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Deciphering The C-Type Lectin Receptor Signaling Pathway In Macrophages In Response To Candida Albicans

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A

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Dedication

My parents, Rebecca and Saeed, for inspiring me to achieve my goals and for always offering to help me in anyway they could.

My brother, Daniel, for keeping me motivated when I needed it the most and for being my best friend.

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Deciphering the C-Type Lectin Receptor Signaling Pathway in Macrophages in Response to *Candida albicans*

Publication No._____

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Candida albicans causes opportunistic fungal infections in humans and is a significant cause of mortality and morbidity in immune-compromised individuals. Dectin-2, a C-type lectin receptor, is required for recognition of *C. albicans* by innate immune cells and is required for initiation of the anti-fungal immune response. We set out to identify components of the intracellular signaling cascade downstream of Dectin-2 activation in macrophages and to understand their importance in mediating the immune response to *C. albicans in vivo*. Using macrophages derived from Phospholipase-C-gamma 1 and 2 (PLCγ1 and PLCγ2) knockout mice, we demonstrate that PLCγ2, but not PLCγ1, is required for activation of NF-κB and MAPK signaling pathways after *C. albicans* stimulation, resulting in impaired production of pro-inflammatory cytokines and reactive oxygen species. PLCγ2-deficient mice are highly susceptible to infections with *C. albicans,* indicating the importance of this pathway to the anti-fungal immune response. TAK1 and TRAF6 are critical nodes in NF-κB and MAPK activation downstream of immune surveillance and may be critical to the signaling cascade initiated by C-type lectin receptors in response to *C. albicans*. Macrophages derived from both TAK1 and TRAF6-deficient mice were unable to activate NF-κB and MAPK and consequently failed to produce inflammatory cytokines characteristic of the response to *C. albicans*. In this work we have identified PLCγ2, TAK1 and TRAF6 as components of a signaling cascade downstream of *C. albicans* recognition by C-type lectin receptors and as critical mediators of the anti-fungal immune response. A

mechanistic understanding of the host immune response to *C. albicans* is important for the development of anti-fungal therapeutics and in understanding risk-factors determining susceptibility to *C. albicans* infection.

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Abbreviations

APC-Antigen Presenting Cell

BCL10- B cell Lymphoma protein 10

BCR- B Cell Receptor

BLNK- B-Cell Linker protein

BMDM- Bone Marrow Derived Macrophages

BTK- Burton's Tyrosine Kinase

CARD- Caspase Recruitment Domain

CARMA-Caspase Recruitment Domain and Membrane Associated Guanylate Kinase-like Domain

DAG-Diacylglycerol

ELISA- Enzyme-linked Immunosorbent Assay

EMSA- Electrophoretic Mobility Shift Assay

ERK- Extracellular Regulated Kinase

IκBα- Inhibitor of NF-κB alpha

IKKα- IκBα Kinase alpha

IKKβ- IκBα Kinase beta

IKKγ- IκBα Kinase gamma

IL-1- Interleukin 1

IL-6- Interleukin 6

IL-10- Interleukin 10

IP- Immunoprecipitate

IP3- Inositol Triphosphate

IRAK1- IL-1 Receptor Associated Kinase 1

JNK- c-Jun N-terminal Kinase

LPS- Lipopolysaccharide

MAGUK- Membrane Associated Guanylate Kinase-like Domain

MALT1- Mucosa Associated Lymphoid Tumor Protein 1

MAPK- Mitogen Activated Protein Kinase

MAP3K- Mitogen Activated Protein Kinase Kinase Kinase

M-CSF- Macrophage Colony Stimulating Factor

MHC- Major Histocombatibility Complex

MyD88- Myeloid Differentiation Primary Response Gene 88

NADPH- Nicotinamide Adenine Dinucleotide Phosphate

NF-κB- Nuclear Factor kappa B

NOD- Non-Obese Diabetic

OCT 1- Octamer protein 1

PKC- Protein Kinase C

PLCγ- Phospholipase-C gamma

PMA- Phorbol 12- Myristate 13-Acetate

RANKL- Receptor Activator of NF-κB ligand

ROS- Reactive Oxygen Species

TAK1- Transforming Growth Factor beta Kinase 1

TCR- T Cell Receptor

TLR- Toll- Like Receptor

TNFα- Tumor Necrosis Factor alpha

TNFR1-TNFα Receptor

TRAF6-TNFR Associated Factor 6

Chapter 1

Introduction

Chapter 1: Introduction

1.1. Opportunistic fungal pathogens

Of 1415 species of known pathogenic infectious organisms for humans, 307 species are fungi (1). The molds, which comprise the majority of fungal species, grow as multicellular filaments. The evolutionary advanced forms of molds grow hyphae containing septa, which divide the filaments. Fungal infections in humans can cause cutaneous, subcutaneous, allergic and systemic diseases. Fungal infections can be divided into two types. The first group is virulent fungi that are pathogenic regardless of an adequate host immune system such as *Blastomyces*, *Histoplasma* and *Paracoccidiodies.* The second group constitutes low virulence fungi that need immunocompromised host or an altered bacterial flora to manifest as an infection (2). The most common opportunistic fungal infections today are the *Aspergillus* and *Candida* species.

Aspergillus species causing invasive infections have an incidence of 5-24% in acute leukaemia and 25-40% in chronic granulomatous disease (3). *Candida* species are the fourth most common bloodstream pathogen found in hospitals (4). *Candida* species are found as colonizers of the genitourinary, gastrointestinal tracts and at lower levels it is found on the skin. Of Candida species, *Candida albicans* is responsible for approximately 95% of vaginal *Candida* infections (5). From approximately 200 species of *Candida* only a few species are known to cause infections in humans. Aside from *C. albicans, C. krusei, C. glabrata, C. parapsilosis* are known for candidiasis (6). The bacterial flora at different body sites is a

major determinant of *Candida* colonization. Although the mechanisms are not well understood, competition for nutrients is thought to play a pivotal role. For example lactobacilli residing in the vagina produce lactic acid and hydrogen peroxide, which creates an environment that is not ideal for *Candida* growth (7). Furthermore the ability of *C. albicans* to adapt to different niches in the host is important for the switch to pathogenicity. In different host niches the accessibility to nutrients and pH varies (8). When colonization by *C. albicans* does occur, the host niche may change by *C. albicans* metabolizing nutrients thereby changing the pH and possibly damaging the host tissues (8).

Candidemia can be a life-threatening infection, especially in immunocompromised and critically ill patients (9, 10). Although *C. albicans* still remains the predominant strain to cause candidemia, in recent years there has been a progressive shift in infections caused by non-*albicans* species (11, 12). The most common non-*albicans* species causing candidemia are *Candida glabrata,* followed by *Candida krusei* and *Candida tropicalis* (13). *Candida* infections can affect any hospitalized patient but are more common in certain populations, including patients with cancer, hematological malignancy or other immunosuppression. Factors predisposing all intensive care unit patients to candidemia include, intravascular catheters, use of broad-spectrum antibiotics, diabetes, transplantation, corticosteroid therapy, acute renal failure, haemodialysis and pancreatitis (14).

