (Tan and Parker, 2003; Guo *et al.*, 2004; Spitaler and Cantrell, 2004).

Expression levels of PKCβ are particularly high in B lymphocytes. The function of PKCβ in B cells appears to be of comparable significance to the above described role of PKCθ in TCR-mediated NF-κB activation (Tan and Parker, 2003). PKCβ is essential for BCR-triggered activation of NF-κB and the survival of B cells (Figure 11). PKCβ is thought to interact with the CBM complex within BCR microdomains for the regulation of IKK and the activation of NF-κB (Guo *et al.*, 2004). PKCβ-deficient (*Prkcb*$^{-/-}$) mice express neither the PKCβI nor the PKCβII isoform. These animals show defects in BCR signaling, B cell survival, and mast cell function. Both PKCβ isoforms are derived from the same transcript and their sequence differs only in the C-terminus. *Prkcb*$^{-/-}$ animals have reduced numbers of splenic B cells, a significantly reduced number of B-1 lymphocytes, and low levels of serum IgM and IgG3 (Leitges *et al.*, 1996; Guo *et al.*, 2004). The IgM-induced proliferation of B cells is also defective in *Prkcb*$^{-/-}$ mice while their T cells are activated normally in response to TCR stimulation (WT T-cells express PKCβ). *Prkcb*$^{-/-}$ cells do not show degradation of IκB and fail to recruit the IKK complex into lipid rafts and to activate it in response to BCR triggering. Thus, PKCβ has been proposed to control the formation of IKK raft complexes in B lymphocytes. Moreover, PKCβ-deficient B cells lack phosphorylated IKKα proteins and the half-life of phosphorylated IKKβ is reduced (Saijo, K. *et al.*, 2002; Tan and Parker, 2003). PKCβ is therefore likely to control NF-κB signaling upstream of IKK activation (Su *et al.*, 2002). Despite their defect in NF-κB activation, the follicular mature B cell pool is reported to be intact in *Prkcb*$^{-/-}$ mice. This suggests the activity of additional pathways, such as TNF-receptor signaling and CD40 activation, that lead to NF-κB activation in those animals as well as a more stringent requirement of PKCβ for NF-κB survival signaling than for other BCR-mediated signals. PKCβ is also not essential for differentiation signals mediated via the BCR, as the development of pre- to mature B cells happens in a largely normal fashion (Su *et al.*, 2002). The impaired B cell activation and the ineffective B cell-dependent immune responses make the phenotype of *Prkcb*$^{-/-}$ mice appear similar to that observed in Bruton's tyrosine

kinase (Btk)-deficient mice and in X-linked immunodeficiency (Xid) mice, carrying a point mutation in Btk (Fruman *et al.*, 2000; Tan and Parker, 2003; Guo *et al.*, 2004). Results from these studies suggest that distinct PKC isoforms mediate BCR- and TCR-induced signal transduction. It appears that the function of PKCβ in antigen receptor-mediated signaling is unique and cannot be compensated for by other isoforms during T cell-independent activation of B lymphocytes (Leitges *et al.*, 1996). Also the NFAT and AP-1 transcription factors respond to BCR engagement but the role of PKCβ seems to be more or less restricted to the NF-κB pathway (Figure 11), despite its critical role in BCR signaling (Su *et al.*, 2002; Guo *et al.*, 2004).

Crosslinking of the BCR induces activation of PKCβ which, in turn, leads to the recruitment of PKCβ and the IKK complex into lipid rafts where they assemble into a signaling complex together with other factors (Su *et al.*, 2002). BCR ligation also causes the translocation of Carma1 into the membrane microdomains, an event which is dependent on the MAGUK domain of Carma1. Activated PKCβ then mediates the phosphorylation of Carma1, causing it to change its conformation and triggering the recruitment of Bcl10 and Malt1. The three proteins assemble and build the CBM complex. This promotes the Bcl10-, Malt1-, TRAF6-, and ubiquitin-conjugating enzyme 13 (Ubc13)-dependent ubiquitination of NEMO (IKKγ) and thereby connects the BCR via PKCβ to canonical activation of NF-κB (Guo *et al.*, 2004; Zhou *et al.*, 2004). As part of a negative feedback loop, PKCβ phosphorylates and inhibits its upstream activator, the Tec kinase Btk, in a site-specific manner, causing a downregulation of PKCβ activity and permitting the fine-tuning of receptor-mediated signaling (Guo *et al.*, 2004). It is therefore not surprising that BCR-dependent cell proliferation and survival are significantly impaired in *Prkcb*$^{-/-}$ mice, since they have defects in the induction of the NF-κB dependent survival genes Bcl2 and Bcl-x_L (Saijo, K. *et al.*, 2002; Su *et al.*, 2002; Tan and Parker, 2003). A recent report connects the PKCβII-dependent activation of NF-κB in bone marrow stromal cells to the survival of malignant B cells in chronic lymphatic leukemia (CLL) patients (Lutzny *et al.*, 2013).

PKCζ has also been reported to be involved in B cell development and activation. PKCζ deficient (*Prkcz*$^{-/-}$) mice have been found to show a BCR signaling defect. The rate of spontaneous apoptosis is increased in B lymphocytes from these animals and the proliferation of B cells in response to IgM cross-linking is impaired. *Prkcz*$^{-/-}$ T cells and thymocytes, in contrast, appear to develop and proliferate normally. The poor survival rate of *Prkcz*$^{-/-}$ B cells is thought to be due to a failure in Erk activation and the inability to express the classical

NF-κB-regulated genes Bcl-x_L, IκB, and IL-6 (Figure 11). *Prkcz*$^{-/-}$ mice were, consequently, not able to respond with an optimal B cell-mediated immune reaction. PKCζ is not directly associated with the AP-1 or NF-κB signaling cascades but it has been shown that PKCζ can directly modulate NF-κB by phosphorylating p65 (RelA) on Ser311, a mechanism that operates independently of IKK (Tan and Parker, 2003; Guo *et al.*, 2004).

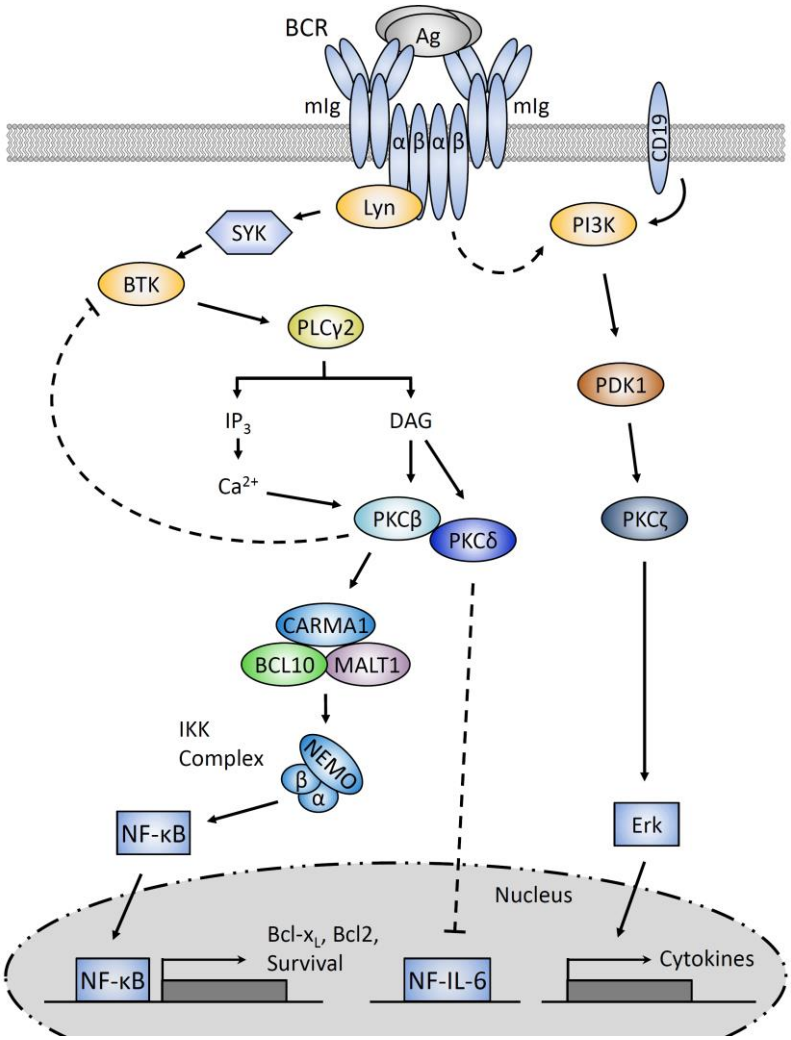

Figure 11: PKC Signaling in B cells.

Schematic representation of the proposed signaling mechanisms in response to BCR stimulation, leading to PKCβ-mediated NF-κB activation and PKCδ-regulated B cell anergy. Ag, antigen; mIg, membrane-bound receptor immunoglobulin. Adapted from Tan and Parker and Guo *et al.* (Tan and Parker, 2003; Guo *et al.*, 2004).

PKCλ has been linked to NF-κB activation during early B cell development via a mechanism that remains to be determined. In pro B cells, PKCλ was suggested to be a target of SFKs, such as Blk, Fyn, and Lyn, and to be required for pre-BCR mediated NF-κB activation (Tan and Parker, 2003; Spitaler and Cantrell, 2004).

The role of PKCβ in B cells is comparable to the specific roles of PKCθ and PKCζ in TCR- and TNF-mediated NF-κB activation, respectively. Therefore, instead of being characterized by overlapping functions, individual PKCs appear to influence NF-κB in pathways downstream of particular receptors and in specific cell types (Su *et al.*, 2002).

1.4.4. PKCs in Innate Immunity

Some PKC isoforms are associated with important functions in the regulation of innate immune responses. It has been shown that several signaling pathways are regulated by PKCs, including innate immune responses to microbial products. Distinct PKC isoforms are involved in the responses of both MPs and DCs to Gram-negative LPS. PKCε has been found to play an important role in MP function and biology and to be involved in LPS induced signaling. PKCε deficient (*Prkce*$^{-/-}$) MPs have a defect in NO production in response to IL-4 stimulation. *Prkce*$^{-/-}$ mice display a MP activation defect. In response to LPS and IFN-γ, PKCε was found to be significantly involved in the production of NO levels, PGE_2, and the mediation of the TNF and IL-1β production through engagement of NF-κB and MAP kinases. *Prkce*$^{-/-}$ MPs were unable to clear infections with either Gram-positive or negative bacteria. A similar role for PKCε in LPS-induced signaling was also described in monocyte-derived DCs. Here PKCε, but not PKCβ or PKCα were essential for IKK directed NF-κB activation and the production of TNF and IL-12, following LPS stimulation of the cells. These results propose a critical role for PKCε in the integration of different signaling pathways, which leads to powerful innate immune responses (Tan and Parker, 2003; Johnson *et al.*, 2007).

In addition to their roles in B cell function, PKCβ and PKCδ have been described to be involved in mast cell degranulation. PKCβ-deficient mast cells showed a significant decrease in degranulation in addition to reduced production of IL-6, while mast cell lacking PKCδ were found to have more sustained Ca^{2+} mobilization and a substantially elevated level of degranulation. These results suggest that PKCδ is a negative regulator of antigen-induced mast cell degranulation and propose opposing functions for these two PKC isoforms, leaving the mechanism yet to be defined (Tan and Parker, 2003).

1.4.5. Protein Kinase C-δ

PKCδ is expressed ubiquitously in most mammalian cells and is considered to be one of the most important isoforms. PKCδ instructs the inhibition of cell growth and proliferation, induces differentiation, and enhances apoptosis in vascular smooth muscle cells as well as in

other cell types (Fukumoto *et al.*, 1997; Li, W. *et al.*, 1998; Li, P.F. *et al.*, 1999; Majumder *et al.*, 2000). PKCδ is the closest related PKC member to PKCθ. The amino acid sequences of the two isoforms diverge significantly only in their V3 (hinge) region (Isakov and Altman, 2012). Moreover, the PKCδ C2-like domain was recently characterized as a novel phosphotyrosine-binding (p-Tyr) domain, and the residues essential for p-Tyr binding are conserved in PKCθ (Benes *et al.*, 2005).

The immune system of PKCδ-deficient (*Prkcd*$^{-/-}$) mice is deregulated in certain aspects. The animals develop splenomegaly and lymphadenopathy, and the onset of autoimmune reactions leads to their premature death. This phenotype is caused by hyperproliferative B and mast cells in the periphery and the production of autoreactive antibodies. The lack of PKCδ causes a B cell anergy (tolerance to self antigen) defect, enabling self-reactive B cells to mature and to differentiate (Mecklenbrauker *et al.*, 2002; Miyamoto *et al.*, 2002). This effect is possibly due to defective NF-κB induction, resulting from insufficient IκB degradation in the cytoplasm, while NF-κB survival signaling was reported to be normal in *Prkcd*$^{-/-}$ B cells (Mecklenbrauker *et al.*, 2002). Miyamoto and colleagues in contrast describe an increased proliferation of PKCδ deficient B cells in response to pro-mitogenic stimuli and suggest a general enhancement of signaling events (Miyamoto *et al.*, 2002). The study shows the induction of NF-κB to be unaffected, while the chromatin-binding capacity of the transcription factor nuclear factor IL-6 (NF-IL-6) and hence the production of the growth-promoting cytokine IL-6 were strongly upregulated in *Prkcd*$^{-/-}$ cells. This indicates a potential negative role for PKCδ in the regulation of B cell growth, mediated through influencing transcriptional activity of the IL-6 gene (Figure 11).

Together, these findings suggest that BCR ligation does not only trigger activation of pro-mitogenic PKCβ, but also induces anti-mitogenic PKCδ and involves both kinases in the specific regulation of B cell immunity, possibly facilitating a fine-tuning of immune responses. The studies also suggest a non-redundant role for PKCδ as a key regulator of essential negative feedback mechanisms, critically required for immune homeostasis of B lymphocytes. A similar inhibitory function for PKCδ has been reported in mast cells but the exact mechanisms are so far not known. (Leitges *et al.*, 2002a; Mecklenbrauker *et al.*, 2002; Miyamoto *et al.*, 2002; Tan and Parker, 2003; Guo *et al.*, 2004). In T lymphocytes PKCδ has been shown to promote negative feedback mechanisms, leading to the downregulation of antigen receptor complexes (Cantrell *et al.*, 1985; Minami *et al.*, 1987; Spitaler and Cantrell, 2004).

1.5. Specific Aims of This Project

C-type lectin receptor induced NF-κB signaling and the transcriptional program activated by this cascade are indispensable mechanisms in enabling cells of the innate immune system to calibrate their responses to microbial insult. Throughout the last years, observations from scientists around the world have contributed to elucidating many of the key molecules and essential processes in both C-type lectin receptor proximal events and NF-κB downstream signaling (Sancho and Reis e Sousa, 2012). Still, defining more precisely the major players and signaling complexes facilitating the induction of NF-κB-dependent transcriptional events and acquiring an improved understanding of their molecular architecture are unchanged needs in this field of research (Baltimore, 2011).

The course of action leading to NF-κB activation in response to antigen receptor stimulation in lymphocytes, in contrast to innate cells, is well elucidated. The phosphorylation of ITAM repeats and the subsequent recruitment and activation of adaptor proteins to the proximity of the receptor intracellular tails are essential events for the transduction of both BCR- and TCR-induced signaling. For TCR signaling the adaptor molecules Lck, linker of activated T cells (LAT), and Ras are constitutively associated with the lipid rafts, whereas PLCγ1, the Zeta-chain-associated tyrosine protein kinase (ZAP)-70, Vav, SLP-76, IKKβ, and PKCθ need to be recruited (Matsumoto *et al.*, 2005; Hara and Saito, 2009). The B cell antigen receptor requires the presence and activity of Lyn, Syk, SLP-65, PI3K, Btk, and Vav together with PLCγ2 and the PKCβ isoform within the membrane microdomains for productive signal transduction (Guo *et al.*, 2000).

It has been suggested that PKCθ directly interacts with and phosphorylates Carma1 in its linker region on Ser552 in response to TCR engagement. Moreover, a Carma1 mutant version with an exchange of Ser552 fails to activate NF-κB (Matsumoto *et al.*, 2005). These findings were extended by further studies. Sommer and colleagues report the Ser564 and Ser657 residues, both located within the Carma1 linker region, to be critically involved in this process as well. They confirm the phosphorylation of Carma1 by PKCθ and in addition show the ability of PKCβ to also phosphorylate Carma1 on the same amino acid residues in response to BCR signaling (Sommer *et al.*, 2005). Shinohara and colleagues find that activated PKCβ mediates the phosphorylation of Carma1 on Ser668 after BCR ligation (Shinohara *et al.*, 2007). PKC-mediated phosphorylation of Carma1 leads to conformational changes in Carma1, enabling the molecule to recruit Bcl10 and Malt1 into lipid microdomains

and allowing for CARD-mediated aggregation and oligomerization (Guo *et al.*, 2004; Sommer *et al.*, 2005; Hayashi and Altman, 2007; Shinohara *et al.*, 2007). The active lymphoid Carma1-Bcl10-Malt1 (CBM) signalosome, in turn, engages the IKK complex, resulting in IκB degradation and finally NF-κB activation and nuclear translocation (Ghosh and Karin, 2002). Together, these results suggest that phosphorylation of Carma1 by PKCθ in response to TCR activation and by PKCβ downstream of BCR signaling are crucial for the activation of NF-κB.