1.2 *Candida* dimorphism

Candida albicans is known to be able to grow in multiple morphological forms. At the epithelial surfaces *Candida* species colonize only as yeasts. *Candida* yeast are ellipsoid

single cells which divide by budding. In order to successfully invade the host tissue *Candida* hyphal growth is necessary which has been demonstrated by *Candida* mutants which are incapable of germinating (15). Hyphal growth starts from a germ tube that extends into a long filament where the cells are separated by septae (16). In between the yeast and hyphal forms *C. albicans* can also grow in a variety of forms referred to as pseudohyphae in which the daughter cell forms a septa but remains attached to the mother cells in an elongated form. The switch from yeast to filamentous form can be triggered by various environmental conditions. Experimentally, hyphal growth can be triggered from yeast cells by adding serum and culturing at temperatures of 37°C. In addition, Lee's media which is nutrient-poor or chemical inducers such as *N*-acetyl-glucosamine, together with culturing at of 37°C can also induce hyphal growth (17). The yeast to hyphae transition is under transcriptional control and genes that regulate the hyphal stage are regulated by a complex network of signaling pathways (18). Serum-induced filamentation is under the control of the transcription factor Tec1 (19). Alkali induced filamentation is controlled by the transcription factor Rim101 (20). In addition to these transcription factors, there are numerous other transcription factors that have been shown to regulate yeast to hyphal transition under certain growth conditions (17).

The cyclic AMP–protein kinase A complex (PKA) pathway is a major pathway in *C. albicans,* which can induce hyphal growth. A range of environmental signals can regulate cAMP-PKA activation, either by direct or indirect activation of adenylyl cyclase (21). When *C. albicans* is grown at high densities, this can lead to the accumulation of the quorum sensing molecule farnesol, that can downregulate cAMP-PKA signaling, thereby inhibiting

hyphal formation. This indicates that cell density at sites of infection can also affect *C. albicans* morphogenesis (22).

In addition to the yeast to hyphal transition, *C. albicans* can also switch from a white round yeast morphology to an elongated form termed opaque, that has an altered cell surface structure (23). Most *C. albicans* strains are *MTL*a/α heterozygous and can produce the a1-α 2 repressor, which inhibits white to opaque switching by inhibiting the expression of WOR1 gene (24). The opaque cells are thought to be the mating-competent form of *C. albicans* and strains that are homozygous at the mating type locus (*MTL***a**/α) are able to switch from the white to opaque form (25).

The white to opaque switch can contribute to better adaptation of *C. albicans* to the host niches. The switch between white and opaque forms occurs at a low frequency. This low frequency of switching allows a partially stable maintenance of the white and opaque forms and ensures that in a population some cells are preadapted to altered environments when encountering new host niches (26). In host niches with lower temperatures, such as the skin, it is thought that *C. albicans* switching from white to opaque may facilitate mating (27). White to opaque switching not only occurs spontaneously but also can be induced by environmental signals. Anaerobic conditions can induce white to opaque switching that is mediated by the transcription factor Czf1p. This anaerobically induced switching can occur in the mammalian gastrointestinal tract at 37° (24). The white to opaque switching can influence virulence as macrophages preferentially phagocytose white cells (28).

1.3 Candida cell surface

Candida cell wall is the first surface of physical interaction of the fungi with its host. The skeletal component of the cell wall is a structure of β-glucans and chitin. Aside from the skeletal components the cell wall matrix is composed of glycosylated proteins, which represent about 40% of the dry weight of the cell wall (29). Mannoproteins, which are used as markers for discriminating between fungal species, are bound to the β-glucan and chitin layer. β-(1,2)-mannans are absent in *S. cerevisiae* but are present in three different types in different *Candida* species (30, 31). From the polysaccharides in the fungal cell wall β-glucans are the most abundant. They are found as β-(1-3)- linked glucose polymers that contain β-(1-6)-linked side chains. In order for the chains of β-(1-3)- glucans to grow, a transglycolysation of glucosyl residues from the UDP-glucose to polysaccharide chains must occur (32). Mannans and glucans can be released by *C. albicans* into the surrounding medium such as infected patients blood and in mouse models these molecules have been shown to induce coronary arthirits and anaphylactic shock (Nakagawa 2003).

The cell wall proteins present in *C. albicans* are two classes. The most abundant class, glycophosphatidylinositol (GPI)-anchored cell wall proteins (GPI-CWPs) are linked to β-1,6- glucans. The second class are named Pir proteins which are directly linked to β-1,3-glucans (33). From (GPI)-anchored cell wall proteins, the agglutinin-like sequence (ALS) gene family are the best characterized. These proteins promote the adhesion of *C. albicans* to host tissues (34, 35). Immunohistochemical approches show the expression of of ALS proteins in *C. albicans* infecting mouse liver, heart, lungs and kidneys (36). Cell wall

proteins can also affect the pathogenicity of *C. albicans*. Many *C. albicans* mutants that lack cell wall proteins have reduced virulence and extracted cell wall proteins can induce arthritis in mice (37).

1.4 Innate immunity

When infections occur, the host immune system has developed specific cellular and molecular mechanisms to fight infections. These reactions typically are accompanied by signs of an inflammatory response, which include heat, swelling and pain. The host anti-fungal immune responses range from innate immune recognition to adaptive immunity. The phagocytic family of innate immune cells consists of neutrophils and macrophages. Innate immune cells can react to pathogens immediately through responses such as phagocytosis, production of reative oxygen species, production of lytic enzymes and nitric oxide (38). Neutrophils are the most abundant leukocyte in the blood and are the very first responders to infections due to their ability to rapidly cross through the vessel endotheliums and phagocytose and kill the pathogens in the infected tissues (39). Furthermore, tissue-resident dendritic cells and macrophages can produce an array of cytokines and chemokines to create a site of local inflammation. These secreted pro-inflammatory cytokines and chemokines alongside antigen presentation from innate immune cells are essential for initiating an adaptive immune response (40). In addition these cytokines and chemokines can interact with endothelial cells in capillary vessels at the sites of infection to loosen endothelial cell-cell contact and produce specific adhesion molecules. These adhesion molecules are

important for the recruitment of phagocytes to endothelial cells and their invasion into infected tissue (41, 42).

The innate immune cells have evolved in a manner that they only recognize structures that are present on pathogens therefore, eliminating the possibility of attacking the host mammalian cells. These structures have been named pathogen-associated molecular patterns (PAMPs) and their respective receptors are now known as pattern recognition receptors (PRRs). Neutrophils express moderate levels of Toll-like receptors (TLR) and high levels of Fc gamma receptors and complement receptors. Monocytes express moderate levels of C-type lectin receptors but high levels of TLRs. Macrophages and dendritic cells express high levels of both TLRs and C-type lectin receptors (43) (Table. 1). In the following sections I will focus on C-type lectin receptors and their prominent role in mediating fungal immunity.

Table 1. Recognition of Fungi by Innate Immune System.