In myeloid cells, the assembly of a CBM-complex is critical for the induction of NF-κB regulated gene transcription downstream of ITAM-coupled receptors such as CLRs. Yet, the expression of Carma1 is restricted to lymphoid cells and myeloid cells employ the Carma1 homolog Card9 instead (Gaide *et al.*, 2002; Hara and Saito, 2009). The myeloid CBM complex therefore consists of Card9, Bcl10, and Malt1 (Figure 7). Card9 is built from a CARD and a coiled-coil domain but unlike Carma1 it does not contain a linker region or MAGUK domain and hence lacks the specific phosphorylation sites targeted by PKCβ or PKCθ (Ruland, 2008). It has been reported though, that the phospholipase PLCγ2 is critically required for the induction of Ca^{2+} flux, the activation of Erk and JNK MAPKs, and the engagement of the transcription factors AP-1, NFAT, and NF-κB as well as the subsequent secretion of cytokines in response to stimulation of the CLR Dectin-1 in DCs (Xu *et al.*, 2009b). The catalytic activities of PLCγ2 and PLCγ1 are essential for the induction of calcium flux and the subsequent activation of PKCβ in B cells and of PKCθ in T cells, respectively. Since the mechanisms leading to AP-1, NFAT, and NF-κB activation in lymphoid and myeloid cells are so similar, this raises the question as to whether one or more PKC isoforms are involved in the CLR-engaged and Card9-mediated activation of NF-κB in myeloid cells.

A major goal within the area of NF-κB research remains to identify protein kinase(s) and/or molecular adaptor(s) that engage the CBM complex and subsequently lead to involvement of the IKKs (Su *et al.*, 2002). In particular the receptor proximal events linking CLR-triggered stimulation to Card9-mediated NF-κB activation in myeloid cells are incompletely understood. The aim of this study was therefore to determine, whether one or more PKC isoforms play a critical role in NF-κB signaling in DCs and if so, to identify which isoform this would be. Thereafter, it was to be analyzed what effects the lack of such a molecule would have on those cells.

2. MATERIAL AND METHODS

2.1. Research Equipment

2.1.1. Laboratory Apparatus

Analytical balance, Denver Summit SI-64	Denver Instrument, Göttingen
Centrifuge, 5417R	Eppendorf, Hamburg
Centrifuge, 5424	Eppendorf, Hamburg
Centrifuge, 5810R	Eppendorf, Hamburg
Centrifuge, Minifuge™	Labnet International, Woodbridge, USA
Circular shaker IKA®-Vibrax®-VXR with VX 2 E 'Eppendorf' attachment	IKA®-Werke, Staufen
CO_2 incubator, Binder C150	Binder, Tuttlingen
CO_2 incubator, HERA cell 150	Heraeus, Thermo Electron Corporation, Langenselbold
Cryo 1°C freezing container	Nalgene™, Schwerte
Dewar carrying flask for liquid N_2, Typ 26 B	KGW-Isotherm, Karlsruhe
Digital camera for microscopy, DS-5Mc	Nikon, Düsseldorf
Digital scale, Kern 440-35N	Kern & Sohn, Balingen
Electrophoresis cell, XCell SureLock® Mini-cell	Invitrogen Life Technologies, Darmstadt
Electrophoresis system, Mini-PROTEAN Tetra	Bio-Rad, München
Flow cytometer, FACS Canto II	BD Biosciences, Heidelberg
Haemocytometer, Neubauer improved	Hartenstein, Würzburg/Versbach
Light microscope, Axiovert 40 C	Carl Zeiss MicroImaging, Göttingen
Light microscope, Eclipse TE2000-S	Nikon, Düsseldorf
Magnetic stirrer, heatable, IKA® RH basic 2	IKA®-Werke, Staufen
Magnetic stirrer, heatable, SLK4	Schott, Mainz
Magnetic stirrer, heatable, Stuart® CB162	Bibby Sterilin, Stone, UK
Microplate reader, Sunrise™-Basic	Tecan Austria, Grödig, Austria
pH meter, inoLab pH Level 1	Wissenschaftlich Technische Werkstätten, Weilheim
Pipettes, HTL Labmate	Abimed, Langenfeld

Pipettes, pipetman®	Gilson International, Limburg-Offheim
Pipettor, 12-channel, 20-200 µl	VWR International, Darmstadt
Pipettor, accu-jet® pro	Brand, Wertheim
Pipettor, PIPETBOY acu	IBS Integra Biosciences, Fernwald
Pipettor, pipetus® Akku	Hirschmann Laborgeräte, Eberstadt
Power supply unit, PowerPac Basic™	Bio-Rad, München
Power supply unit, PowerPac HC™	Bio-Rad, München
Power supply unit, Standard Power Pack P25	Biometra, Göttingen
Steam autoclave, Systec V95	Systec, Wettenberg
Sterile hood, Holten LaminAir 1.8	Holten, Gydewang, Denmark
Thermomixer comfort	Eppendorf, Hamburg
Trans-Blot SD Semi-Dry Transfer Cell	Bio-Rad, München
Tube mixer, RM5, horizontally rotating and swaying	Karl Hecht, Sondheim
Tumbling table, WT 12	Biometra, Göttingen
Vacuum pump, Eco Vac	Schuett-biotech, Göttingen
Vortex mixer, VORTEX GENIE® 2	Scientific Industries, Bohemia, USA
Water bath, WB14	Memmert, Schwabach
X-ray film processor, Optimax	Protec, Oberstenfeld
Yeast incubator, Binder BD-115	Binder, Tuttlingen

2.1.2. Molecular Biology Supplies

Annexin V-PE apoptosis detection kit	BD Pharmingen, Heidelberg
BD OptEIA™ ELISA sets (IL-1β, IL-2, IL-10, and TNF)	BD Pharmingen, Heidelberg
Blot paper, extra thick, Protein® II xi Size	Bio-Rad, München
Cell scraper (24 cm, 30 cm)	TPP FAUST Laborbedarf, Schaffhausen, Switzerland
Cell strainer, nylon (70 µm, 100 µm)	BD Falcon™, Heidelberg
CL-XPosure™ Film	Thermo Scientific, Bonn
Cryovials, 2 ml	Sarstedt, Nümbrecht
CytoTox 96® non-radioactive cytotoxicity assay	Promega, Mannheim
ELISA Ready-SET-Go!® (TNF)	eBioscience, Frankfurt/Main
FACS tubes, round bottom, 5 ml	BD Falcon™, Heidelberg

Hybond™-P PVDF transfer membrane	Amersham Biosciences, Freiburg
Inoculation loop, 10 µl	Greiner-Bio-One, Frickenhausen
MaxiSorp 96-well plates	Nunc, Langenselbold
Nitrocellulose Transfer Membrane, BA85 Protran® 0.2 µm and 0.45 µm pore size	Whatman®, Dassel
NuPAGE® 4-12% Bis-Tris Gels 1.5 mm x 15 well	Invitrogen Life Technologies, Darmstadt
Parafilm "M"	Pechiney Plastic Packaging, Chicago, USA
Petri dishes (10 cm)	Josef Peske Medizintechnik/ Laborbedarf, Aindling-Arnhofen
Pipette barrier tips, pre sterilized, (ART® 10 Reach, ART® 20P, ART® 200, ART® 1000)	Molecular BioProducts, Thermo Fisher Scientific, Bonn
Pipette tips, Omnitip™ (10 µl, 200 µl, 1000 µl)	ULPlast, Warsaw, Poland
Reaction tubes (15 ml, 50 ml)	BD Falcon™, Heidelberg Sarstedt, Nümbrecht TPP FAUST Laborbedarf, Schaffhausen, Switzerland
Reaction tubes, safe-lock (0.5, 1.5, 2.0 ml)	Eppendorf, Hamburg
Serological pipettes, Cellstar®, sterile (10, 25, 50 ml)	Greiner-Bio-One, Frickenhausen
Serological pipettes, sterile (1, 2 ml)	BD Falcon™, Heidelberg
Sterile needles, sterican (22 G x 1¼'', 24 G x 1'')	B. Braun Melsungen, Melsungen
Sterile syringes, Injekt® (2 ml, 20 ml)	B. Braun Melsungen, Melsungen
Tissue culture plates (6, 10, 15 cm; 6-, 12-, 24-, 96-well)	TPP FAUST Laborbedarf, Schaffhausen, Switzerland
Tissue culture plates (48-well)	BD Falcon™, Heidelberg
WB Substrate, Lumigen™ TMA-6	GE Healthcare Europe, Freiburg
WB Substrate, Pierce® ECL	Thermo Scientific, Bonn

2.2. Reagents

2.2.1. Chemicals

7-AAD viability staining solution	eBioscience, Frankfurt/Main
Adenosintriphosphate (ATP)	Sigma-Aldrich, Taufkirchen
Albumin Fraction V, bovine (BSA)	Roth, Karlsruhe
Albumin Fraction V, bovine (BSA), purified, 100x (10 mg/ml)	New England Biolabs, Frankfurt/Main
Ammoniumperoxodisulfate (APS)	Fluka Sigma-Aldrich, Taufkirchen
Aqua *ad iniectabilia* Delta Select	Delta Select, Pfullingen
Aqua B. Braun	B. Braun Melsungen, Melsungen
Bisindolylmaleimide I (Gö6850, panPKC inhibitor)	Calbiochem Merck, Darmstadt
Bromophenol blue	Fluka Sigma-Aldrich, Taufkirchen
CHAPS	Sigma-Aldrich, Taufkirchen
Chloroform	Sigma-Aldrich, Taufkirchen
Citric acid monohydrate, *p.a.*	Merck, Darmstadt
CpG oligodeoxynucleotide (CpG-DNA)	Cayla InvivoGen, Toulouse, France
Curdlan	Wako Chemicals, Neuss
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Taufkirchen
Disodium phosphate (Na_2HPO_4)	Roth, Karlsruhe
Ethanol, *p.a.*	Merck, Darmstadt
Ethylenediamine tetraacetic acid (EDTA, 0.5 M, pH 8.0)	Promega, Mannheim
Glycerol	Sigma-Aldrich, Taufkirchen
Glycine	Roth, Karlsruhe
Glycine hydrochloride	Sigma-Aldrich, Taufkirchen
Gö6976 (PKCα and PKCβ selective inhibitor)	Calbiochem Merck, Darmstadt
Hydrochloric acid, fuming 37% (HCl)	Merck, Darmstadt
Hydrogen peroxide, 30% (H_2O_2)	Riedel-de-Haën, Honeywell Speciality Chemicals Seelze, Seelze
Lipopolysaccharide (LPS)	Cayla InvivoGen, Toulouse, France
2-Mercaptoethanol (2-ME)	Fluka Sigma-Aldrich, Taufkirchen

Methanol, *p.a.*	J.T. Baker, Avantor Performance Materials, Center Valley, USA
Nuclease-free water	Promega, Mannheim
NuPAGE® Antioxidant	Invitrogen Life Technologies, Darmstadt
NuPAGE® MES SDS Running Buffer (20x)	Invitrogen Life Technologies, Darmstadt
(5Z)-7-Oxozeaenol (TAK1 inhibitor)	Calbiochem Merck, Darmstadt
panPKC LMWI (PKC inhibitor)	ALTANA Pharma, Konstanz
Phosphate buffered saline (PBS) Dulbecco, solide	Biochrom AG, Berlin
Poly(I:C)	Cayla InvivoGen, Toulouse, France
Polyoxyethylenesorbitan monolaurate (Tween® 20)	Sigma-Aldrich, Taufkirchen
PonceauS Solution	Sigma-Aldrich, Taufkirchen
Potassium chloride (KCl)	Fluka Sigma-Aldrich, Taufkirchen
Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Darmstadt
2-Propanol	Roth, Karlsruhe
Protein assay dye reagent concentrate	Bio-Rad, München
Rothiphorese® Gel 30 (37.5:1)	Roth, Karlsruhe
Skim milk powder	Fluka Sigma-Aldrich, Taufkirchen
Sodium azide (NaN_3)	Sigma-Aldrich, Taufkirchen
Sodium chloride (NaCl)	Roth, Karlsruhe
Sodium dihydrogen phosphate (NaH_2PO_4)	AppliChem, Darmstadt
Sodium dodecyl sulfate (SDS)	Serva Electrophoresis, Heidelberg
Sulfuric acid, 96% (H_2SO_4)	Roth, Karlsruhe
Synthetic triacylated lipoprotein (Pam_3CSK_4)	Cayla InvivoGen, Toulouse, France
3,3',5,5'-Tetramethylbenzidine dihydro-chloride (TMB)	Sigma-Aldrich, Taufkirchen
N,N,N',N'-tetramethylethylendiamine (TEMED)	Sigma-Aldrich, Taufkirchen
Titrisol®, hydrochloric acid (HCl, 1 N)	Merck, Darmstadt
Titrisol®, sodium hydroxide solution (NaOH, 1 N)	Merck, Darmstadt
D-(+)-trehalose 6,6'-dibehenate (TDB)	Avanti Polar Lipids, Alabaster USA
Tris-(hydroxymethyl)-aminomethane	Roth, Karlsruhe
Trypan blue	Serva Electrophoresis, Heidelberg

Zymosan	Sigma-Aldrich, Taufkirchen
Zymosan, FITC-conjugated	Molecular Probes®, Invitrogen Life Technologies, Darmstadt

2.2.2. Solutions and Buffers

Antibody incubation and blocking buffer	5% skim milk powder (w/v) in TBST, pH 7.4
alternatively	5% BSA (w/v) 0.1% Sodium azide in TBST, pH 7.4
CHAPS lysis buffer	30 mM Tris/HCl, pH 7.5 150 mM NaCl 1% CHAPS (w/v)
ELISA blocking buffer (assay diluent)	PBS 10% FBS
ELISA coating buffer	0.2 M Sodium phosphate (Na_2HPO_4 and NaH_2PO_4) pH 6.5
ELISA substrate buffer	0.2 M Na_2HPO_4 0.1 M Citric acid monohydrate 1 tablet TMB/10 ml buffer 2 µl 30% H_2O_2/10 ml buffer
ELISA wash buffer	PBS, pH 7.4 0.05% Tween 20
FACS buffer	PBS 3% FBS (v/v)

Laemmli buffer (5x)	250 mM Tris/HCl, pH 6.8 25% Glycerol (v/v) 10% 2-Mercaptoethanol (v/v) 5% SDS (w/v) 0.1% Bromophenol blue (w/v)
PBS	137 mM NaCl, pH 7.4 2.7 mM KCl 10 mM Na_2HPO_4 x $2H_2O$ 2 mM KH_2PO_4
SDS-PAGE running buffer	25 mM Tris, pH 8.3 2 M Glycine 1% SDS (w/v)
Stripping buffer	0.2 M Glycine/HCl 0.05% Tween 20 (v/v) pH 2.5 sterile-filtered
TBS	20 mM Tris, pH 7.4 137 mM NaCl
TBST	TBS, pH 7.4 0.025% Tween 20 (v/v)
Transfer buffer	50 mM Tris, pH 8.5 40 mM Glycine 0.03% SDS (w/v) 20% Methanol (v/v) (freshly added)
Trypan blue quenching buffer (5x)	250 mM Citric acid monohydrate 1.25 mg/ml Trypan blue 600 mM NaCl

2.2.3. Material and Media for Microbiological Culture

BBL™ CHROMagar Candida Medium (plates)	BD Biosciences, Heidelberg
C. albicans strain SC5314	obtained from Dr. Rudolf Rupec
Columbia Agar with 5% Sheep Blood (plates)	BD Biosciences, Heidelberg

2.2.4. Media and Supplements for Mammalian Cell Culture

Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Taufkirchen
Dulbecco's Phosphate buffered saline (DPBS), 1x	Gibco Life Technologies, Darmstadt
Dulbecco's Phosphate buffered saline (DPBS), 10x	Gibco Life Technologies, Darmstadt
Fetal bovine serum (FBS)	PAA Laboratories, Pasching, Austria
Fetal bovine serum (FBS), HyClone®	Thermo Scientific, Bonn
L-Glutamine (L-Glut), 200 mM	Gibco Life Technologies, Darmstadt
2-Mercaptoethanol (2-ME), 50 mM	Gibco Life Technologies, Darmstadt
Pen/Strep, Penicillin (10,000 U/ml)/ Streptomycin (10,000 µg/ml)	Gibco Life Technologies, Darmstadt
RBC lysis buffer, G-Dex™ II	iNtRON Biotechnology, Seongnam, Korea
Recombinant murine Granulocyte/Macrophage Colony-Stimulating Factor (GM-CSF)	Peprotech, Hamburg
RPMI 1640, cell culture media	Gibco Life Technologies, Darmstadt
Sodium pyruvate, 100 mM	Gibco Life Technologies, Darmstadt
Trypan blue stain, 0.4%	Gibco Life Technologies, Darmstadt

2.2.5. Antibodies

Anti-Actin, rabbit polyclonal IgG	Sigma-Aldrich, Taufkirchen
Anti-Bcl10 (C-17), goat polyclonal IgG	Santa Cruz Biotechnology, Heidelberg
Anti-Bcl10 (C78F1), rabbit monoclonal IgG	Cell Signaling Technology, Frankfurt/M.
Anti-Card9 (H-90), rabbit polyclonal IgG	Santa Cruz Biotechnology, Heidelberg
Anti-caspase-1 p10 (M-20), rabbit polyclonal IgG	Santa Cruz Biotechnology, Heidelberg
Anti-CD11c, PE-conjugated, armenian hamster polyclonal IgG	eBioscience, Frankfurt/Main
Anti-CD16/32 (blocks Fc binding), rat monoclonal IgG2a	eBioscience, Frankfurt/Main