Type	Component	Function
Barriers/ effector proteins	Skin Musocal epithelial cells Defensins Collectins Complement system	Protection barrier, Killing, Opsonic recognition
Effector cells	Neutrophils Dendritic cells Macrophages/Monocytes	Phagocytosis, Killing, Antigen presentation
Cytokines	TNF-α IL- 1β IL-12 IL-10 IL-2	Inflammation, T helper cell differentiation, Immune cell recruitment

1.5 C-type lectin receptors

C- type lectin- like receptors are a family of proteins that have been characterized by containing one or more C-type lectin-like domains. The C-type lectin receptors are pattern recognition receptors that recognize carbohydrate structures with their extracellular carboxy-terminal domains (44). On the basis of their domain organization and phylogeny, C-type lectin receptors have been divided into 17 families (45) but members of the group II and V have become of particular interest for their roles in mediating fungal immunity (45). The C-type lectin receptors that are known to recognize *C. albicans* are Dectin-1, Dectin-2, Mincle and galectin-3.

Dectin-1 is a fungal recognition receptor first identified on dendritic cells that signals using a non-classical immunoreceptor tyrosine-based activation motif (46). Dectin-1 is primarily expressed in myeloid lineage cells such as dendritic cells, macrophages and neutrophils (47). Dectin-1 is a glycosylated type II transmembrane protein, containing a extracellular C-type lectin domain (CTLD), a stalk region that links the CTLD through a transmembrane region to an intracellular tail containing a ITAM-like motif (47). Dectin-1 couples to Syk to induce the classical NF-κB pathway (48). Dectin-1 can also activate the non-classical NF-κB pathway by activating RelB (49). Dectin-1 can recognize β-1, 3 glucans and is required for phagocytosis (50, 51). Upon zymosan stimulation Dectin-1 and Syk are both required for ROS production (52-54). The importance of Dectin-1 in mediating antifungal immunity is highlighted in Dectin-1-deficient mice which display high mortality rates in systemic

infections with *C. albicans* and are impaired in cytokine production compared to WT controls (54). In a macrophage-specific Dectin-1-deficient mouse model, Dectin-1 is necessary to control fungal gastrointestinal infection (55). In humans polymorphisms in the Dectin-1 gene, which result in an early stop codon, implicate an important role for this receptor in antifungal immunity in humans. Patients homozygous for this mutation are more susceptible to mucocutaneous candidiasis and are defective in cytokine production (56). This indicates that the immune mechanisms recognizing the invasive hyphal form of *Candida* are intact in these patients.

Aside from *C. albicans,* Dectin-1 can recognize *Aspergillus fumigatus,* which is a frequent fungal infection in immunosuppressed individuals causing invasive pulmonary aspergillosis (57). The recognition of *A. fumigatus* was dependent on the morphology since germ tubes were phagocytosed and killed by ROS but conidia could only induce these responses upon swelling that reveals concealed β-glucans (58). Dectin-1-deficient mice show increased mortality rates and impaired cytokine production *in vivo* in response to *A. fumigatus* infection (59).

Dectin-2. The Dectin-2 receptor was first identified in a murine langerhans cell line (60). The Dectin-2 receptor shows specificity for mannose structures (61)**.** This receptor primarily recognizes hyphal forms of fungi (62). Dectin-2 contains a short N-terminal intracellular domain that associates with FcγR chain through the arginine residue in its cytoplasmic domain to induce its signaling (63). The generation of Dectin-2 knockout mice shows that Dectin-2 is the receptor recognizing α-mannans found in *C. albicans* cell wall. Interestingly

Dectin-2 does not recognize β-mannan because BMDCs stimulated with cell wall mannans that were depleted from β-mannans could still induce similar levels of cytokines compared to natural *Candida* mannans and in both cases Dectin-2-deficient BMDCs could not produce cytokines (64). Furthermore, Dectin-2 is the only Syk-coupled C-type lectin receptor to be asscociated with helminth infections. Dectin-2 can recognize soluble components from the eggs of *Schistosoma mansoni*, thereby activating Syk and the NLRP3 inflammasome (65). Allergens from dust mites (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*) can also be recognized by Dectin-2, triggering the production of cysteinyl leukotriene (66).

Mincle The macrophage inducible C-type lectin (Mincle) is characterized as a type II transmembrane protein, which has been shown to recognize *C. albicans*. Mincle- deficient mice appear to be more susceptible to *C. albicans in vivo* (67). Similar to the Dectin-2 receptor, Mincle signaling depends on FcγR chain for its signaling and is thought to mediate its signaling through the Syk-CARD9 cascade (68). Although Mincle was not found to be involved in phagocytosis it has been shown to localize to the phagocytic cup (67).

Galectin-3 can recognize β-1,2 linked mannosides (69) and direct binding of Galactin-3 to *C. albicans* has been shown to kill *C. albicans* (70). Galectin-3 can synergize with the Dectin-1 receptor in co-immunoprecipitation experiments. In macrophages in which

Galectin-3 has been knocked down there is a decrease in TNF-α production in response to *C. albicans* stimulation (71, 72).

Dendritic cell-specific ICAM3-grabbing nonintegrin (DC-SIGN) is a receptor that contains a non-classical ITAM that recognizes high mannose structures. In addition DC-SIGN has been shown to induce cellular responses namely phagocytosis (73). The role of this receptor in immune responses to fungi has not been elucidated but it has been proposed to modulate TLR-mediated responses through the Raf-kinase pathway (74).

1.6. NF-κB activation

The Nuclear Factor kappa B (NF-κB) is one of the most extensively studied inducible transcription factors. NF-κB was first described by Sen and Baltimore who identified it in B cells as a transcription factor that binds to the promoter of the kappa light chain of immunoglobulins (75). The NF-κB transcription factors consist of RelA, Rel B, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100). These transcription factors play a crucial role in regulating the innate and adaptive immune system by controlling various processes that range from regulating the immune response to various stimuli to development of lymphocytes (76). The immune system responds to pathogens by activating the NF-κB pathway, which regulates the transcription of inflammatory genes.

The NF-κB signaling pathway is classified into two major pathways: the classical and non-classical NF-κB signaling pathways. The classical NF-κB pathway signaling

pathway is composed of p50: RelA and p50: c-Rel hetermodimers. These transcription factors translocate to the nucleus through the activation of the trimeric IκB kinase (IKK) complex, which consists of the catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ. The IKK complex becomes activated by the phosphorylation of the IKKα and IKKβ subunits (77) and the K63-linked ubiquitination of IKKγ (78-80). The mechanistic detail of how the IKK complex is activated differs between one receptor to another (81). In non-stimulated cells inhibitors of NF-κB, named IκB proteins retain NF-κB dimers in the cytoplasm. When receptors are engaged the IKK complex becomes activated and phosphorylates IκBα on Ser36 and Ser32. Phosphorylated IκBα then becomes ubiquitinated by the E3 ligase β-TrCP, leading to its subsequent K48-linked ubiquitination and degradation enabling the nuclear translocation of NF-κB (82, 83).