Anti-Dectin-2, rat monoclonal IgG2a	AbD serotec, Düsseldorf
Anti-IκBα, rabbit polyclonal IgG	Cell Signaling Technology, Frankfurt/M.
Anti-IKKα, mouse monoclonal IgG1	Upstate Millipore, Schwalbach
Anti-IKKβ, mouse monoclonal IgG1	Upstate Millipore, Schwalbach
Anti-MHC Class II, FITC-conjugated, rat monoclonal IgG2b, kappa	eBioscience, Frankfurt/Main
Anti-mouse IgG HRP-linked, horse	Cell Signaling Technology, Frankfurt/M.
Anti-p44/42 MAPK (Erk1/2), rabbit polyclonal IgG	Cell Signaling Technology, Frankfurt/M.
Anti-Phospho-IκBα (Ser32/36) (5A5), mouse monoclonal IgG1	Cell Signaling Technology, Frankfurt/M.
Anti-Phospho-IKKα/β (Ser176/180) (16A6), rabbit monoclonal IgG	Cell Signaling Technology, Frankfurt/M.
Anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), rabbit polyclonal IgG	Cell Signaling Technology, Frankfurt/M.
Anti-Phospho-PKCδ (Tyr311), rabbit polyclonal	Cell Signaling Technology, Frankfurt/M.
Anti-Phospho-PLCγ2 (Tyr759), rabbit polyclonal	Cell Signaling Technology, Frankfurt/M.
Anti-Phospho-Syk (Tyr525/526), rabbit polyclonal	Cell Signaling Technology, Frankfurt/M.
Anti-Phospho-TAK1 (Thr184/187), rabbit polyclonal	Cell Signaling Technology, Frankfurt/M.
Anti-Phospho-Tyrosine (p-Tyr-100), mouse monoclonal IgG1	Cell Signaling Technology, Frankfurt/M.
Anti-PKCα, rabbit polyclonal	Cell Signaling Technology, Frankfurt/M.
Anti-PKCβ II (C-18), rabbit polyclonal	Santa Cruz Biotechnology, Heidelberg
Anti-PKCδ, rabbit polyclonal	Cell Signaling Technology, Frankfurt/M.
Anti-PKCθ, rabbit polyclonal	Cell Signaling Technology, Frankfurt/M.
Anti-PLCγ2, rabbit polyclonal	Cell Signaling Technology, Frankfurt/M.
Anti-rabbit IgG, HRP-linked, goat	Cell Signaling Technology, Frankfurt/M.
Anti-Syk, rabbit polyclonal IgG	Cell Signaling Technology, Frankfurt/M.

2.3. Methods

2.3.1. Cultivation of *C. albicans*

Candida albicans strain SC5314 was used for all experiments described here. Frozen *C. albicans* stored at -20°C were thawed, plated onto selective BBL™ CHROMagar Candida Medium plates, and incubated for 2 to 3 days at 30°C in a yeast incubator. 1 to 2 days prior to an experiment, a single colony was taken from a CHROMagar plate, resuspended in 200 µl of 1x PBS, re-plated onto Columbia Agar plates with 5% Sheep Blood, and incubated at 30°C. *Candida* colonies were harvested by carefully flushing and scraping them off the Columbia Agar plates, using 1x PBS and cell scrapers. The harvested suspension was centrifuged at 1.000 x g and 4°C for 3 minutes, the supernatant discarded, and the remaining pellet resuspended in one pellet volume 1x PBS (~125 µl). This yielded *C. albicans* suspensions with a density of 1 x 10^9 cells/ml. For differentiation of *Candida* cells to the hyphal form, an aliquot of the harvested and washed cells was resuspended in 10 ml B cell-medium (RPMI1640, 10% FBS, 1% L-Glut, 1% Pen/Strep, 0.1% 2-ME) and incubated in a water bath at 37°C for 3 hours (Bi *et al.*, 2010).

For experiments requiring a *C. albicans* negative control, an aliquot of cells was resuspended in a total volume of 500 µl 1x PBS and the fungi were killed by heat inactivation in a heating block at 95°C for 10 minutes. As final preparation prior to an experiment, differentiated or heat killed *Candida* were centrifuged at 1.000 x g and 4°C for three minutes, washed twice with 1x PBS, pelleted, and resuspended in one pellet volume 1x PBS. The resulting *Candida* solutions at densities of 1 x 10^9 cells/ml were then employed in stimulation experiments.

2.3.2. Mammalian Cell Culture

Cells were always handled under sterile conditions. In preparation for an experiment, cells were kept on ice at all times, unless indicated differently. All centrifugation steps were carried out at 400 x g, 4°C, and for 5 minutes if not otherwise stated. Incubation of cells was carried out in a humidified cell culture incubator at 37°C and with a constant level of 5% CO_2.

2.3.2.1. Bone Marrow Stem Cell Extraction

For the extraction of bone marrow stem cells (BMSCs), femur and tibia from the hind limb of mice were prepared and removed. After two disinfection steps in 70% ethanol and two washes

each in both 1x PBS and medium, the bones were stored in ice cold R10-medium (B cell-medium + 1% sodium pyruvate) until further processing. Medullar channels were flushed with R10-medium using 22G x 1¼ size needles or smaller and the gained cells were passed through a 100 µm cell strainer into a 50 ml Falcon tube. BMSC were pelleted and the cell pellet was resuspended in 1 to 2 ml of red blood cell (RBC) lysis buffer. After incubation for 5 minutes at RT, the reaction was stopped by adding 10 ml R10-medium and cells were passed through a 70 µm cell strainer into a fresh 50 ml Falcon tube. Followed by another round of centrifugation, BMSC pellets were resuspended in 10 ml Medium and counted under the microscope using a haemocytometer (counting chamber).

2.3.2.2. Freezing Bone Marrow Stem Cells

For long term storage, the pelleted BMSC of one mouse were resuspended in 900 µl pure FBS. The cell suspension was supplemented with 100 µl DMSO (cell culture quality), directly transferred into a cryovial, stowed in a cryo freezing container, and transferred immediately into a -80°C deep freezer. After 24 hours, the cryovials were transferred into a liquid nitrogen storage tank and kept there for future use.

2.3.2.3. Thawing Cells

Cells stored in liquid nitrogen were thawed by immersing the sealed cryovial into a water bath at 37°C for 20 seconds. Cells were then transferred immediately into 10 ml RPMI medium or 1x PBS and centrifuged. After aspiration of the supernatant, cells were resuspended in 10 ml RPMI medium with supplements and taken into culture as described in the following section.

2.3.2.4. Dendritic Cell Culture

DCs were generated by differentiation starting from BMSC. To this purpose, freshly prepared or thawed BMSC were counted, resuspended in DC-medium (RPMI1640, 10% heat inactivated FBS, 1% L-Glut, 1% Pen/Strep, 0.1% 2-ME and 20 ng/ml recombinant murine GM-CSF) at a final density of 1x 10^5 cells per ml and plated in 10 ml aliquots onto non-tissue culture treated 100 mm Petri dishes. On day 4 of differentiation, 10 ml of fresh DC-medium were added to each dish. The degree of differentiation was assessed by FACS-analyses (7-AAD$^-$, CD11c$^+$, MHCII^{+low}) on day 6 or 7 and immature DCs were taken into stimulation experiments.

2.3.3. Functional Assays

2.3.3.1. Stimulation of Dendritic Cells

DCs were harvested by flushing them off the petri dishes, dishes were washed with 1x PBS, and the remaining adherent cells incubated with 5 ml of 5 mM EDTA in 1x PBS per dish at 37°C in a cell culture incubator for 5 to 15 minutes. The reaction was stopped by addition of 5 ml RPMI-medium supplemented with 10% FBS, and the remaining cells were carefully removed with a cell scraper. All collected cells were centrifuged, resuspended in 10 ml B cell- or DC-medium, and counted. Cell density was adjusted to 1 x 10^6 cells/ml and cells were seeded at 2.5 x 10^5 per reaction in tissue culture treated 48-well plates. Thereafter, cells were allowed to rest for 30 to 60 minutes and stimuli and/or inhibitors were added as indicated for each experiment. Stimulations were carried out for the times indicated. Where indicated, cells were pre-treated for 60 to 120 minutes with the PKC inhibitors Gö6850, Gö6976, or panPKC LMWI, or with the TAK1 Inhibitor (5Z)-7-Oxozeaenol.

2.3.3.2. Cytokine Measurement

The production of intracellular cytokines as well as their release into the cell culture supernatant before and after stimulation was measured by means of plate-bound ELISA, so called "sandwich-ELISAs" (ELISA Ready-SET-Go!®, eBioscienec or BD OptEIA, BD Biosciences Pharmingen). This method works with special micro titer plates (Maxisorp 96-well plates) in a 96-well format, which are first coated with a defined concentration of a primary or capture antibody (50 µl per well) and incubated overnight. The next day the solution of capture antibodies was removed from the wells by inverting the plates into a sink or appropriate container and tap drying. Then, the wells were blocked to avoid unspecific binding by adding 400 µl of assay diluent per well and incubation for at least 60 minutes at RT. Thereafter, plates were washed three times in ELISA wash buffer and 50 µl of the samples or of a serial dilution of the appropriate standard were added to each well. Samples were laid out and analyzed in triplicate. Incubation of standard and samples were carried out overnight and plates were washed for 5 times the following day to remove excess and unbound antigen or cytokines. Next, the wells were incubated with 50 µl each of a working detector antibody mix, consisting of biotinylated antibodies raised against the cytokine of interest, linked to horseradish peroxidase (HRP) via streptavidin. The working detector antibody mix was incubated for 2 to 6 hours at RT in the dark (HRP is light sensitive). Plates were then washed for 7 times in order to thoroughly remove unbound antibodies and HRP and

fresh wash buffer was used after every second wash. Subsequently each well was incubated with 100 µl of TMB containing substrate buffer (tetramethylbenzidine in phosphatecitrate buffer) and the reaction was terminated by adding 100 µl of 2N H_2SO_4 per well as soon as the reaction mix started turning turquoise (15 to 60 minutes, depending on the cytokine being measured). The resulting, yellow colored, reaction mixtures were photometrically analyzed in a Sunrise microplate reader using Magellan software V 5.03 and measuring absorption at 450 nm (reference wavelength 570 nm). Reagents and buffers required for this method were prepared according to manufacturer's recommendations.

2.3.3.3. LDH-release Assay

In order to determine the potential toxicity of chemical agents administered to DCs during stimulations and inhibitor experiments, the release of lactate dehydrogenase (LDH) from treated cells was measured by means of a micro titer plate based assay in a 96-well format (Promega's CytoTox 96 Assay). The assay is designed to detect a coupled enzymatic reaction involving the conversion of iodonitrotetrazoliumchloride salt (INT) into a formazan product. This reaction is catalyzed by LDH release from the cultured and treated cells in addition to diaphorase present in the assay substrate mixture. Formazan concentrations are then determined by measuring optical absorbance at 492 nm in a 96-well format. The release of LDH into the cell culture supernatant correlates with the amount of cell death and membrane damage, providing an accurate measure of the cellular toxicity induced by the tested substance (Allen and Rushton, 1994). In brief, supernatants of stimulated cells were collected and 25 µl of each replicate were transferred into one well of a 96-well plate. As controls, freeze-thaw treated DCs (positive control, maximum LDH release), an internal LDH positive control provided by the manufacturer, and DC-medium (to define background signal) were integrated into each assay. Each vial of substrate-mix was reconstituted in 12 ml of assay buffer and 50 µl of this solution were added to each well. After 30 minutes incubation at RT in the dark, 50 µl of Promega Stop-solution were added to each well and absorption was measured at 492 nm using a Sunrise microplate reader (reference 0 nm). To calculate cytotoxicity, experimental LDH release (absorption measured minus background) was divided by maximum LDH release (absorption measured for freeze-thaw DCs minus background) and expressed as percent.

2.3.3.4. Flow Cytometry

FACS analyses were done to check for differentiation of BMSC to DCs or MPs, to analyze the expression of cell surface markers of interest, or to investigate potential pro-apoptotic effects of inhibitors administered to the cells. For each sample, 1 x 10^6 cells were transferred into a FACS tube and washed twice with FACS buffer. Cells were resuspended in 100 µl of FACS buffer and incubated with a 1:200 dilution of anti-CD16/32 (blocks Fc binding) antibody for 5 minutes at RT, to avoid unspecific binding of the FACS antibodies to the Fc receptors of the cells. Cells were washed once more with FACS-buffer and then incubated with the appropriate staining antibodies. Antibodies were incubated in the dark for 20 minutes at 4°C, washed, and additionally stained with 7-AAD to distinguish live and dead cell populations throughout the analyses. Samples were recorded on a FACS CantoII flow cytometer and analyzed using FlowJo (Tree Star, Inc.) software.

2.3.3.5. Internalization of Zymosan

For phagocytosis experiments cells were harvested as described above, washed, and resuspended in FACS buffer or RPMI-medium. 1 x 10^6 DCs were used for each replicate. FITC-conjugated Zymosan particles were added to final concentrations of 10 µg/ml, 30 µg/ml, or 100 µg/ml or cells were left untreated. After a short centrifugation step to synchronize phagocytosis, cells were incubated in a heating block at 37°C for 30 minutes to 3 hours, or kept on ice for the same period of time (negative control). Thereafter, cells were washed twice with ice cold 1x PBS, one time with ice cold FACS-buffer, and then stained against CD11c or left untreated. All cells were incubated with trypan blue quenching buffer for 5 minutes at RT in order to reduce false positive signals from FITC-labeled Zymosan adhering to the cell surface, followed by washing with ice cold FACS-buffer. Stained cells were analyzed by FACS measurement and the percentage of FITC positive cells within the $CD11c^{+high}$ population was displayed. Unstained cells were analyzed by fluorescent microscopy using a Nikon Eclipse TE2000-S microscope, together with a Nikon DS-5Mc digital microscope camera, a Nikon DS-U2 USB controller, and NIS Elements BR 3.10 imaging software.

2.3.4. Protein Analyses

2.3.4.1. Precipitation of Proteins from Cell Culture Supernatants

Cells were laid out on 24-well plates and stimulated in duplicate. Stimulation was carried out in a final volume of 550 µl for 6 hours and the plates were transferred to and kept on ice thereafter. Supernatants were harvested by transferring them into 1.5 ml reaction tubes, followed by centrifugation at 425 x g and 4°C for 5 minutes. From each sample/each tube 250 µl supernatant were transferred onto a 96-well plate and subjected to cytokine analyses. Duplicates of the remaining supernatants were pooled into one reaction tube and 500 µl MeOH and 100 µl Chloroform were added to each tube. After vigorous vortexing samples were centrifuged at ~20,000 x g and RT for one minute, leading to a separation of the mixture into phases: a chloroform phase at the bottom of the tube, a protein layer in the middle, and a phase consisting of methanol and medium at the top. The top aqueous phase was removed by aspiration without disturbing the interphase. To precipitate the proteins 500 µl of MeOH were added to each tube, followed by vortexing and centrifugation at ~20,000 x g and RT for one minute. The majority of the supernatant above the protein pellet on the floor of the reaction tube was removed carefully by aspiration and the pellet was dried under a chemical vapor hood for 30 minutes. 50 µl of 1x Laemmli buffer were added to each pellet and boiled at 95°C for 5 minutes. 20 to 25 µl of each sample were analyzed by SDS-PAGE and immunoblot, as described below.

2.3.4.2. Generation of Total Protein Lysates

Stimulated and unstimulated DCs were pelleted, washed with ice cold 1x PBS, and centrifuged at maximum speed (~20,000 x g, 4°C) for 1 minute. Supernatants were discarded and cell pellets snap frozen in liquid N_2. DCs were lysed by carefully pipetting them up and down in 100 µl of CHAPS lysis buffer and subsequent incubation on ice for 20 to 30 minutes. The lysates were centrifuged again at maximum speed and 4°C for 5 minutes in order to separate cell debris from the protein lysates. Supernatants were transferred to fresh 1.5 ml reaction tubes and aliquots taken for measurement of protein concentration (see Bradford assay). One volume 2x Laemmli buffer was added to each sample and the mixture was incubated at 95°C in a heating block for 5 minutes to denature the proteins. Afterwards samples were either directly transferred to SDS-PAGE for immunoblot analysis or stored at -20°C for future use.

2.3.4.3. Determination of Protein Concentration (Bradford Assay)

In order to provide equal loading of SDS gels for immunoblot analyses, the concentrations of protein lysates were determined by means of the Bradford assay. To this purpose, a serial dilution of 100x BSA was created using CHAPS lysis buffer, yielding standard concentrations of 5 µg/µl, 2.5 µg/µl, 1.25 µg/µl, 0.625 µg/µl, 0.3125 µg/µl, and 0 µg/µl (= blank). Standard and samples were analyzed in duplicate by pipetting 1 µl aliquots per reaction into one well of a 96-well microtiter plate and after adding 100 µl of 1:5 diluted Bio-Rad protein assay dye reagent concentrate per well. Absorbance was measured photometrically at 570 nm wavelength (reference 0 nm), using a Sunrise microplate reader.