The non-classical NF-κB pathways is activated by the TNF receptor superfamily members such as lymphotoxin β or CD40 causing the activation of NF-κB-inducing kinase (NIK) which in turn activates the IKKα subunits leading to the phosphorylation of p100. p100 can then be processed into p52 leading to the translocation of the p52/RelB dimer into the nucleus (84). The nuclear translocation of NF-κB enables this transcription factor to cooperate with other transcription factors, leading to the transcriptional induction of various genes, including growth factors, proinflammatory genes and anti-apoptotic proteins. The extracellular signaling that induces NF-κB is primarily received through transmembrane receptors of the TNF receptor superfamily such as Tumor Necrosis Factor alpha (TNFα), G-protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), T cell and B cell antigen receptors (TCR, BCR) and Toll-like receptors (TLRs).

Although there has been a great deal of progress in understanding NF-κB signaling, the mechanistic details of how different receptors can activate the IKK complex still remains to be fully elucidated. Scaffold proteins have been shown to play receptor and tissue-specific roles in mediating NF-κB signaling (85, 86). A family of Caspase recruitment domain (CARD)-containing scaffold proteins which have been termed CARD-and membrane –associated guanylate kinase –like domain-containing proteins (CARMA), have been shown to regulate the IKK complex (87, 88). The CARMA family consists of five members that exhibit specific patterns in tissue distribution. CARMA1 is primarily expressed in hematopoietic tissues; CARD9 is expressed in the myeloid lineage of hematopoietic cells; CARMA3 is not expressed in hematopoietic cells but has a broad range of expression in other tissues; and CARMA2 is expressed in placenta (89, 90). These proteins have a similar structure in that they all contain a N-terminal CARD domain followed by a coiled-coil domain. CARMA1, CARMA2 and CARMA3 contain an associated guanuylate kinase –like domain (MAGUK) domain, which is thought to be important for plasma membrane localization. However, the CARD9 family member lacks the MAGUK domain (88). The CARMA family members can form a complex with BCL10 and MALT, which is referred to as the CBM complex, leading to NF-κB activation (91, 92). In our studies we have focused on understanding how the NF-κB signaling pathway is activated downstream of C-type lectin receptors (CLRs).

1.7 NF-κB activation by antigen receptor signaling

In the antigen receptor signaling pathway activation of the T cell receptor and B cell receptors lead to NF-κB activation. Upon receptor activation the tyrosines on the immunoreceptor tyrosine-based activation motifs (ITAMs) become phosphorylated by Src family kinases, Lck and Fyn. Syk tyrosine kinases Zap70 and Syk then get recruited and phosphorylate SLP-76 and LAT. Phosphorylation of SLP-76 in TCR signaling induces its binding to Vav1. The Vav1-SLP76-Tec family tyrosine kinase Itk complex activates PLCγ1, which can lead to the release of diacylglycerol (DAG), thus stimulating PKCs (93, 94). It is proposed that PKCθ can phosphorylate CARMA1, facilitating its associating with BCL10 (93). The activated CARMA1-BCL10-MALT1 complex leads to IKK activation. Recently some components that were thought to be primarily involved in TLR/IL-1 receptor mediated NF-κB activation such as TAK1 and TRAF6 have been shown to be involved in TCR-mediated IKK activation. The TRAF6-MALT1 interaction can lead to the K63-linked polyubiquitination of IKKγ and IKK complex activation (95).

1.8 Dectin-1 and Dectin-2 signaling

Dectin-1 and Dectin-2 are characterized as type II glycosylated transmembrane proteins containing single CRDs that are highly conserved in humans and mice. Dectin-1 receptor contains a tyrosine-based activation motif (ITAM)-like motif or hemITAM in the intracellular region of this receptor whereas Dectin-2 does not have any known signaling motif in its cytoplasmic regions (96). Although ITAM associated receptor signaling has been well defined in T cells and B cells, in myeloid cells the components for this type of receptor

signaling was not known until recently. Studies by Rogers and colleagues (48) showed that Syk is the kinase in myeloid cells that is recruited to the ITAM in the intracellular region of Dectin-1, where it is phosphorylated and accumulates at the phogocytosis cup.

The Dectin-2 receptor utilizes Syk for its signal transduction. BMDCs stimulated with α-mannan or Dectin-2 monoclonal antibodies induced the production of TNF, IL-10 and IL-2 cytokines that were decreased in Syk-deficient BMDCs compared to WT (64, 97). Downstream of Syk the adaptor protein CARD9 can form a complex with BCL10 and MALT1 (98). The adaptor protein CARD9 is critical for cytokine production downstream of Dectin-1 and Dectin-2. Furthermore the role of CARD9 in mediating anti-fungal immunity is shown *in vivo* with CARD9-deficient mice exhibiting higher susceptibility to *C. albicans* compared to WT mice (99, 100).

In humans, the role of CARD9 in immune protection against *Candida* infection is demonstrated in patients harboring mutations in CARD9 by increased susceptibility to infections with *C. albicans* (101). In our recent study we found that CARD9 is required for Dectin-2 but not Dectin-1 mediated NF-κB activation. In addition CARD9 is regulating the IKK complex by the polyubiqutination of IKKγ and does not affect IKKα/β phosphorylation status (62) (Figure 1). In addition to the Syk-dependent classical NF-κB pathway the non-canonical NF-κB subunit RelB can be activated downstream of Dectin-1 but in a Syk-independent manner (49). Ligation of Dectin-1 activates Raf-1, promoting p65 activation and repressing RelB activation.