2.3.4.4. SDS-PAGE and Immunoblot Analyses

Protein lysates were heated to 56°C for 5 minutes in order to avoid precipitation of glycerol in the loading buffer. Subsequently, 5 to 15 µg total protein per sample were loaded into one lane of an SDS-gel (4 to 12%) and proteins were separated by applying a voltage between 60 and 200 V until the blue front created by the loading dye had reached the bottom end of the gel. Gels were run in SDS-PAGE running buffer or in NuPAGE MES SDS Running Buffer with subsequent equilibration in transfer buffer at RT for 5 to 15 minutes. Nitrocellulose and polyvinylidene fluoride (PVDF) membranes were activated in distilled water or methanol, respectively, and kept in transfer buffer thereafter until assembly of the blot sandwich. In the next step, the separated proteins were transferred onto protein-binding nitrocellulose or PVDF membranes by immunoblot. This transfer was achieved by applying the semi-dry method at 25 V (equal to 150 mA per gel-membrane sandwich) for 60 minutes. Membranes were then stained with PonceauS reagent to control for equal loading. The stained membranes were photocopied and scanned on an Epson perfection 4990 photo scanner for later digital processing and documentation. The PonceauS reagent was removed from the membranes by four consecutive washes in 1x TBST for an average of 30 minutes. Bio-Rad and/or Invitrogen devices and equipment were used for casting and running gels, as well as for protein transfer.

2.3.4.5. Immunochemical Detection of Transferred Proteins

After the transfer, membranes were washed and incubated with a 5% solution of skim milk powder in 1x TBST for 90 to 120 minutes in order to avoid unspecific binding of proteins to the membranes. Since milk may contain phosphorylated proteins (e.g. casein) membranes were blocked in 5% BSA in TBST when antibodies directed against phosphorylated proteins were used. After blocking of unspecific binding, the membranes were incubated with specific

mouse or rabbit primary antibodies raised against an epitope within the protein of interest. Primary antibodies were used according to manufacturers' recommendations in either a 5% solution of skim milk or a 5% solution of BSA in 1x TBST and usually at a standard dilution of 1:1000. Incubation of the primary antibodies was done overnight at 4°C and the membranes with the antibody solutions were kept in motion on a rotating tube mixer. The next day, membranes were washed four times for 10 to 15 minutes each and then incubated with the appropriate HRP-linked secondary antibodies, directed against the corresponding IgG Fc region. This incubation was carried out for 2 hours at RT and using a standard antibody dilution of 1:2000 in 1x TBST. Membranes were washed again four times for 10 minutes each in 1x TBST and then incubated with ECL-solution. Detection of the signal was achieved by exposing highly sensitive x-ray film to the membranes for time intervals between 2 seconds and 15 minutes. The x-ray film was processed in a developing machine and subsequently scanned on an Epson perfection 4990 photo scanner for digital processing and documentation.

2.3.4.6. Removal of Antibodies (Stripping Membranes)

For sequential detection of multiple proteins on a single membrane, antibodies were removed by incubation in 10 ml stripping buffer for 20 minutes at 80°C in a water bath. Membranes were subsequently washed three times for 5 minutes with 1x TBST and then blocked for at least 60 minutes at RT with either 5% skim milk or 5% BSA in 1x TBST. This treatment was followed by incubation and detection with antibodies against the next protein of interest, as described in section 2.3.4.5.

3. RESULTS

3.1. Dectin-1 Signaling Depends on PKC Activity

3.1.1. Inhibitor Influence on Cell Survival

DCs play a central role in the detection of foreign invaders and express a wide range of PRRs, including several different CLRs (Banchereau *et al.*, 2000). To find out more about the potential involvement of PKC isoforms in myeloid CLR signaling, three different PKC inhibitors were tested in respect to their effects on primary bone marrow-derived dendritic cells (BMDCs). The indolocarbazole Gö6976 specifically targets conventional Ca^{2+}-dependent PKCs, whereas Gö6850 (also known as Bisindolylmaleimide I) functions as a panPKC inhibitor and impairs multiple isoforms in their activity (Toullec *et al.*, 1991; Martiny-Baron *et al.*, 1993). panPKC LMWI is a highly selective maleimide-based inhibitor that specifically blocks classical and novel but not atypical PKC isoforms (Hermann-Kleiter *et al.*, 2006). Such bisindolylmaleimide compounds are derived from the anti-fungal alkaloid staurosporine, which is produced by the bacterium *Lentzea albida* (formerly known as *Streptomyces staurosporeus*).

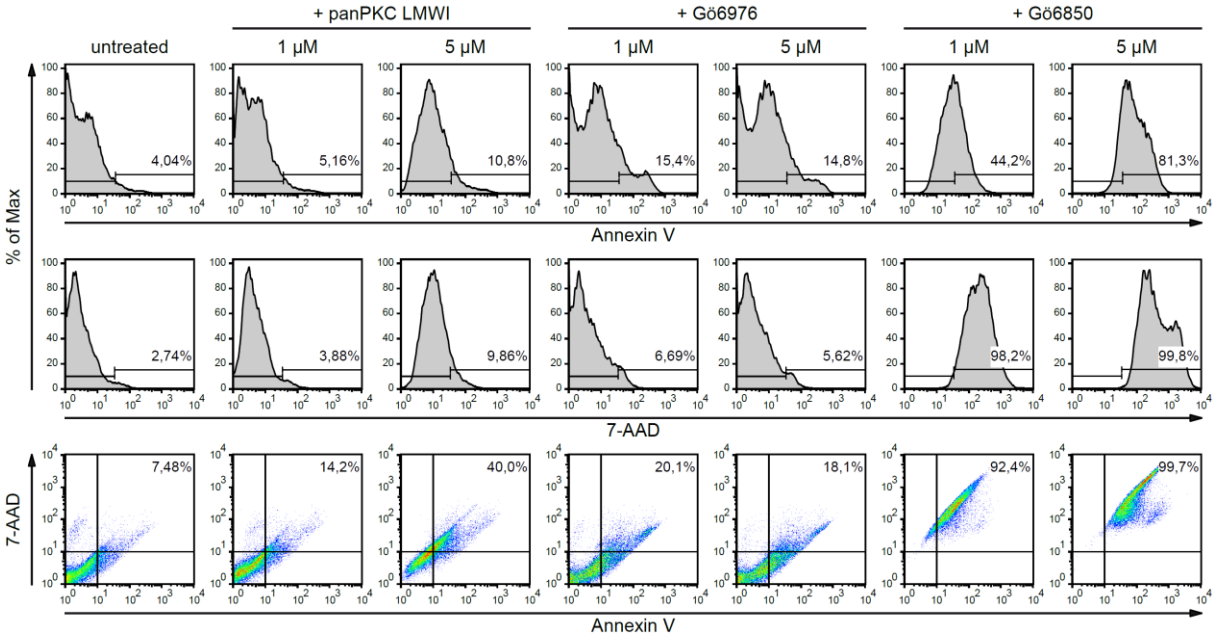

Figure 12: Unspecific Cytotoxic Effects of PKC Inhibitors.

BMDCs were left untreated or incubated with the indicated concentrations of panPKC LMWI, Gö6976, or Gö6850 for 8 hr without stimulation. Cells were stained with PE-Annexin V and 7-AAD followed by FACS

analysis to reveal demising cells. The frequencies of PE-Annexin V or 7-AAD positive cells (histograms) or double-positive cells (dot plots) are indicated. Double negative cells are alive and not undergoing detectable apoptosis. PE-Annexin V positive but 7-AAD negative populations represent cells undergoing apoptosis. Double positive cells are either in the late stage of apoptosis or necrosis or already dead.

In vitro studies performed in lymphocytes report that these substances block the PKC-dependent activation of NF-κB and the subsequent production of cytokines (Spitaler and Cantrell, 2004). Yet, the inhibitors function by competing with ATP and have to be employed at relatively high and thus possibly toxic concentrations in order to be efficient, as the intracellular concentration of ATP is ~1 mM (Isakov and Altman, 2012). To assess the influence of these substances on cell viability, BMDCs from wild-type (WT) mice were incubated with different concentrations of Gö6976, Gö6850, or panPKC LMWI and analyzed for apoptosis markers by flow cytometry. Cells treated with panPKC LMWI appeared largely unaffected and Gö6976 was found to have moderate cytotoxic effects. Gö6850, in contrast, strongly induced apoptosis of BMDCs and was therefore excluded from further experiments (Figure 12).

3.1.2. Inhibitor Effects on PRR signaling

In order to understand whether PKC isoforms are generally required for CLR signaling in myeloid cells, the inhibitors were next used in stimulation experiments. BMDCs from WT mice were pretreated with Gö6976 or panPKC LMWI and then exposed to either one of the CLR agonists zymosan or curdlan, or the TLR ligands LPS or CpG-DNA. Zymosan is a fungal cell wall preparation that consists primarily of β-glucans and functions as a strong activator of Dectin-1. In addition, it contains ligands for Dectin-2 and TLR2 (Brown and Gordon, 2001; Gross *et al.*, 2006; Taylor *et al.*, 2007; Robinson *et al.*, 2009). The pure β-(1,3)-glucan polymer curdlan, in contrast, selectively engages Dectin-1 (LeibundGut-Landmann *et al.*, 2007). panPKC LMWI inhibited the production of TNF, IL-10, and IL-2 in response to both CLR stimuli, whereas cytokine secretion induced by TLR4 stimulation with LPS or TLR9 stimulation with CpG-DNA was not impaired (Figure 13A). Pretreatment of the cells with Gö6976 lead to an inhibition of both CLR and TLR signaling, suggesting that conventional PKC isoforms might be components in the signaling cascades downstream of both classes of PRRs (Figure 13B). In parallel, LDH release assays were performed with cell culture supernatants from the same BMDCs, to validate that the measured differences in cytokine production between untreated and inhibitor-treated cells were not due to cytotoxic effects of the inhibitors. It was confirmed that BMDCs survive equally well under all tested

conditions (data not shown). Further, LDH release assays were conducted with supernatants from all following stimulation experiments shown in this study and equal cell survival was confirmed for the conditions applied (data not shown).

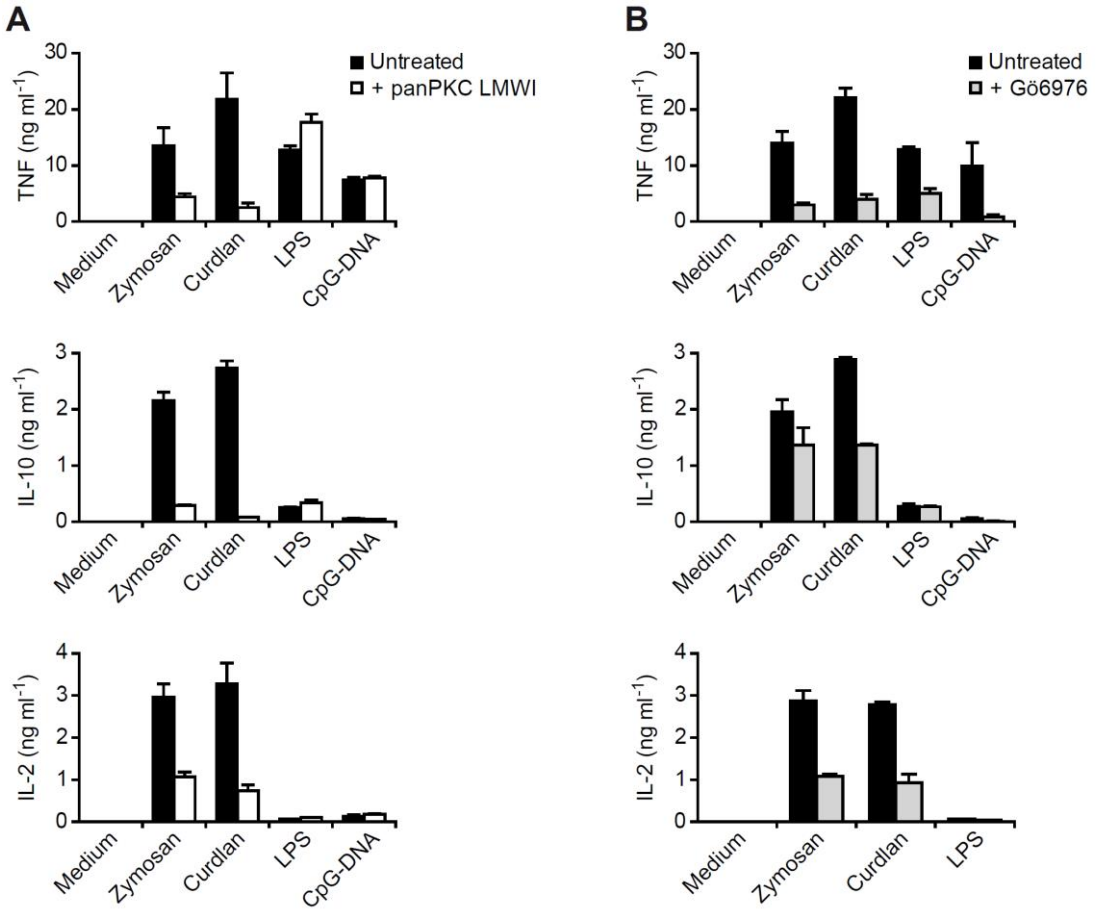

Figure 13: Dectin-1 Signaling Depends on PKCs.

(A) BMDCs were left untreated or preincubated with the PKC inhibitor panPKC LMWI (5 µM) and stimulated with zymosan (20 µg ml^{-1}), curdlan (400 µg ml^{-1}), LPS (200 ng ml^{-1}), or CpG-DNA (2 µM) for 6 hr. TNF, IL-10, and IL-2 concentrations in the supernatants were assayed by ELISA.

(B) The experimental setup described in (A) was repeated with the cPKC-specific inhibitor Gö6976 (500 nM). Data are expressed as means + SD of triplicate samples and were reproduced in independent experiments.

3.2. PKCδ Is Essential for CLR-Mediated Cytokine Production

3.2.1. Identification of the Relevant Isoform

The inhibitors Gö6976 and panPKC LMWI both have been reported to target several different PKC isoforms and it was therefore necessary to dissect the functions of individual PKCs in

CLR signaling (Martiny-Baron *et al.*, 1993; Hermann-Kleiter *et al.*, 2006). To this end, a genetic approach was chosen and BMDCs from mouse strains that are deficient in either PKCα (encoded by *Prkca*) (Leitges *et al.*, 2002b), PKCβ (encoded by *Prkcb*) (Leitges *et al.*, 1996), PKCβ and PKCθ (encoded by *Prkcq*) (Pfeifhofer *et al.*, 2003), or PKCδ (encoded by *Prkcd*) (Leitges *et al.*, 2001) were generated and used for stimulation experiments. BMDCs deficient in PKCα, PKCβ, or PKCβ and PKCθ produced regular amounts of TNF and IL-10 in response to zymosan treatment or upon specific Dectin-1 stimulation with curdlan (Figure 14A and data not shown).

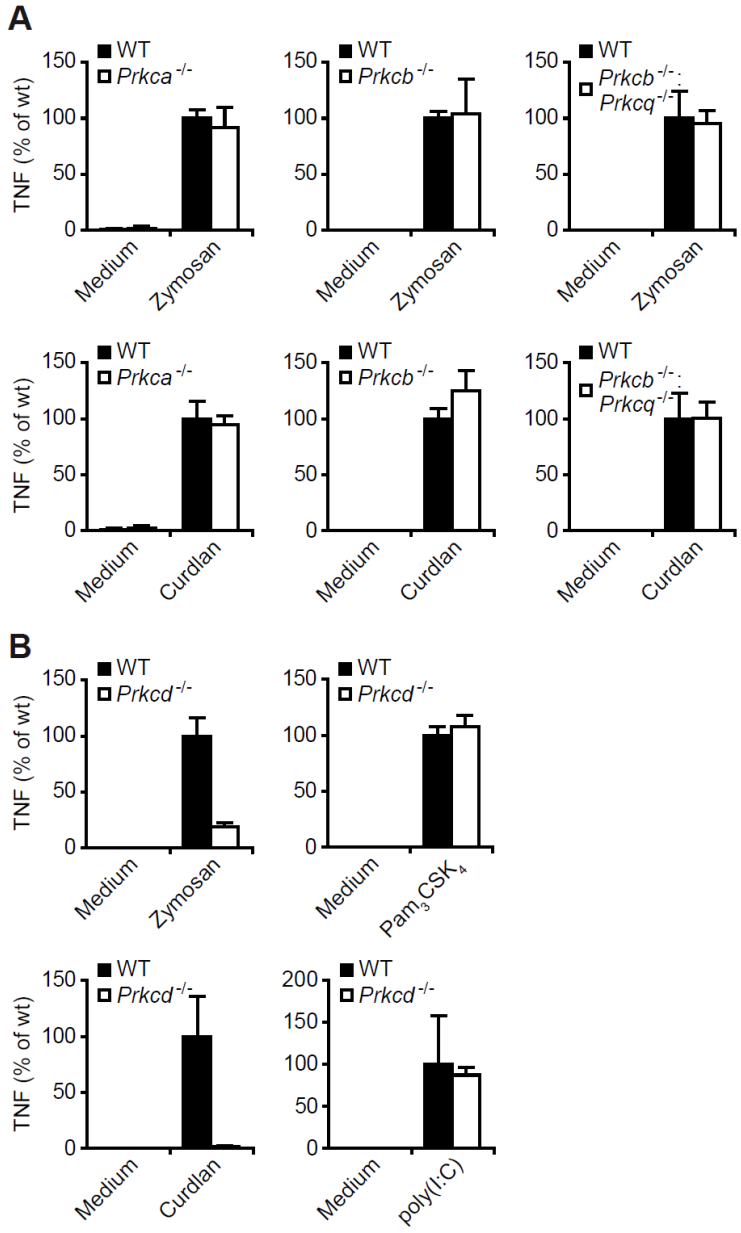

Figure 14: Productive Dectin-1 Signaling Critically Involves PKCδ.

(A) TNF production in *Prkca*$^{-/-}$, *Prkcb*$^{-/-}$, or *Prkcq*$^{-/-}$ BMDCs that were left untreated (Medium) or stimulated with zymosan (20 µg ml^{-1}) or curdlan (400 µg ml^{-1}) for 6 hr as indicated.

(B) *Prkcd*$^{-/-}$ BMDCs were stimulated as in (A) or with Pam_3CSK_4 (30 ng ml^{-1}) or poly(I:C) (30 µg ml^{-1}) and TNF production was analyzed by ELISA. Data are expressed as percent of WT + SD, derived from stimulations in triplicates and were reproduced at least three times in independent experiments.

In sharp contrast, *Prkcd*$^{-/-}$ BMDCs were severely impaired in zymosan- or curdlan-induced cytokine production, although responses to TLR1-TLR2 stimulation with Pam_3CSK_4 or TLR3 triggering with long poly(I:C) were not reduced (Figure 14B). Interestingly, BMDC differentiation was not impaired by the lack of any of these PKC isoforms as confirmed by FACS analysis (data not shown). Together, these findings indicate an essential and specific role for PKCδ in the Dectin-1 pathway.

To characterize the involvement of PKCδ in the Dectin-1 cascade in more detail, dose-response experiments with different PRR agonists were performed. Again, a critical requirement for PKCδ in zymosan- or curdlan-mediated TNF (Figure 15A) and IL-10 (Figure 15B) production was observed. Conversely, cytokine production in response to TLR4 stimulation with LPS was not affected by the deletion of PKCδ (Figure 15A).

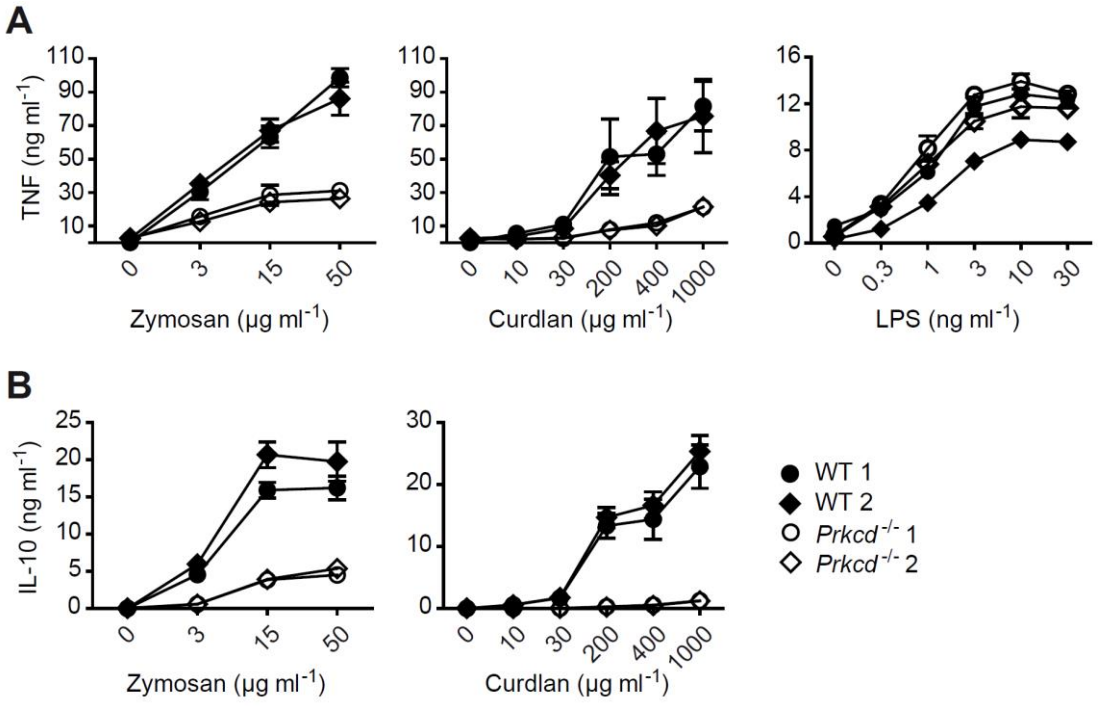

Figure 15: Selective Impairment of Dectin-1 Signaling in *Prkcd*$^{-/-}$ BMDCs.

WT and *Prkcd*$^{-/-}$ BMDCs were incubated with the indicated concentrations of zymosan, curdlan, or LPS. (A) TNF and (B) IL-10 concentrations in the supernatants were assayed by ELISA. Data from at least three independent experiments are expressed as means ± SD of triplicates.

3.2.2. Phagocytosis Functions Independently of PKCδ in BMDCs

Dectin-1 signaling triggers a strong cytokine response in BMDCs within a few hours only but not solely. Activation of the receptor further engages the uptake machinery of the cells to induce phagocytosis of fungal components, as well as the production of ROS. Therefore, the role of PKCδ in these pathways was studied. In contrast to cytokine production, phagocytosis of zymosan particles was found to be independent of PKCδ, as assessed by either fluorescence microscopy or flow cytometric quantification (Figure 16A and 16B). Additional experiments show that also zymosan-induced ROS generation was not significantly impaired in *Prkcd*$^{-/-}$ BMDCs (Strasser *et al.*, 2012).

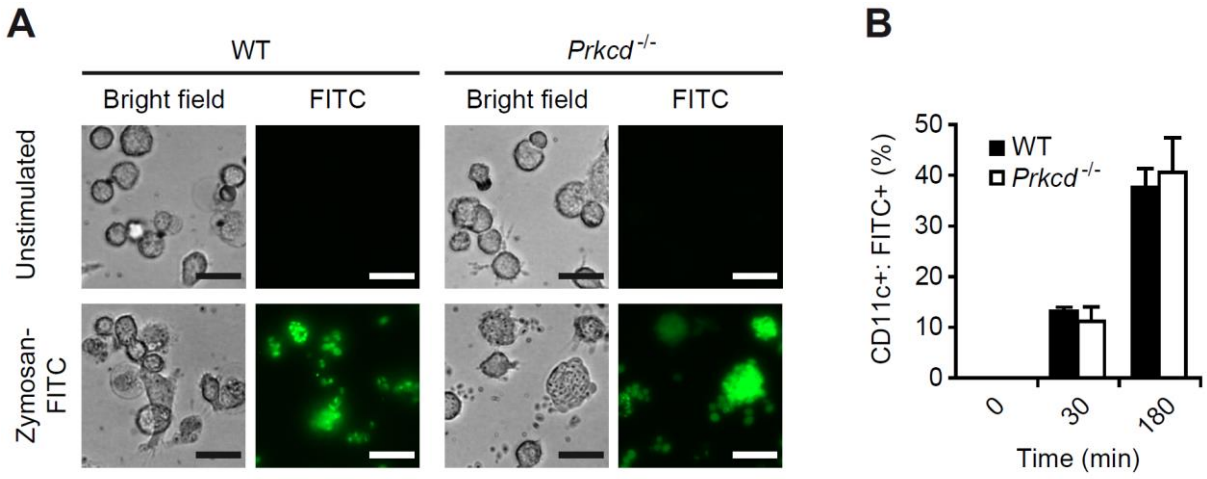

Figure 16: Phagocytosis Is Not Affected by Lack of PKCδ.

(A) BMDCs from WT and *Prkcd*$^{-/-}$ mice were incubated for 2 hr with FITC-zymosan particles (100 µg ml^{-1}). FITC-zymosan internalization was visualized by fluorescence microscopy (scale bars represent 20 µm).

(B) BMDCs were incubated with FITC-zymosan as in (A) for the times indicated. The frequencies of CD11c^{+} cells containing zymosan-FITC particles were quantified by FACS analysis. Results are representative of at least three independent experiments.

3.2.3. Several Syk-Coupled CLRs Depend on PKCδ for Signaling

In the next step, it was hence to be investigated, whether PKCδ functions also in Dectin-1-independent CLR responses. To this end, *Prkcd*$^{-/-}$ cells were stimulated with agonistic antibodies against Dectin-2 or with TDB, a synthetic adjuvant analog of the mycobacterial cord factor that functions as a selective and specific agonist for Mincle (Schoenen *et al.*, 2010). Similar to the Dectin-1-mediated responses, the absence of PKCδ also significantly impaired the production of IL-10, both after Dectin-2 or Mincle stimulation (Figure 17).

Together with the results from the phagocytosis experiments above, these findings indicate that PKCδ plays a general role in Syk-coupled CLR response pathways, where it is specifically required for the control of cytokine synthesis.

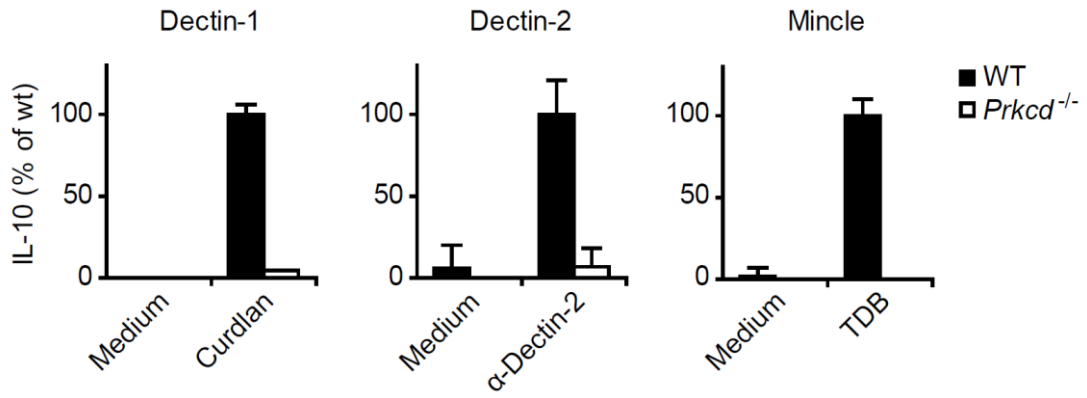

Figure 17: Syk-Coupled CLRs Require PKCδ for Signaling.

WT and *Prkcd*$^{-/-}$ BMDCs were stimulated through Dectin-1, Dectin-2, or Mincle with curdlan (20 µg ml^{-1}), plate-bound Dectin-2 antibody, or TDB (100 µg ml^{-1}), respectively. IL-10 concentrations in the cell-culture supernatants were quantified by ELISA. Data are expressed as percent of WT + SD, derived from stimulations in triplicates.

3.3. Zymosan Stimulation Triggers Tyrosine Phosphorylation of PKCδ

To learn more about the mechanisms of Dectin-1 signaling and how they depend on and involve PKCδ, the focus was shifted again to zymosan stimulation and selective Dectin-1 triggering. As expected, zymosan stimulation of BMDCs from WT mice induced tyrosine phosphorylation of multiple target proteins (Figure 18A). Subsequently, the specific phosphorylation of PKCδ in response to zymosan stimulation was analyzed in lysates of these cells. The experiments were performed by probing the samples with different phosphospecific antibodies raised against PKCδ serine (Ser) 643, threonine (Thr) 505, or tyrosine (Tyr) 311. While the phosphorylation status of PKCδ Ser643 and Thr505 remained unaltered in response to zymosan stimulation, a robust phosphorylation of PKCδ in its Tyr311 residue was detected, both in a dose- and time-dependent manner (Figure 18B, 18C, and data not shown). These results are in line with another study that describes PKCδ Tyr311 to be specifically required for activation of the kinase (Konishi *et al.*, 2001). In further experiments that were recently published, pharmacological inhibitors were employed to block Src and Syk signaling. The analyses showed that such a treatment of the cells prior to stimulation impedes zymosan-induced PKCδ phosphorylation in Tyr 311 (Strasser *et al.*, 2012). Taken together, these

results strongly suggest that innate recognition of zymosan activates PKCδ through a Src and Syk tyrosine kinase-dependent mechanism.

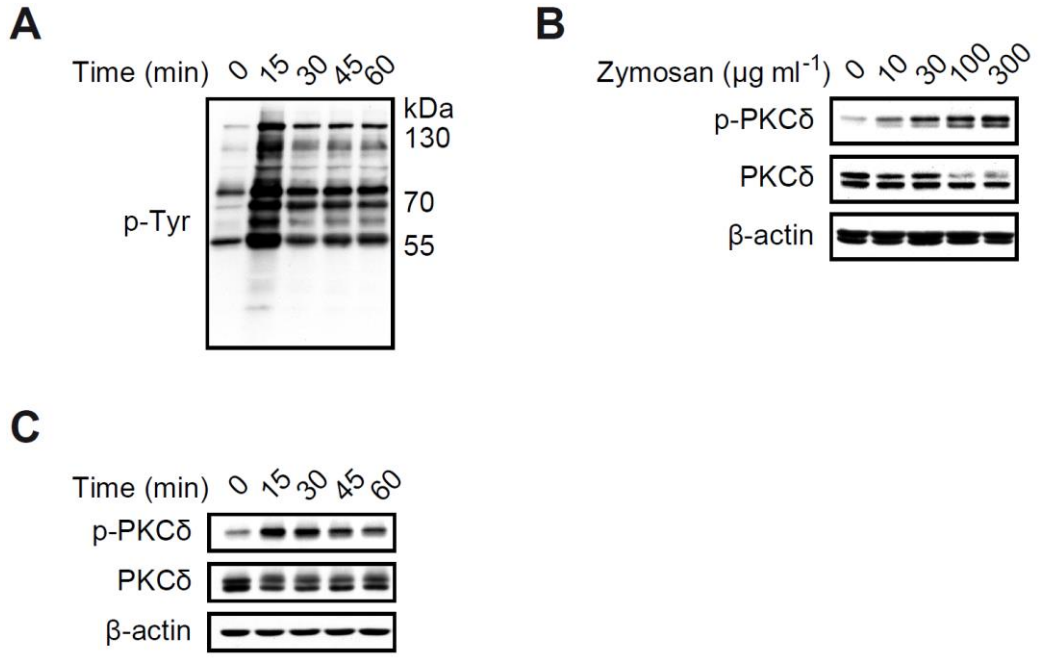

Figure 18: Dectin-1 Engagement Triggers Dose- and Time-Dependent PKCδ Tyrosine Phosphorylation.
(A) WT BMDCs were stimulated with zymosan (300 µg ml^{-1}) for the times indicated. Cellular lysates were subjected to immunoblotting and analyzed with phospho-Tyrosine (p-Tyr) specific antibodies.
(B) BMDCs from WT mice were incubated with increasing doses of zymosan. Activation of PKCδ was determined by immunoblot with antibodies against phospho-PKCδ (Tyr311). Immunoblotting with PKCδ and β-actin antibodies confirms equal sample loading.
(C) BMDCs were left untreated or stimulated with zymosan as in (A) at different time points. Lysates were analyzed by immunoblotting with antibodies against phospho-PKCδ, PKCδ, or β-actin.

3.4. PKCδ Regulates Dectin-1-Mediated NF-κB Signaling

3.4.1. NF-κB Signaling Is Compromised in PKCδ-Deficient BMDCs

The following set of experiments was designed to define the molecular function of PKCδ in CLR signaling more precisely (Figure 19A, 19B, and Figure 20). The overall tyrosine phosphorylation pattern, the activation of Syk, as well as the phosphorylation of the Dectin-1 signal transducer PLCγ2 (Xu *et al.*, 2009b) did not differ substantially between zymosan- or curdlan-stimulated WT and *Prkcd*$^{-/-}$ cells (Figure 19 and data not shown). Moreover, PKCδ

was, to a large extent, dispensable for Erk1 and Erk2 MAPK activation, as shown by the fact that Erk1 and Erk2 phosphorylations were only slightly reduced in *Prkcd*$^{-/-}$ cells (Figure 19B).

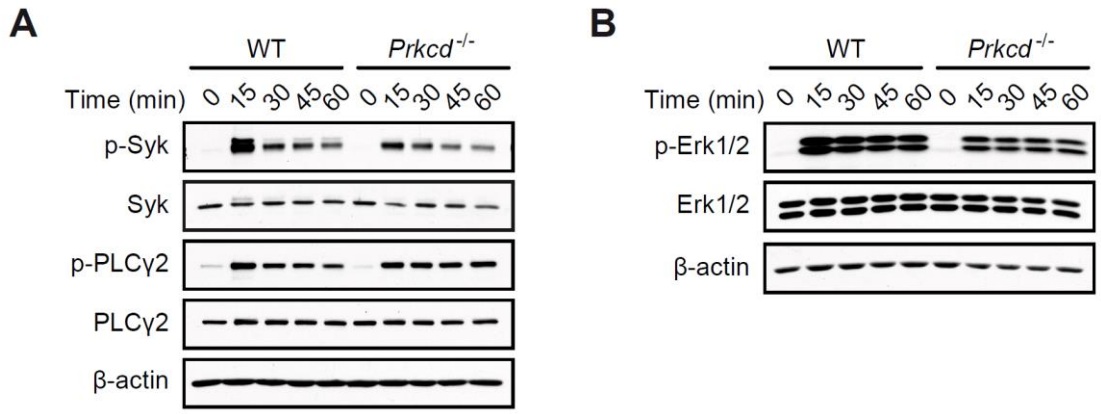

Figure 19: Syk, PLCγ2, and Erk MAPKs are Activated Normally in the Absence of PKCδ.