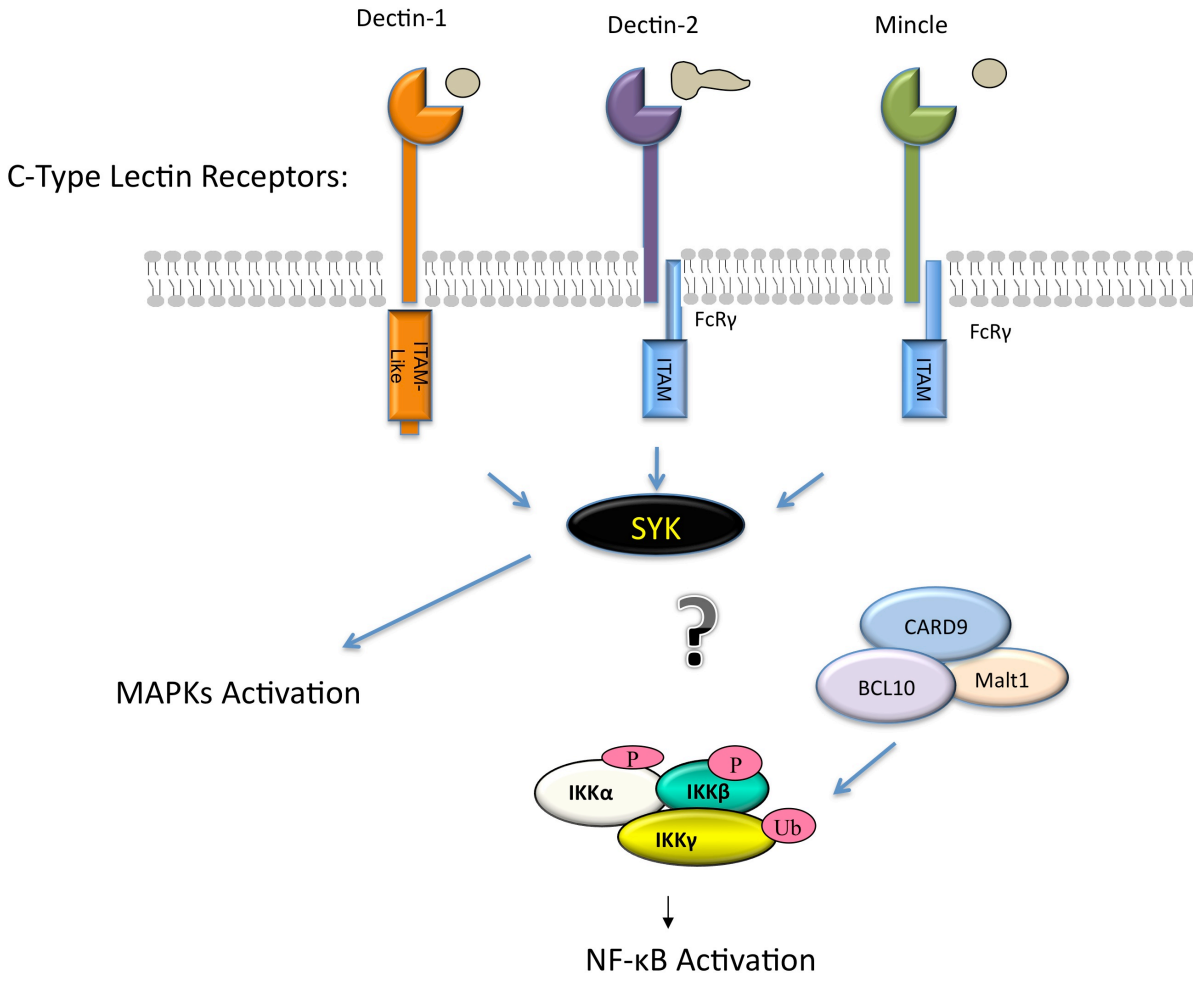

Figure 1. C-type lectin induced NF-κB activation in myeloid cells. The C-type lectin receptors Dectin-1, Dectin-2 and Mincle utilize ITAMs either directly or indirectly to recruit Syk, leading to the activation of the CARD9-BCL10-MALT1 complex and the subsequent activation of the IKK complex.

1.9 Adaptive anti-fungal immune response

The C-type lectin receptors Dectin-1 and Dectin-2 trigger innate immune responses, leading to adaptive immune responses, which include Th17, Th1 and cytotoxic T-cells. The differentiation of Th17 cells is induced by cytokines such as IL-1β, IL-6, TGF-β, IL-23 and IL-21 in addition to transcription factors such as γt and retinoic acid receptor-related orphan nuclear receptors (102). Stimulation of Dectin-1 by purified β-glucans on antigen presenting cells was shown to be able to induce the differentiation of Th1 and Th17 CD4+ T-cells in addition to CD8+ cytotoxic T cell responses (49, 103, 104). The signaling components in innate immune cells that are a driving force for Th17 development are not fully characterized but the Syk-CARD9 and the Raf-1 signaling pathways are shown to regulate Th17 inducing cytokine production such as IL-23 IL-1β, IL-6 and dampen the production of Th-2 cytokines such as IL-12 (49, 105).

There is emerging evidence for the role of Dectin-1 in regulating IL-17 production in humans during fungal infections. The identification of a polymorphism in the Dectin-1 receptor in humans, which prevents the expression of Dectin-1 on the cell surface, showed that these individuals are defective in the production of cytokines such as IL-17 and are susceptible to mucocutaneous fungal infections (56). In a separate study Dectin-1 polymorphism is linked to an increase in the incidence of graft-versus-host disease associated with increased *Candida* colonization (106). Recent studies demonstrate that the Dectin-2 receptor plays a prominent role in inducing Th17 and Th1 responses. Upon *C. albicans* infection, Dectin-2 by signaling through the Syk-CARD9 cascade, induces Th-1

and Th17 T helper cell differentiation (64, 97). In these studies, stimulation of dendritic cells with fungal particles leads to the induction of cytokines, which include IL-6, TNF, IL-1β and IL-23.

1.10 Tissue invasion and mucosal colonization

C. albicans hyphae predominates at the primary site of infiltration into epithelial cells layers, whereas yeast cells are found either on the epithelial cell surface or emerge from penetrating hyphae that are infiltrating tissues (107). Aside from phagocytic cells, *C. albicans* interacts with mucosal epithelial cells. Mucosal epithelial cells can recognize fungi and respond by producing cytokines (108, 109). In healthy individuals where small yeast numbers are present, epithelial cells are not damaged to trigger the production of cytokines. Upon crossing tissue surfaces, *C. albicans* can cause invasive infections. Hyphae can cause more epithelial damage than yeast through the production of lytic enzymes such as secreted asparyl proteinases (SAPs) (110, 111). Both yeast and hyphae can induce endocytosis by epithelial cells (112, 113), although hyphae are thought to be more efficient in stimulating this process (113). Epithelial cells discriminate between yeast cells and invasive hyphae by a two-phase MAPK pathway activation process (114). Epithelial cells in the first phase of MAPK activation activate the transcription factors FOS and JUN through p38 MAPKs. This initial MAPK activation is independent of the morphological status of *C. albicans*. The second phase of MAPK activation can only be induced by hyphae and not by the yeast form. In the second phase the transcription factor MAPK phosphatase 1 is activated by ERK1/2

and p38 MAPKs. In addition to epithelial cells, DCs and tissue resident macrophages constantly sample the contents of mucosal flora. However, the mechanisms that allow these cells to discriminate between yeast and hyphal forms need further investigation. One proposed model for the discrimination between the yeast and hyphal forms of *C. albicans* by mucosal immune cells is the ability to activate the NLRP3 inflammasome in these cells. In macrophages only hyphae can induce the activation of the NLRP3 inflammasome to induce IL-1β secretion (114). Under normal conditions of *C. albicans* colonization, no substantial activation of the NLRP3 inflammasome occurs and therefore low levels of pro-IL-1β is processed by DCs only. However, the germination of yeast cells into hyphae triggers NLRP3 inflammasome activation in macrophages and DCs, leading to high levels of IL-1β processing (115). Secretion of IL-1β by mucosal immune cells leads to the induction of Th17 cells, which produce IL-17 and IL-22 cytokines. IL-22 can induce the production of defensins by epithelial cells (116) and IL-17 recruits neutrophils to phagocytose and kill hyphae (117) . Furthermore damaged epithelial cells can release ATP which also in a known activator of NLRP3 inflammasome (118).