(A) BMDCs from WT or *Prkcd*$^{-/-}$ mice were stimulated with zymosan for the indicated times. Syk and PLCγ2 activation was determined by immunoblot with phospho-Syk or phospho-PLCγ2 antibodies. Immunoblotting with Syk, PLCγ2, and β-actin antibodies indicates equal protein loading.

(B) WT or *Prkcd*$^{-/-}$ BMDCs were stimulated with zymosan as indicated. Activation of the MAP kinases Erk1 and Erk2 was determined by immunoblot with phospho-Erk1/2 antibodies. Immunoblotting with Erk1/2 and β-actin antibodies indicates equal protein loading.

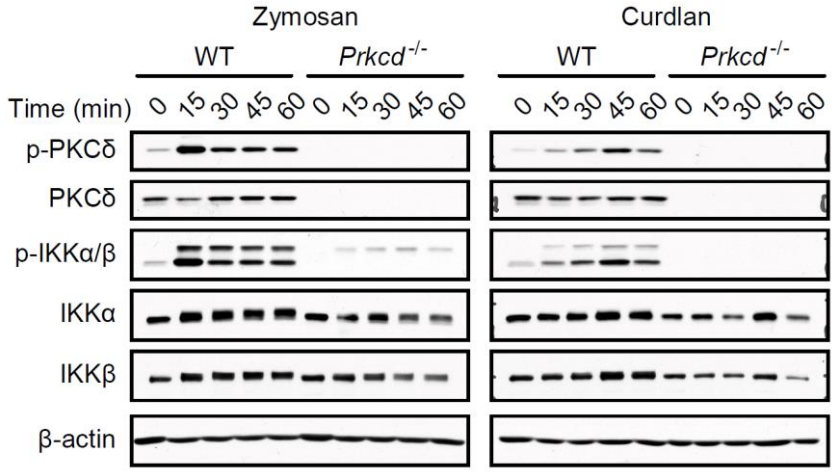

Figure 20: Defective NF-κB Signaling in *Prkcd*$^{-/-}$ BMDCs.

Cells were stimulated with zymosan or curdlan for the amount of time shown. Lysates were analyzed by immunoblot with phospho-PKCδ, phospho IKKα/β and PKCδ, IKKα, IKKβ, and β-actin antibodies.

In sharp contrast, signaling to the canonical NF-κB pathway, a key driver of cytokine production, was almost completely blocked in zymosan-treated or Dectin-1-stimulated

Prkcd$^{-/-}$ BMDCs, as indicated by the lack of phosphorylation in the activation loops of IKKα and IKKβ (Figure 20).

3.4.2. Card9 Is Activated Independently of PKCδ

As mentioned above, Dectin-1-Syk signaling engages the Card9 adaptor protein for canonical IKK-dependent NF-κB activation (Gross *et al.*, 2006; Mocsai *et al.*, 2010). To study the potential involvement of PKCδ in the Card9 signaling cascade, *Card9*$^{-/-}$ BMDCs (Gross *et al.*, 2006) were stimulated with zymosan and analyzed for PKCδ tyrosine phosphorylation. PKCδ was found to be activated normally in the absence of Card9 (Figure 21).

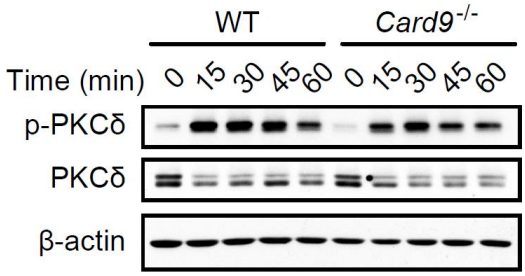

Figure 21: PKCδ is Activated Normally in *Card9*$^{-/-}$ Cells.
WT and *Card9*$^{-/-}$ BMDCs were incubated with zymosan (300 µg ml^{-1}) for different time intervals, as indicated. Lysates were immunoblotted and PKCδ engagement was analyzed with phospho-PKCδ antibodies. Immunoblotting against PKCδ and β-actin confirms equal protein loading.

In a recently published article the possibility was tested that PKCδ might directly phosphorylate Card9. Using *in vitro* kinase assays, the authors were able to demonstrate that PKCδ can phosphorylate Card9. Beyond that, they identified Thr231 as the critical residue out of a group of three predicted PKC phosphorylation sites and were able to show that PKCδ mediates Thr231 phosphorylation of Card9 and that this phosphorylation is essential for Card9 function (Strasser *et al.*, 2012).

3.5. PKCδ Activates TAK1 via Card9-Bcl10 Complex Formation

Upon cellular stimulation, Card9 forms a signaling complex with its adaptor protein Bcl10, leading to canonical IKK-dependent NF-κB signaling (Gross *et al.*, 2006; Mocsai *et al.*, 2010). To investigate the requirement for PKCδ in these events, Card9-Bcl10 complex assembly was analyzed in *Prkcd*$^{-/-}$ cells. To this end, Bcl10 was immunoprecipitated from

zymosan-stimulated WT and *Prkcd*$^{-/-}$ cells and its association with Card9 subsequently studied by protein immunoblot. Card9-Bcl10 complexes assembled only in zymosan-stimulated WT BMDCs but not in cells lacking PKCδ (Figure 22), indicating an essential function for PKCδ activity in Card9-Bcl10 complex formation.

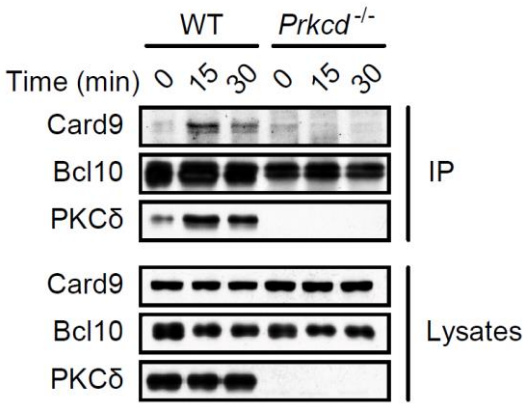

Figure 22: PKCδ Controls Card9-Bcl10 Complex Assembly.

WT and *Prkcd*$^{-/-}$ BMDCs were stimulated with zymosan for the indicated times and lysates subjected to immunoprecipitation with Bcl10-specific antibodies. Immunoprecipitates and total lysates were immunoblotted as indicated. The experiment depicted in this figure was performed by Hanna Bergmann. Adapted from Strasser *et al.* (Strasser *et al.*, 2012).

How the Card9-Bcl10 signalosome activates IKKs has not been well defined. However, given that Bcl10 can utilize the kinase TAK1 for T cell receptor-mediated NF-κB activation (Sun, L. *et al.*, 2004), one option to be considered was that TAK1 could also be activated through Card9-Bcl10 complex during innate immune responses.

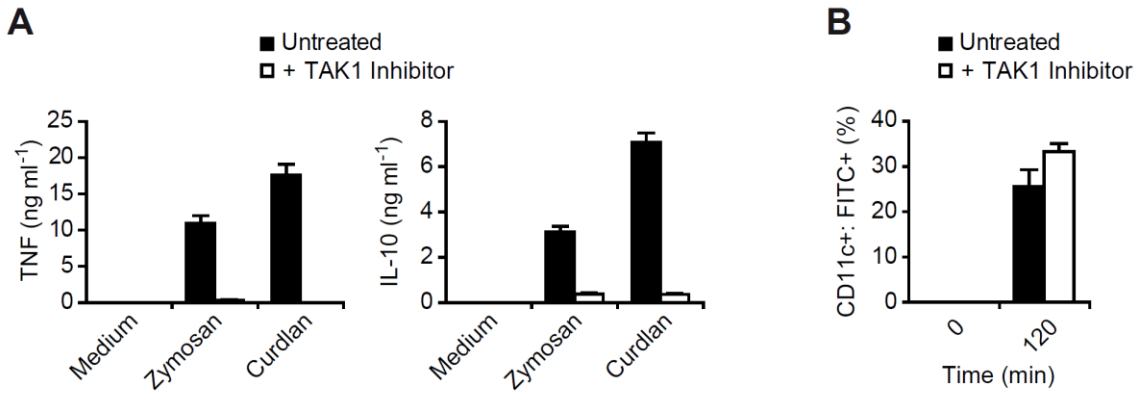

Figure 23: CLR Signaling but Not Phagocytosis Requires TAK1 Activity.

(A) WT BMDCs were incubated with the selective TAK1 inhibitor (5Z)-7-Oxozeaenol (250 nM) 30 min prior to stimulation with zymosan or curdlan. Cytokine concentrations in the cell culture supernatants were measured by ELISA 6 hr later. Data are expressed as means + SD of samples in triplicate.

(B) Cells from WT mice were pretreated with (5Z)-7-Oxozeaenol for 30 min and then incubated for 2 hr with FITC-Zymosan particles (100 µg ml^{-1}) or left unstimulated. FACS analysis was performed to quantify the frequencies of $CD11c^{+}$ BMDCs, which had internalized FITC-zymosan.

To test this hypothesis, WT BMDCs were incubated with the selective chemical TAK1 inhibitor (5Z)-7-Oxozeaenol (Ninomiya-Tsuji *et al.*, 2003) prior to stimulation. This treatment strongly impaired the production of TNF and IL-10 in response to zymosan or curdlan stimulation, whereas phagocytosis of FITC-labeled zymosan particles was not affected by TAK1 inhibition (Figure 23).

Thereafter, BMDCs lacking either Card9 or PKCδ were stimulated with zymosan and TAK1 phosphorylation was investigated. Indeed, zymosan-induced TAK1 phosphorylation was strictly dependent on Card9 (Figure 24A). Moreover and in line with the finding that *Prkcd*$^{-/-}$ BMDCs were defective in Card9-Bcl10 activation described above, zymosan-induced TAK1 activation was also abrogated in PKCδ-deficient cells (Figure 24B). To further investigate the function of TAK1 in zymosan-induced NF-κB signaling on a biochemical level, BMDCs were pretreated with (5Z)-7-Oxozeaenol before cell stimulation. Although TAK1 inhibition did not block zymosan-induced PKCδ activation, it prevented the activation of IKKs (Figure 24C).

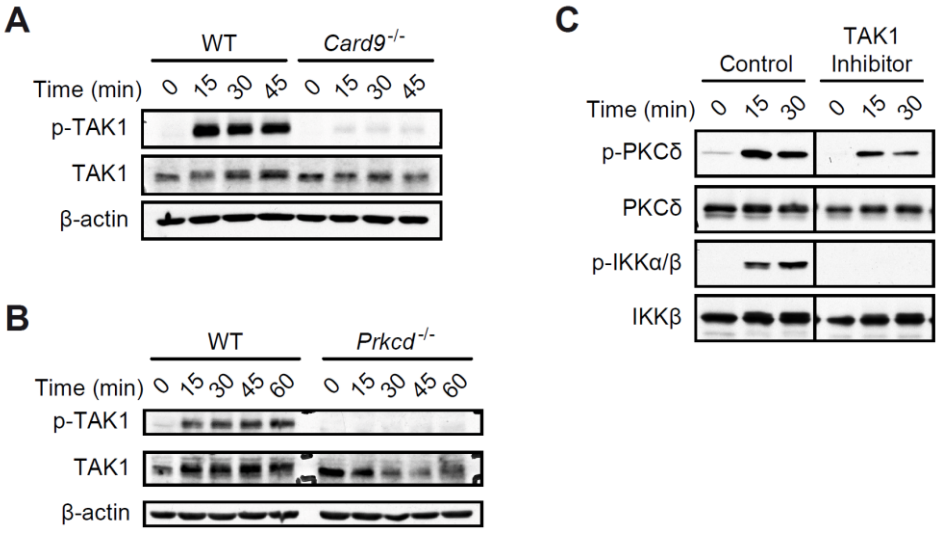

Figure 24: PKCδ Triggers Card9-Dependent TAK1 Activation.

(A) Card9-dependent TAK1 activation. WT or *Card9*$^{-/-}$ cells were stimulated with zymosan as indicated. Lysates were immunoblotted with antibodies against phospho-TAK1, TAK1, or β-actin.

(B) PKCδ mediates TAK1 activation. BMDCs from WT or *Prkcd*$^{-/-}$ mice were stimulated as in (A). Lysates were analyzed by immunoblot with antibodies against phospho-TAK1, TAK1, or β-actin.

(C) TAK1 signaling is critical for IKK activation. WT BMDCs were pretreated for 30 min with DMSO (control) or (5Z)-7-Oxozeaenol (2 µM) and stimulated with zymosan. Lysates were analyzed by protein immunoblotting for PKCδ and IKK activation with antibodies against p-PKCδ, PKCδ, p-IKKα/β, and IKKβ. The experiments depicted in this figure were performed by Hanna Bergmann. Adapted from Strasser *et al.* (Strasser *et al.*, 2012).

Together, these findings indicate that PKCδ plays an essential role upstream of the Card9-Bcl10 module, which is critical for the subsequent activation of TAK1 and the engagement of the canonical NF-κB pathway.

3.6. PKCδ Is Essential for Innate Anti-fungal Immune Defense

After uncovering the molecular functions of PKCδ in CLR signaling, it was intriguing to analyze its role in a pathophysiologically relevant setting. For these experiments *C. albicans* was chosen as a model pathogen. The opportunistic pathogenic fungus is of significant clinical importance and most pertinent in the context of this study. Its PAMPs are recognized by Dectin-1 (Taylor *et al.*, 2007), Dectin-2 (Robinson *et al.*, 2009), and Mincle (Wells *et al.*, 2008) and they drive Syk- and Card9-mediated innate immunity and host defense (Gross *et al.*, 2006; LeibundGut-Landmann *et al.*, 2007; Glocker *et al.*, 2009; Robinson *et al.*, 2009). Although *C. albicans* infection of WT BMDCs induced a robust and dose-dependent production of the cytokines TNF, IL-10, and IL-1β, production of these cytokines was almost completely abolished in the absence of PKCδ (Figure 25).

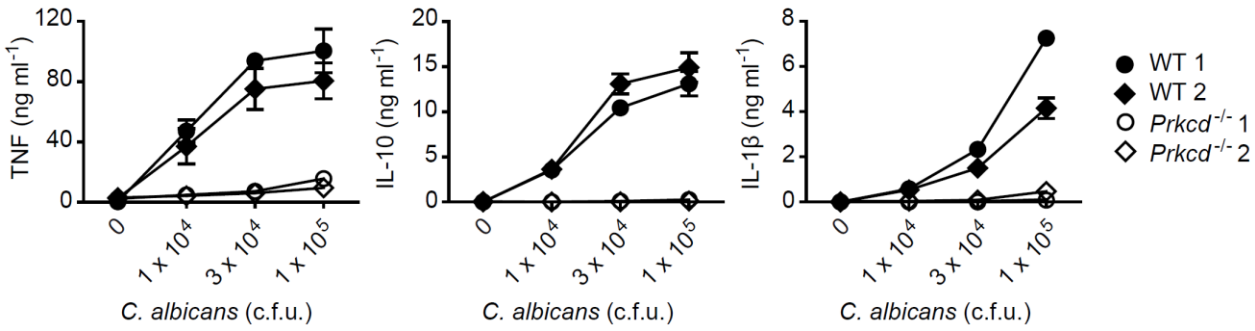

Figure 25: PKCδ Is Critical for *C. albicans*-Induced Cytokine Production.

BMDCs from WT and *Prkcd*$^{-/-}$ mice were incubated with increasing doses of live *C. albicans* hyphae. Concentrations of TNF, IL-10, and IL-1β in the culture supernatants were determined 6 hr later. Results are means ± SD of triplicates.

Assembly of the Nlrp3 inflammasome leads to caspase-1-mediated cleavage of pro-IL-1β and is hence a prerequisite for the secretion of mature IL-1β. This mechanism has been shown to be essential for host defense against *C. albicans* (Gross *et al.*, 2009). Moreover, activated cells release a caspase-1 precursor together with the active p10 fragment of the enzyme during this process. Therefore, WT and *Prkcd*$^{-/-}$ BMDCs were stimulated with *Candida* hyphae, followed by precipitation of the cell culture supernatants and immunoblot analysis. The enzyme precursor, as well as active caspase-1 p10, were released in comparable amounts by cells of both genotypes. These results, together with those from the experiment above in which cytokine levels produced by *C. albicans* stimulated BMDCs were measured, indicate that the production of pro-IL-1β requires PKCδ, whereas Caspase-1 is activated by a PKCδ-independent mechanism (Figure 26).