1.11 Evasion of *Candida* from the host defense

One mechanism, which *Candida* uses to escape phagocytosis, is the shielding of important PAMPs from being recognized by pattern recognition receptors. β-glucan is shielded by the outer cell wall components, which prevents its recognition by Dectin-1 (53). In heat-killed *C. albicans*, which the architecture of the cell wall is disrupted, significant

amounts of cytokines are induced compared to live *C. albicans,* due to the exposure of β-glucans (119). An important process for killing of *C. albicans* is the fusion of the phagosome with the lysosomes. *Candida* can inhibit the formation of phagolysosomes and wild-type *C. albicans* compared to nonfilamentous mutants has a higher capacity to control phagosomal composition (120). The production of ROS is an important antifungal mechanism is phagocytes. *Candida* species have evolved several defense strategies to counteract ROS. For example *C. albicans* strains that are defective in catalase production are less virulent and are cleared faster compared to WT strains in an experimental model (121). Although the recognition of fungal cell wall components are necessary for ROS production, *Candida* viability is necessary for the suppression effect (122). The quorum-sensing molecule farnesol is shown to decrease macrophage viability by inducing ROS (123). Furthermore, farnesol can protect *C. albicans* from oxidative stress by upregulating SOD2, SOD4 and CAT1 (124). It also has been demonstrated that macrophages produce higher levels of IL-6 when stimulated with WT *C. albicans* compared to farnesol-deficient strain (125). Although the majority of studies have focused on the mechanisms which *Candida* induces cytokine production in the host, little is known about how *C. albicans* exploits host cytokine production. Recently, it was shown that *C. albicans* could actively inhibit the host's IL-17 production by altering host tryptophan metabolism. In this study *C. albicans* could shift tryptophan metabolism by inhibiting indoleamine 2,3-dioxygenase (IDO) expression, leading to more 5-hydroxytryptophan metabolites, subsequently inhibiting host IL-17 production (126).

1.12 Phospholipase gamma family

In response to stimuli, activated cells amplify incoming signals by producing second messengers that link receptors to intracellular pathways. Inositol-1,4,5-trisphosphate (Ins (1,4,5)P_3) and diacylglycerol (DAG) are essential second messengers that are produced by phospholipase C (PLC). The PLC enzymes contain a N-terminal PH domain, a catalytic triose phosphate isomerase (TIM) barrel, four EF-hands, and a C2 domain (127).

In mammals there are two PLC-γ isoenzymes (PLCγ1 and 2). PLC-γ1 is expressed ubiquitously whereas PLC-γ2 is primarily expressed in hematopoietic cells (128) . The PLC-γ isoforms are structurally different from other PLC enzymes due to a unique split PH domain, a SH3 domain and two SH2 domains. Most growth factor receptors that contain tyrosine kinase activity (RTKs) use PLC-γ isozymes, which cause dimerization and autophosphorylation of RTKs, creating docking sites for the SH2 motifs of PLC-γ isozymes (129, 130). In addition, PLC-γ members can be activated downstream of receptors lacking intrinsic tyrosine kinase activity such as B-cell and T cell immunoreceptors (131, 132). In these cells PLC-γ gets activated by immunoreceptor tyrosine-based activation motifs (ITAMs), which are coupled to the receptors. In T cells upon activation of the T cell receptor, Zap70 is recruited into the signaling complex by its SH2 domains (133). Zap70 then tyrosine phosphorylates LAT and SLP-76, which are adaptor proteins (134). PLC-γ1 is then able to bind to SLP-76 and LAT by its N-terminal SH2 and SH3 domains respectively. In hematopoietic cells, PLC-γ signaling has been studied in the B-cell antigen receptor (BCR) pathway. In B cells upon phosphorylation of ITAMs, the spleen tyrosine kinase (Syk)

is recruited to the complex where it phosphorylates SLP-65 (135). SLP65 can interact with PLC-γ2 and Burton tyrosine kinase. Thus, the kinases Syk and Btk are thought to be responsible for the phosphorylation of PLC-γ2 (136).

The goal of our study was to identify components of the intracellular signaling cascade downstream of C-type lectin receptor activation in macrophages and to understand their importance in mediating the immune response to *C. albicans in vivo*. A mechanistic understanding of the host immune response to *C. albicans* is important for the development of anti-fungal therapeutics and in understanding risk-factors determining susceptibility to *C. albicans* infection.

Chapter 2

Methods and Materials

Chapter 2: Methods and Materials

2.1 Generation of Bone Marrow Derived Macrophages (BMDM)

Primary cultures of bone marrow-derived macrophages (BMDMs) were prepared as previously described (100, 137, 138). Briefly, bone marrow cells were harvested from the femurs and tibias of mice. Erythrocytes were removed from cells samples by subjecting the samples to ACK buffer, which is a hypotonic solution. Cells were cultured for 7 days in DMEM containing 20% FBS, 55 μM β-mercaptoethanol, streptomycin (100 μg/ml), penicillin (100 U/ml), and 30% conditioned media from L929 cells overexpressing macrophage colony-stimulating factor (M-CSF) and plated on 150 cm petri dishes. Non-adherent cells were removed and after 7 days of differentiation the cultures were confluent and the cells were used for experiments. . Flow cytometry analysis indicated that the harvested cell population contained 86–95% $CD11b^+$ $F4/80^+$cells as assessed.

2.2 Lentivirus-encoded shRNA knockdown of Dectin-2 in BMDMs

The lentiviral vectors for Dectin-2 were purchased from Sigma. Lentiviral particles for infection were prepared by transfection of 293T cells (62). Mouse bone marrow cells were infected with virus on days 1 and 3 by spinoculation followed by incubating the cells for 4 hours with the prepared lentivirus and selected with 2 μg/ml puromycin on day 5 of culture.

2.3 *C. albicans* preparation

Candida albicans (strain SC5314) (139) was kindly provided by Dr. Michael C. Lorenz (Department of Microbiology and Molecular Genetics, University of Texas Medical School at Houston). A single colony of *C. albicans* was grown overnight at 30°C in yeast peptone dextrose media. The cells were washed three times with PBS and then used as live yeast. For hyphae, the washed yeast cells were re-suspended in DMEM with 10% FCS and grown at 37°C for 3 hours, and washed in PBS. The hyphae were then used for live stimulations. For heat-killed yeast the cells were incubated at 65°C for one hour.

2.4 *In vivo C. albicans* infection

For *in vivo C. albicans* infection**,** male mice aged at 8-14 weeks were injected with 2 x 10^5 or 1x10^6 live *C. albicans* in 0.3 ml of PBS (pH 7.4). The mice infected were monitored daily to determine survival curves. Candidal burden was determined by killing the mice 42 hours after infection and harvesting and homogenizing their kidneys, lungs, livers, and spleens. Fungal colony formation units were quantified by plating serial dilutions of the homogenized organs on yeast extract peptone dextrose (YPD) agar. For histological analysis, the tissue sections were cut and stained for glycogen using Periodic Acid-Schiff or hematoxylin and eosin.

2.5 Reactive oxygen species (ROS) production assay

For ROS production evaluation, $1x10^5$ BMDMs were washed with Hank's balanced salt solution without phenol red. Then 100 μL Hank's balanced-salt solution containing 100 μM luminol and 5 units of horseradish peroxidase (Sigma) were added to the cells and the cells were incubated for 10 minutes at 37°C, The cells were then stimulated with either hyphae (multiplicity of infection [MOI]=2) or phorbol myristate acetate (PMA). ROS production was measured every 3 minutes using a luminometer.