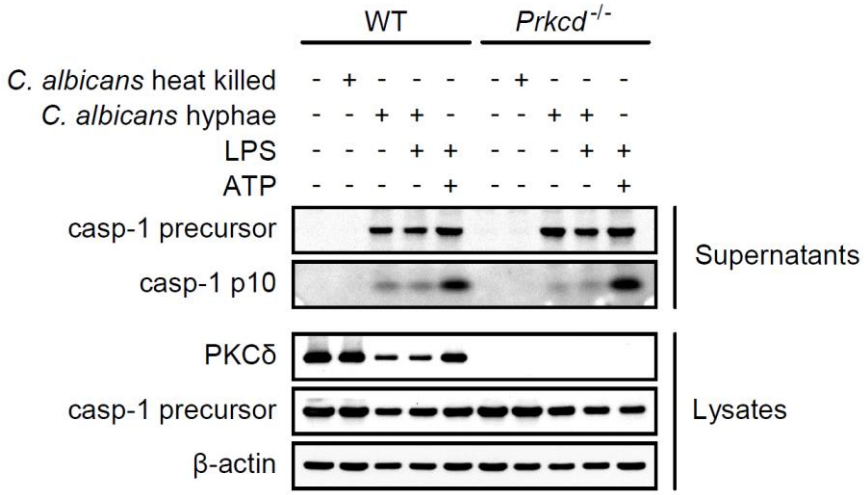

Figure 26: Caspase-1 Activation Functions Independently of PKCδ.
WT and *Prkcd*$^{-/-}$ BMDCs were exposed to *C. albicans* cells that had been heat inactivated (95°C for 10 min), or to live *C. albicans* hyphae (MOI = 3), LPS (5 ng ml^{-1}), and ATP (5 mM), for 6 hr as indicated. Cellular lysates and methanol-precipitates from cell culture supernatants were subjected to immunoblotting and subsequent detection of the relevant proteins with antibodies against the inactive caspase-1 (casp-1) precursor, the active enzyme fragment of approximately 10 kDa (casp-1 p10), or β-actin.

Further analysis on the biochemical level led to the observation that, in spite of normal Syk phosphorylation, *Prkcd*$^{-/-}$ BMDCs had severe deficiencies in activating NF-κB signaling after stimulation with *C. albicans* hyphae, a potent agonist of Dectin-2 (Saijo, S. *et al.*, 2010). In line with the results described above, *Prkcd*$^{-/-}$ cells furthermore showed defects in IKK activation, as well as in IκBα phosphorylation and degradation (Figure 27) after *C. albicans* recognition.

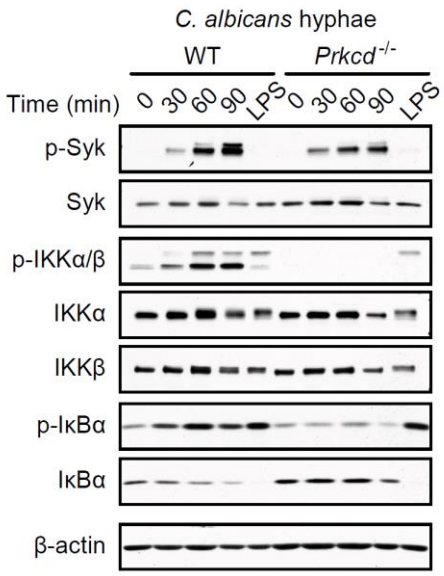

Figure 27: NF-κB Signaling in Response to *C. albicans* Infection Depends on PKCδ.
BMDCs were stimulated for the indicated times with *C. albicans* hyphae or for 30 min with 100 ng ml^{-1} LPS. Lysates were analyzed by immunoblot with antibodies against phospho-Syk, Syk, phospho-IKKα/β, IKKα, IKKβ, phospho-IκBα, IκBα, or β-actin.

In a recently published article, these studies were extended to investigate the relevance of PKCδ for host protection *in vivo*, by infecting *Prkcd*$^{-/-}$ mice with *C. albicans*. The authors found that, compared to the wild-type, PKCδ-deficient mice exhibited significantly greater weight loss upon infection and had much lower survival rates. In an independent set of experiments, they sacrificed the animals 8 days after infection and assessed intravital fungal growth. Consistent with an essential role for PKCδ in innate resistance *in vivo*, they observed massive fungal infiltration in the kidneys of *Prkcd*$^{-/-}$ mice by histopathology and detected significantly higher titers of *C. albicans* in the kidneys, livers, small intestines, and spleens of those animals (Strasser *et al.*, 2012).

4. DISCUSSION

Billions of patients suffer from medical conditions caused by pathogenic fungi every year and the mortality rate in cases of fatal infections ranges with that of malaria and tuberculosis. Incidences are predicted to further increase due to modern medical regimens and procedures or diseases, such as AIDS, that compromise the immune system. Hence, proper diagnostics in addition to safe, efficient, and affordable anti-fungal medication need to be developed or improved (Brown *et al.*, 2012). Such innovations require a profound mechanistic understanding of immune responses to fungal invaders as well as the identification of molecular targets that provide a lever for future anti-fungal drugs and vaccines. The results of the present study define an essential role for PKCδ in the activation of CLR-mediated, Card9-dependent innate immune responses. The findings are in line with the established functions of CLRs and Card9 in anti-fungal defense (Gross *et al.*, 2006; Taylor *et al.*, 2007; Ferwerda *et al.*, 2009; Glocker *et al.*, 2009; Saijo, S. *et al.*, 2010) and show an essential activity of PKCδ in host resistance against fungal pathogens.

4.1. Dectin-1-Syk Signaling Specifically Requires the PKCδ Isoform

The detection of fungal particles by Dectin-1 and the subsequent activation of Syk trigger various intracellular signaling pathways. Those include, most importantly, the central NF-κB cascade, signal transduction to the transcription factors NFAT and AP-1, and the engagement of MAP kinases (Mocsai *et al.*, 2010; Kerrigan and Brown, 2011; Osorio and Reis e Sousa, 2011). The results presented in this study show that the recognition of zymosan by Dectin-1 elicits phosphorylation of PKCδ at Tyr311 in both a time- and dose-dependent manner. A recent publication further reports that this particular phosphorylation event requires the concerted activity of Src-family kinases and of the kinase Syk (Strasser *et al.*, 2012). Altogether, these data suggest that PKCδ activation occurs downstream of Syk.

The Card9-Bcl10-Malt1 signalosome has been shown to function as the central hub of Dectin-1 signaling (Roth and Ruland, 2013). The results of this study here clearly illustrate that *Prkcd*$^{-/-}$ BMDCs are impaired in Card9-Bcl10 complex assembly, as well as in NF-κB control, in spite of normal Syk activation. *Card9*$^{-/-}$ mice, in contrast, were shown to phosphorylate and activate PKCδ normally after Dectin-1 stimulation. These data demonstrate that PKCδ acts upstream of Card9. Taken together with the findings described above, this indicates that PKCδ operates as a missing link between Syk signaling and Card9 complex

formation for the activation of innate immunity. As shown by the fact that only *Prkcd*$^{-/-}$ BMDCs, and not cells lacking PKCα, PKCβ, or PKCθ, were defective in Dectin-1-induced cytokine production, this study has identified PKCδ as the specific PKC isoform for signaling in the Dectin-1 pathway.

4.2. Signaling Through PKCδ Is Critical for Card9 and TAK1 Engagement

Here it is reported that the treatment of BMDCs with either one of two different small molecule PKC kinase inhibitors blocked zymosan- or curdlan-induced cytokine production in those cells. This finding strongly suggests that the enzymatic serine-threonine kinase activity, and not merely a scaffolding function of PKCδ, is responsible for its influence on Dectin-1-mediated signaling. In a recently published article, *in vitro* kinase assays lead to the discovery that Card9 is a direct substrate of PKCδ and, in addition, define the Card9 threonine residue Thr231 to be phosphorylated by PKCδ (Strasser *et al.*, 2012). Using *Card9*$^{-/-}$ cells in reconstitution experiments the authors identified PKCδ-mediated phosphorylation of Card9 Thr231 to be absolutely required for downstream signaling and cytokine production. The authors further suggest that PKCδ most likely targets additional Card9 residues as a Card9(T231A) mutant, which they had generated, was found to be still substantially phosphorylated *in vitro*. In this context, they propose Card9 Thr95 to be a putative additional PKCδ target site (Strasser *et al.*, 2012) and their finding is in line with results presented in other studies (Choudhary *et al.*, 2009). Moreover, they speculate that Thr95 phosphorylation of Card9 might be involved in protein stability (Strasser *et al.*, 2012).

The results of the work presented here report that PKCδ signaling is essentially required for Card9-Bcl10 complex assembly as well as for Card9-dependent TAK1 activation. Together with the above mentioned *in vitro* phosphorylation data from other studies, these findings allow for the postulation of a molecular model in which Syk-induced PKCδ activity mediates direct Card9 phosphorylation, resulting in Card9-Bcl10-complex assembly and subsequent TAK1 activation (Figure 28). TAK1 then most probably mediates Dectin-1-induced IKK activation, in a fashion similar to its mode of triggering NF-κB activity in response to stimuli from other immune receptors such as TLRs (Vallabhapurapu and Karin, 2009). This would be consistent with the observation that pharmacological blocking of TAK1

with (5Z)-7-Oxozeaenol inhibited zymosan- and curdlan-induced cytokine production and IKK activation but not phagocytosis (Figure 28).

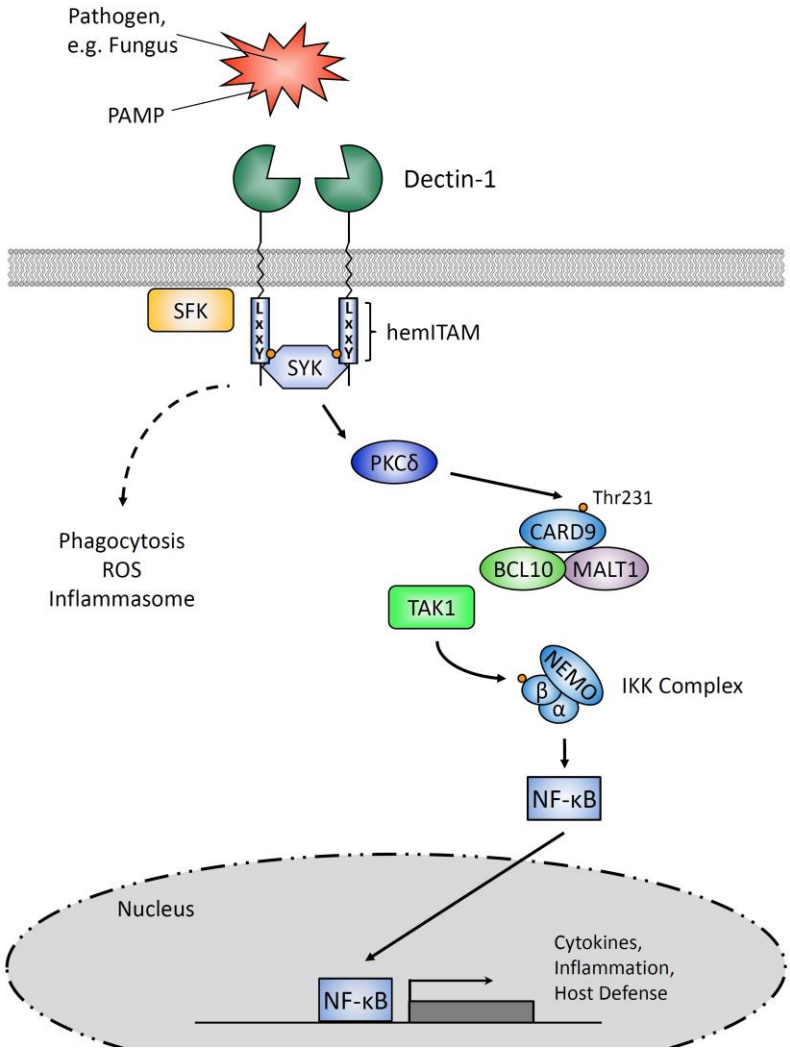

Figure 28: Molecular Model for the Role of PKCδ in the Dectin-1 Signaling Cascade.
Dectin-1 stimulation with activatory ligands causes phosphorylation of a tyrosine residue located within the hemITAM structure of the receptor's intracellular tail. SFKs are thought to mediate this activating modification, which allows for Syk recruitment and signaling to PKCδ. PKCδ, in turn, phosphorylates Card9 on Thr231, thereby triggering TAK1 engagement and the subsequent phosphorylation of IKKβ which ultimately results in the induction of NF-κB regulated gene transcription. Adapted from Roth and Ruland (Roth and Ruland, 2013).

4.3. PKCδ Selectively Regulates Dectin-1-Signaling Outcomes

Intriguingly, although the Card9 signaling pathway is severely impaired in *Prkcd*$^{-/-}$ BMDCs, the present study shows phagocytosis of zymosan particles to be unaffected by lack of PKCδ.

Moreover, the activation of Erk MAP kinase signaling was found to be only slightly reduced. A recently published article further reports the production of ROS to be largely independent of PKCδ (Strasser *et al.*, 2012). Taken together, these results indicate that PKCδ controls only specific subsets of the Dectin-1 responses. Another study shows that Dectin-1 ligation can activate the serine-threonine kinase Raf-1 through alternative mechanisms (Gringhuis *et al.*, 2009). This raises the possibility that Raf-1 might be responsible for Dectin-1-triggered and PKCδ independent Erk activation. Such a model would be consistent with the role of Raf-1 in activating MAPK pathways in numerous settings (Galabova-Kovacs *et al.*, 2006). Interestingly, it has been further reported that *Prkcd*$^{-/-}$ mice are similar to *Card9*$^{-/-}$ mice, as animals from both genotypes were found to be highly susceptible to fungal infections (Gross *et al.*, 2006; Strasser *et al.*, 2012). Therefore, it can be concluded that the specific PKCδ-Card9 effector response downstream of CLRs is absolutely critical for host defense.

Two genetic studies further underscore the aforementioned pivotal functions of Dectin-1 and Card9 in human anti-fungal immunity, as they report severe and chronic incidences of candidiasis in families that carry mutations in the respective genes (Ferwerda *et al.*, 2009; Glocker *et al.*, 2009). Of note, three articles that investigate the consequences of genetic PKCδ defects in humans have been published very recently (Belot *et al.*, 2013; Kuehn *et al.*, 2013; Salzer *et al.*, 2013). One of these studies describes the absence of PKCδ to be a so far unknown cause of common variable immunodeficiency-like B-cell deficiency, combined with severe lupus-like autoimmunity in one patient. From their findings, the authors conclude that PKCδ may function as an essential factor in the control of immune homeostasis and prevention of autoimmunity (Salzer *et al.*, 2013). The second publication characterizes a loss-of-function mutation in the human *PRKCD* gene (encoding the human PKCδ protein) to inflict chronic benign lymphadenopathy, in combination with self-reactive antibodies, and dysfunctional NK cells in patients. The authors further report this PKCδ deficiency to impair the control of proliferation and apoptosis in B lymphocytes and to interfere with the cytolytic activity of NK cells (Kuehn *et al.*, 2013). In the third study, Belot and colleagues examine three female consanguineous patients who carry a PKCδ missense mutation (Belot *et al.*, 2013). They confirm PKCδ as the cause for the systemic lupus erythematosus (SLE)-like phenotype described by Salzer *et al.* as well as the increased B cell expansion reported by Kuehn and coworkers. The findings presented in the articles by Kuehn *et al.* and Belot *et al.* therefore describe a phenotype which is comparable to that of a *Prkcd*$^{-/-}$ mouse strain (Miyamoto *et al.*, 2002).

4.4. PKCδ Functions as a General Mediator of CLR Signaling

Many of the experiments that were performed for this study aimed at identifying the principle mechanisms of CLR signaling. For this purpose, zymosan stimulation or selective Dectin-1 engagement with curdlan were utilized. Moreover it was found that in the absence of PKCδ, activation of the NF-κB pathway as well as cytokine production were also severely impaired in response to intact *C. albicans* cells. Recently published studies have indicated that *C. albicans* cells, and particularly *C. albicans* hyphae, are potent activators of Dectin-2 signaling (Saijo, S. *et al.*, 2010). Furthermore, it has been suggested that fungal hyphae additionally activate other CLRs such as the Mincle receptor (Wells *et al.*, 2008). Based on the combined insights from previous studies and the results presented in this work, it seems likely that PKCδ also couples signals from those other CLRs to the Card9-controlled, canonical NF-κB pathway (Figure 29). This hypothesis is in line with the observation presented here that *Prkcd*$^{-/-}$ BMDCs are defective in cytokine responses to selective agonists for Dectin-2 and Mincle, and formally establishes PKCδ as a general integrator of CLR function.

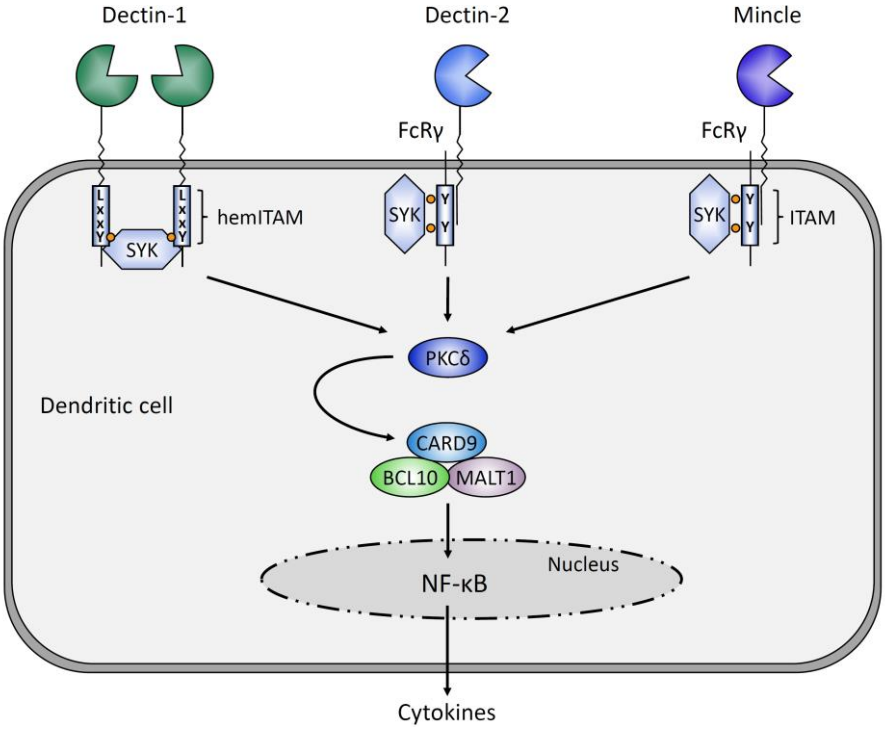

Figure 29: Schematic Representation of PKCδ Involvement in General CLR Signaling.

Several CLRs contain ITAMs or ITAM-like modules (hemITAMs) within their intracellular domains or associate with ITAM-containing adaptors, such as FcRγ, for signal transduction. Activation of such receptors consistently leads to the phosphorylation of tyrosine residues within the ITAMs or ITAM-like structures, which in turn allows for Syk kinase recruitment. Syk then channels the signal to the CBM complex, leading to NF-κB

activation and the production of cell-type specific and stimulus-dependent cytokine compositions. Adapted from Drummond *et al.* and Roth and Ruland (Drummond *et al.*, 2011; Roth and Ruland, 2013).

4.5. PKCδ in Host Defense Against Non-fungal Pathogens

The findings reported in the present study in all probability have implications beyond anti-fungal immunity, as the receptors analyzed are required for responses to an array of microbial invaders, parasites, and self molecules. Mincle and Dectin-1 both detect ligands on mycobacteria, in addition to recognizing mold-derived antigens (Rothfuchs *et al.*, 2007; Ishikawa *et al.*, 2009). Moreover, *Card9*$^{-/-}$ mice have been shown to be impaired in their ability to mount an inflammatory response following Mincle stimulation with the mycobacterial cord factor TDM (Werninghaus *et al.*, 2009; Schoenen *et al.*, 2010). Animals of this genotype further succumb rapidly to aerosol lung infection with *M. tuberculosis* (Dorhoi *et al.*, 2010). The tropical helminth parasite, *Schistosoma mansoni*, in contrast, has been characterized to activate Dectin-2 for signaling via Card9 (Ritter *et al.*, 2010) and viruses such as the Dengue virus induce inflammatory responses through the ITAM-coupled CLR Clec5a (Chen, S.T. *et al.*, 2008). Also, the recognition of self ligands has been reported for many CLRs. Dectin-1 binds to endogenous structures on T cells that remain to be identified in detail (Ariizumi *et al.*, 2000), the Syk-coupled CLR Clec9a recognizes ligands which become exposed upon cellular necrosis (Sancho *et al.*, 2009), and Mincle triggers Card9-dependent inflammatory responses upon binding to SAP130 from necrotic cells under non-infectious conditions (Yamasaki *et al.*, 2008). Together with the results from this study, it can therefore be postulated that PKCδ may also mediate innate responses to bacteria, parasites, or viruses or could be involved in immune responses to conditions of sterile cell damage and tissue injury. Yet, these hypotheses need to be tested.

4.6. Linking Innate to Adaptive Immunity via PKCδ

Finally, CLR-triggered Card9 signaling is not solely designed to regulate immediate innate anti-microbial responses. Importantly, the cascade also couples innate pathogen recognition to the activation of adaptive immunity (Kerrigan and Brown, 2011; Osorio and Reis e Sousa, 2011). For this purpose, triggering of the Card9 pathway by Dectin-1, Dectin-2, or Mincle ligands instructs professional APCs to synthesize a distinct combination of cytokines. This

characteristic cytokine milieu, in turn, potentiates the development of antigen-specific T_H17 cell responses and additionally induces T_H1 cell-mediated immunity (LeibundGut-Landmann *et al.*, 2007; Robinson *et al.*, 2009; Werninghaus *et al.*, 2009; Saijo, S. *et al.*, 2010). T_H17 cell responses are often associated with autoimmunity and Card9 polymorphisms are recurrently detected in human inflammatory conditions including Crohn's disease, ulcerative colitis, or the inflammatory arthritis subtype ankylosing spondylitis (Roth and Ruland, 2013). All of these inflammation-related disorders ultimately arise from aberrant NF-κB activity, yet, due to its broad implications in normal physiology, NF-κB itself may not be the ideal therapeutic target (Smale, 2011). As such, it will be important to test whether PKCδ signaling in innate cells favors T_H17 cell-mediated immune reactions and if aberrant activity of CLR-induced PKCδ-Card9 signaling contributes to human inflammatory disease. In that respect, PKCδ should be considered as a valid candidate for drugable targets and PKC inhibitors are already being applied in clinical trials. The findings presented in this work thus raise the possibility of therapeutically manipulating CLR-mediated Card9 signaling by altering PKCδ function.

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LIST OF ABBREVIATIONS

°C	Degree centigrade (degree Celsius)
(v/v)	Volume per volume
(w/v)	Weight per volume
7-AAD	7-aminoactinomycin D
A	Alanine, adenine
AIDS	Acquired immunodeficiency syndrome
AIM2	Absent-in-melanoma 2
Akt	V-akt murine thymoma viral oncogene homologue
ANK	Ankyrin repeat module
AP-1	Activator protein-1
APC	Antigen presenting cell
aPKC	Atypical PKC
APS	Ammoniumperoxoidsulfate, ammoniumpersulfate
ATP	Adenosine triphosphate
β-glucan	β-(1,3) or β-(1,6)-linked glucan
β-TrCP	β-transducin repeat containing protein
BAD	Bcl2-associated death promoter
BAFF	B cell activating factor
Bcl	B cell lymphoma
Bcl-x_L	Bcl-extra large
BCR	B cell receptor
Bim	Bcl2-interacting mediator
Bimp	Bcl10-interacting MAGUK protein
BIR	Baculovirus inhibitor of apoptosis protein repeat
Bis-Tris	Bisamino-trismethane
Blk	B lymphocyte kinase
BLNK	B cell linker, aka SLP-65
BMDC	Bone marrow-derived dendritic cell
BMSC	Bone marrow stem cell
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
C	Cysteine, cytosine
C. albicans	*Candida albicans*
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CARD	Caspase-associated recruitment domain
Card9	Caspase recruitment domain-containing protein 9
Card9$^{-/-}$	Card9-deficient
Carma	CARD-containing MAGUK protein
Casp-1	Caspase-1
Caspase	Cysteine-dependent aspartate-directed protease
Casp-L	Caspase-like
Cbl	Casitas B-lineage lymphoma
CBM	Carma1- or Card9-Bcl10-Malt1 containing complex
CBP	CREB-binding protein
CC	Coiled-coil
CD	Cluster of differentiation

CD28RE	CD28 response element
CD40L	CD40 ligand
C/EBPβ	CCAAT/enhancer binding protein β
c.f.u.	Colony forming units
CHAPS	Cholamidopropyl-dimethylammonio-propanesulfonate
cIAP	Cellular inhibitor of apoptosis
c-Jun	Subunit of the AP-1 transcription factor
Clec	C-type lectin-like receptor
CLL	Chronic lymphatic leukemia
CLR	C-type lectin receptor
c-Myc	Cellular myelocytomatosis protein
CNS	Central nervous system
CO_2	Carbon dioxide
COX-2	Cyclooxygenase 2
CpG-DNA	Cytosine-phosphate-guanine oligodeoxynucleotides (CpG-ODN)
cPKC	Conventional PKC
CR2	Type 2 complement receptor
CRD	Carbohydrate recognition domain
CREB	cAMP response element-binding protein
CRP	C-reactive protein
C-terminal/-terminus	Carboxy-terminal/-terminus
CTL	Cytotoxic or cytolytic T lymphocyte
CTLD	C-type lectin-like domain
C-type	Ca^{2+}-dependent type
CXCL	C-X-C motif ligand
Cyld	Cylindromatosis protein
D	Aspartic acid
DAG	Diacylglycerol
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DD	Death domain
Dectin	DC-associated C-type lectin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNGR-1	DC NK lectin group receptor-1
ds	Double-stranded
E	Glutamic acid
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
e.g.	*exempli gratia*, for example
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
Erk	Extracellular signal-regulated kinase
et al.	*et alteri*, and others
EYA4	Eyes absent 4
FACS	Fluorescence-activated cell sorting
FADD	FAS-associated death domain-containing protein
FBS	Fetal bovine serum
Fc	Fragment crystalizable
FcRγ	Fc-receptor γ chain

FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
g	Guanine, gravitational force
G	Glycine, guanine
GLK	GSK-like kinase
GM-CSF	Granulocyte macrophage colony-stimulating factor
GRR	Glycine-rich region
GSK	Germinal center kinase
GTP	Guanosine triphosphate
GUK	Guanylate kinase
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HMGB1	High-mobility group box 1 protein
HRP	Horseradish peroxidase
HTLV-1	Human T-lymphotropic/T-cell leukemia virus type 1
I	Isoleucine
ICAM-1	Intercellular adhesion molecule-1
i.e.	*id est*, that is
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of κB
IKAP	IKK complex-associated protein
IKK	IκB kinase
IL	Interleukin
IL-1R	IL-1 receptor
iNOS	Inducible nitric oxide synthase
INT	Iodonitrotetrazoliumchloride
IP_3	Inositol 1,4,5-trisphosphate
IPS-1	IFN-β-promoter stimulator-1
IRAK	IL-1R-associated kinase
IRF	IFN-regulatory factor
IRS	Insulin receptor substrate
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JNK	c-Jun N-terminal kinase
K	Lysine
kb	Kilo bases, 1000 bp
kDa	Kilo Dalton (unit indicating mass on an atomic or molecular scale)
KIR	Killer cell Ig-like receptor
L	Leucine
LAT	Linker of activated T cells
Lck	Lymphocyte-specific protein tyrosine kinase
LDH	Lactate dehydrogenase
LFA-1	Lymphocyte function-associated antigen-1
L-Glut	L-Glutamine
LGP2	Laboratory of genetics and physiology 2
LMWI	Low molecular weight inhibitor
LOX-1	Low-density Lipoprotein Receptor-1
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat

LT-β	Lymphotoxin-β
LZ	Leucine-zipper
µg	10^{-6} gram (microgram)
µl	10^{-6} liter (microliter)
µm	10^{-6} meter (micrometer)
µmol	10^{-6} mol (micromol)
µM (µmol/l)	10^{-6} molar (micromolar)
M (mol/l)	Molar
mA	10^{-3} Ampere (milliampere)
MAGUK	Membrane-associated guanylate kinase, aka PDZ-SH3-GUK
Mal	MyD88 adaptor-like
Malt	Mucosa-associated lymphoid tissue lymphoma translocation protein
MAPK	Mitogen-activated protein kinase
MBL	Mannose-binding lectin
MD2	Myeloid differentiation factor 2
Mda5	Melanoma differentiation-associated gene 5 protein
2-ME	β-mercaptoethanol
MEKK	MAPK/Erk kinase kinase
MeOH	Methanol
mg	10^{-3} gram (milligram)
MHC	Major histocompatibility complex
MICL	Myeloid C-type lectin-like receptor
mIg	Membrane-bound receptor immunoglobulin
Mincle	MP-inducible C-type lectin
ml	10^{-3} liter (milliliter)
mm	10^{-3} meter (millimeter)
mM (mmol/l)	10^{-3} molar (millimolar)
MOI	Multiplicity of infection
MP	Macrophage
MR	Mannose receptor
M. tuberculosis	*Mycobacterium tuberculosis*
MyD88	Myeloid differentiation primary response gene 88
N	Any nucleotide (adenine, guanine, thymidine, or cytosine)
N (mol/l)	Normal
N_2	Nitrogen
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NAK	Synonym for TBK1
NAP1	NAK-associated protein 1
NEMO	NF-κB essential modulator, aka IKKγ
NES	Nuclear export signal
NF-κB	Nuclear factor κB
NFAT	Nuclear factor of activated T cells
NF-IL-6	Nuclear factor IL-6
ng	10^{-9} gram (nanogram)
NIK	NF-κB-inducing kinase
NK cell	Natural killer cell
NKG2	NK group 2
N-linked glycosylation	Attachment of a sugar molecule to a nitrogen atom of an amino acid
NLR	Nod-like receptor

Nlrp3	NLR family, pyrin domain containing protein 3
NLS	Nuclear localization signal
nm	10^{-9} meter (nanometer)
nM (nmol/l)	10^{-9} molar (nanomolar)
NO	Nitric oxide
Nod	Nucleotide-oligomerization domain
nPKC	Novel PKC
N-terminal/-terminus	Amino-terminal/-terminus
O_2^-	Superoxide anion
P	Proline
p.a.	*pro analysi*, indicates a substance of high chemical purity
PAGE	Polyacrylamide gel electrophoresis
Pam_3CSK_4	Tripalmitoylated lipopeptide (cysteine-serine-lysine$_4$)
PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
PDK1	Phosphoinositide-dependent kinase 1
PDZ domain	Post-synaptic density-95/discs large/zonula occludens-1 domain
PDZ-SH3-GUK	aka MAGUK
PE	Phycoerythrine
Pen	Penicillin
PEST	Proline-, glutamic acid-, serine-, and threonine-rich region
PGE_2	Prostaglandin E_2
pH	*pondus Hydrogenii*
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKB	Protein kinase B, also known as Akt
PKC	Protein kinase C
PKD	Protein kinase D
PLCγ	Phospholipase C γ
Poly(I:C)	Polyinosinic polycytidylic acid
Prkca$^{-/-}$	PKCα deficient
Prkcb$^{-/-}$	PKCβ deficient
Prkcd$^{-/-}$	PKCδ deficient
Prkce$^{-/-}$	PKCε deficient
Prkcq$^{-/-}$	PKCθ deficient
Prkcz$^{-/-}$	PKCζ deficient
PRR	Pattern recognition receptor
PS	Pseudosubstrate domain or motif
PVDF	Polyvinylidene fluoride
R	Any purine (adenine or guanine)
Raf-1	Rapidly accelerated fibrosarcoma-1
Ras	Rat sarcoma
RBC	Red blood cell
Rel	Reticuloendotheliosis oncogene
RHD	Rel homology domain
RICK	Receptor-interacting serine-threonine kinase, aka RIP2
RIG-I	Retinoic acid-inducible gene I
RIP	Receptor-interacting protein
RLR	RIG-I-like receptor
RNA	Ribonucleic acid

RNF125	RING finger protein 125
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
S	Serine
SAP130	Sin3A-associated protein of 130kDa
SAPK/JNK	Stress-activated protein kinase/c-Jun N-terminal kinase
SD	Standard deviation
SDS	Sodium dodecyl sulfate
Ser	Serine
SFK	Src family protein tyrosine kinase
SH2	Src homology 2
SH3	Src homology 3
SIGN	Specific intercellular adhesion molecule-3-grabbing non-integrin
SIGNR1	SIGN-related gene-1
SINTBAD	Similar to NAP1/TBK1 adaptor
SLE	Systemic lupus erythematosus
SLP-65	SH2 domain containing leukocyte protein of 65 kDa, aka BLNK
SLP-76	SH2 domain containing leukocyte protein of 76 kDa
Sp1	Simian virus 40 promoter factor 1
SPAK	STE20-SPS1-related proline-alanine-rich protein kinase
S-phase	Synthesis phase
Src	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
SRR	Signal responsive region
Strep	Streptomycin
Syk	Spleen tyrosine kinase
T	Threonine, thymine
TAB	TAK1-binding protein
TAD	Transcriptional activation or transactivation domain
TAK1	TGF-β-activated kinase 1
TANK	TRAF family member-associated NF-κB activator
TAP	Transporter of antigenic peptides
T-bet	T box expressed in T cells
TBK1	TANK-binding kinase 1
TBP	TATA-binding protein
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
TCR	T cell receptor
TDB	Trehalose 6,6’-dibehenate
TDM	Trehalose 6,6’-dimycolate
TEMED	Tetramethylethylenediamine
TFIIB	Transcription factor IIB
TGF-β	Transforming growth factor β
T_H cell	T helper cell
T_H1	Type 1 T_H cell
T_H2	Type 2 T_H cell
T_H17	IL-17 secreting T_H cell
Thr	Threonine
TIR domain	Toll/IL-1R homology domain
TIRAP	TIR domain-containing adaptor protein

TLR	Toll-like receptor
TMB	Tetramethylbenzidine dihydrochloride
TNF	Tumor necrosis factor
TNFR	TNF receptor
TRADD	TNFR-associated death domain protein
TRAF	TNFR-associated factor
TRAM	TRIF-related adaptor molecule
T_{reg}	Regulatory T cell
TRIF	TIR domain-containing adaptor inducing IFN-β
TRIM25	Tripartite motif-containing 25 E3 ubiquitin ligase
Tris	Tris-(hydroxymethyl)-aminomethane
TUM-GS	Graduate School der Technischen Universität München
Tween 20	Polyoxyethylenesorbitan monolaurate
Tyr	Tyrosine
U	Unit
Ubc13	Ubiquitin-conjugating enzyme 13
UV	Ultra violet
V	Volt
VCAM-1	Vascular cell adhesion molecule-1
WASP	Wiskott-Aldrich syndrome protein
WB	Western blot = immunoblot
WIP	WASP-interacting protein
WT	Wild-type
n x g	Number (*n*) multiplied with g = relative centrifugal force (RCF)
X, x	Arbitrary amino acid or nucleotide
Xid	X-linked immunodeficiency
Y	Tyrosine, any pyrimidine (thymine or cytosine)
ZAP-70	Zeta-chain-associated tyrosine protein kinase 70

LIST OF FIGURES AND TABLES

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