2.6 Electrophoretic Mobility Shift Assay (EMSA)

BMDMs ($7x10^6$) were lysed in 250 μL of lysis buffer containing 10 mM KCl, 10 mM HEPES, 0.1 mM EDTA, 0.4% Nonidet P-40, 0.5 mM PMSF, 1 mM dithiothreitol and 1% protease inhibitor cocktail followed by a minute centrifugation at 13000 RPM at 4°C. The supernatant were removed and the nuclear pellet was washed once with lysis buffer. Nuclear extraction (30 μL) buffer containing 0.4 M NaCl, 20 mM HEPES, 1 mM EDTA and 0.5 mM PMSF, 1% protease inhibitor and 1 mM dithiothreitol was added to the nuclear pellet and the pellet was vortexed for 30 minutes at 4°C. The nuclear proteins were collected by centrifugation at 13000 RPM for 10 minutes at 4°C and protein concentration was assessed by Bio-Rad protein assay (Bio-Rad). Nuclear extracts (5 μg) were incubated with ^{32} P-labeled NF-κB or Oct-1 probe (Promega) for 15 minutes at 25°C. The mixtures were then

run on a 6% polyacrylamide gel for 85 minutes at 220 volts at room temperature. The gels were dried for 1 hour at 85°C on a vacuum gel dryer and exposed to X-ray fim.

2.7 Cytokine production

The amount of secreted TNF-α, IL-2, IL-6, IL-10, IL-12p40 and IL-1β in the culture supernatants was measured using ELISA according to the manufacturers recommendation (eBioscience, San Diego, USA). In order to increase sensitivity the supernatants were incubated on the ELISA plates coated with the primary antibody overnight at 4°C.

2.8 Western blot analysis and immunoprecipitation

To prepare proteins lysates for western blot analysis cells were lysed in 150 or 250 mM NaCl lysis buffer containing 50 mM HEPES, 1 mM EDTA, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1 mM dithiothreitol and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) for 20 minutes at 4°C on a rocker. The lysates were centrifuged for 15 minutes at 13000 RPM at 4°C. The supernatants were collected and denatured with 4X SDS loading dye (20% β-mercaptoehanol, 240 mM Tris-Cl pH 6.8, 8% SDS, 0.2% bromophenol blue, 40% glycerol). In experiments where immunoprecipitation was performed cell lysates that were prepared as described above were added to either protein A, G, FLAG or HA conjugated sepharose beads (Roche) that had been washed with lysis buffer and incubated with antibody for 10 minutes on ice in the cases were protein A or

G sepahrose beads were used. The samples were rotated at 4°C for 4-16 hours. The beads were then washed 4 times with lysis buffer. In experiments where elution of the target protein from FLAG-conjugated sepharose beads was needed the beads were incubated with elution buffer (50 mMTris-HCl, pH 7.5, 150 mM NaCl, 0.5 % NP40) and 0.5 mg/ml of FLAG peptide for 30 minutes then pelleted and fresh elution buffer containing FLAG peptide was added. This step was repeated 4 times. After immuoprecipitation and the washing steps, equal amount of 4X SDS loading dye was added to the beads to elute the target immunoprecipitated protein. Samples were then subjected to electrophoresis. The SDS-PAGE gels were either stained directly using sypro ruby or silver staining methods or they were transferred to a nitrocellulose membrane for at least 1 hour at 90 volts. Membranes were blocked with TBST containing 5% milk or BSA for 30 minutes. Primary antibodies were diluted in TBST containing 1% BSA overnight or for one hour. Secondary antibodies were diluted in TBST containing 5% milk or BSA for 1 hour. The membranes were washed 3 times for 15 minutes each and were incubated with ECL substrate and exposed to the autoradiography film.

2.9 Mass spectrometry

Sypro ruby stained gels were visualized on a transilluminator by UV fluorescence. Gradient precast gels were purchased from BioRad and bands were excised from the gel using an ethanol rinsed scalpel. To reduce keratin contamination all procedures were performed by regularly changing gloves. The acrylamide gel slices were sent to the proteomics and mass

spectrometry facility according to the facility procedures. In gels stained by silver staining all procedures were done according to the manufactures protocols (Invitrogen).

2.10 Calcium phosphate transfection

HEK 293T cells were plated at 4.5 x10^4 /cm^2 in DMEM containing 10% FBS and 1% penicillin/ streptomycin overnight. DNA (5 μg) was the maximum amount used per 10^6 cells which was mixed with 2x HBSS (280 mM NaCl, 10 mM KCl, Na_2HPO_4 ,12 mM Dextrose, 59 mM HEPES), then slowly mixed with 2 M $CaCL_2$. After 30 minutes of incubation at room temperature, the mixture was gently added to the cells.

2.11 Cell culture and transfection

Raw264.7 cells were cultures in RPMI containing 10% FBS and 1% penicillin/ streptomycin. Raw264.7 was stably infected with retrovirus. Cells were rested for three days followed by puromycin (2 μg/ml) drug selection.

Chapter 3

Phospholipase C γ2 is a critical component of the Dectin-2 signaling pathway and mediates innate antifungal immune responses.

Chapter 3: Phospholipase Cγ2 is a critical component of the Dectin-2 signaling pathway and mediates innate antifungal immune responses.

3. 1. Background

In humans with immunocompromised immune systems the dimorphic fungus *C. albicans* causes a range of infection manifestations, from superficial and mucosal to lethal bloodstream infections. The innate immune cells such as neutrophils and macrophages play an essential role in antifungal host defense (43). The C-type lectin receptors Dectin-1 and Dectin-2 are important pattern recognition receptors for recognizing C. *albicans* cell wall components and initiating an immune response. β-glucan carbohydrate structures are recognized by Dectin-1 receptor (53, 140). These β-glucan structures are buried by mannoproteins in the yeast form of *C. albicans* but they can be exposed when the yeast transforms into the hyphal form (141) thereby providing an opportunity for the Dectin-1 receptor to interact with β-glucan to induce cytokine production. The Dectin-2 receptor recognizes the mannan coat in the cell wall of *C. albicans* hyphae (63, 64). Although the signaling pathway components utilized by Dectin-1 and Dectin-2 are not fully characterized, Syk is the major kinase in this pathway leading to multiple signaling cascades (48, 97, 142-144). Syk is not only important in C-type lectin receptor signaling pathways but also in B cell receptor signaling. In B cell receptor signaling, PLCγ2 plays an essential role to mediate downstream receptor signaling. PLCγ can hydrolyze phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-triphosphate (145). In the cells of the hematopoietic

system, both isoforms of PLCγ, PLCγ1 and PLCγ2 are expressed. Studies performed in dendritic cells show that in response to β-glucan found in zymosan and curdlan preparations both isoforms of PLCγ can be activated through the Dectin-1 receptor (146, 147). In our studies, we demonstrate that macrophages specifically utilize PLCγ2 but not PLCγ1 downstream of the Dectin-2 receptor in response to *C. albicans* infection. Using mouse models deficient in PLCγ1or PLCγ2, we demonstrate the key role of the PLCγ2 isoform in mounting an immune response by the production of essential cytokines and the generation of reactive oxygen species, which are essential to host defense to *C. albicans*.

3.2. Results

3.2.1 Hyphae stimulation leads to PLCγ2 phosphorylation through the Dectin-2 receptor

A previous study in bone marrow derived dendritic cells showed that in response to zymosan, which is a fungal cell wall extract derived from *Saccharomyces cerevisiae* both PLCγ1 and PLCγ2 are phosphorylated. To determine whether *C. albicans* can activate PLCγ2 in macrophages, we examined PLCγ2 phosphorylation in BMDMs after infecting them with *C. albicans* hyphae. Stimulation of BMDMs with *C. albicans* led to an inducible phosphorylation of PLCγ2 (Figure 3.2.1 A). This result indicated that PLCγ2 becomes activated in response to *C. albicans* infection. Since both C-type lectin receptors Dectin-1 and Dectin-2 are involved in antifungal innate immune responses, we evaluated whether PLCγ2 activation in response to hyphae is induced through these receptors. We utilized BMDMs from Dectin-1 knockout mice or BMDMs, which were previously treated with shRNA to Dectin-2 during their differentiation process. Consistent with previous findings from our lab which showed that hyphae-induced signal transduction primarily was initiated through the Dectin-2 receptor and not Dectin-1; we observed that PLCγ2 phosphorylation was dependent on Dectin-2 but not Dectin-1 (Figure 3.2.1 B and Figure 3.2.2). These results indicate that PLCγ2 is an important component of the Dectin-2 signaling pathway in innate immune cells.

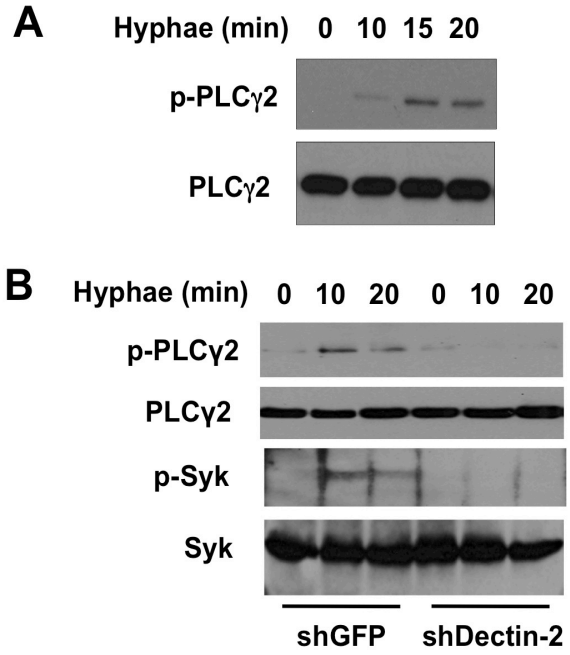

Figure 3.2.1. Dectin-2 is required for *C. albicans*-induced PLC γ2activation. *A*, wild-type macrophages were stimulated with *C. albicans* hyphae (MOI: 1) and the phosphorylation of PLCγ2 was examined by Western blotting. *B*, BMDMs were infected with lentivirus encoding Dectin-2 shRNA or GFP shRNA, then selected with puromycin, and stimulated on day 9 with *C. albicans* hyphae (MOI: 1) at different times. Cell lysates were subjected to Western blotting analysis using indicated antibodies.

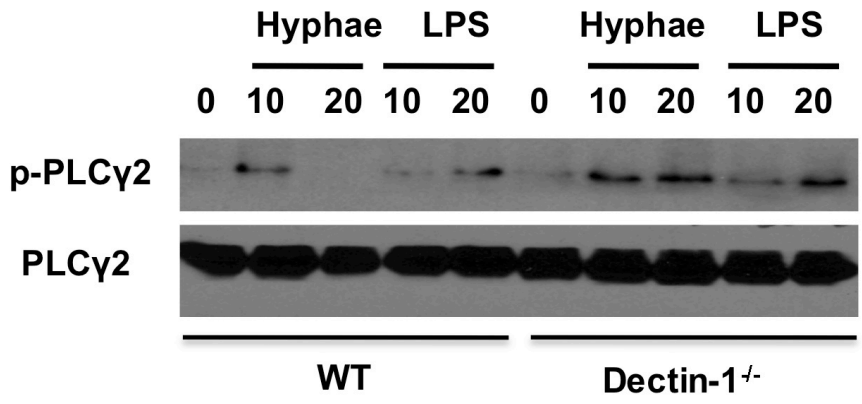

Figure 3.2.2. Hyphae induced phosphorylation of PLCγ2 is not mediated through Dectin-1. WT and Dectin-$1^{-/-}$ BMDMs were stimulated with *C. albicans* hyphae (MOI: 1) or LPS (100 ng/ml) for the indicated times. Cell lysates were subjected to Western blotting analysis using the indicated antibodies.

3.2.2 PLCγ2 is required for *C. albicans* infection-induced NF-κB activation

We next sought to identify the PLCγ2-dependent signaling pathways in macrophages and determine whether these signaling pathways are redundant in response to yeast and hyphal forms of *C. albicans*. Both the hyphal and yeast forms of *C. albicans* activate the transcription factor NF-κB. We stimulated PLCγ2-deficient BMDMs with the yeast and hyphal form of *C. albicans* and evaluated the DNA-binding activity of NF-κB transcription factor. We found that in response to both yeast and hyphal forms of *C. albicans* (Figure 3.2.3 A and B) PLCγ2-deficient macrophages were defective in NF-κB activity compared to WT macrophages. The dectin-1 receptor has been shown to cooperate with TLR2 to induce cytokine production in response to fungal infections (52, 148). Therefore, we examined the role of MyD88 in regulating NF-κB activation in response to hyphal and yeast forms of *C. albicans*. In contrast to PLCγ2 -deficiency, loss of MyD88 did not affect NF-κB activation (Figure 3.2.3 B and D).

Considering that in the BCR signaling pathway both Syk and PLCγ2 are critical components for transducing signals, we investigated whether other components of BCR signaling, which mediate PLCγ2 activation, are required for fungal-induced NF-κB activation. Burtons tyrosine kinase (BTK) is known to be essential to activate PLCγ2 in the BCR signaling pathway (149), we evaluated the role of BTK in fungal-mediated NF-κB activation. We used the BTK inhibitor LFM-A13 to pretreat macrophages before challenging with *C. albicans* hyphae. We found that the BTK inhibitor did not affect PLCγ2 phosphorylation and NF-κB activation in response to *C. albicans* hyphae (Figure 3.3.4).

This finding indicates that although PLCγ2 is both used in BCR signaling and innate fungal recognition, these signaling pathways are not redundant. The IKK complex is regulated by two signaling events. The ubiquitination of Nemo is regulated by the CBM complex (62, 150) whereas the phosphorylation of IKKα/β is mediated through an independent signal. The adaptor protein CARD9 regulates Nemo ubiquitination, whereas Syk mediates IKKα/β phosphorylation. We next examined how PLCγ2 regulates the IKK complex. We found that unlike CARD9-deficient macrophages, PLCγ2-deficient macrophages were defective in the phosphorylation of IKKα/β upon stimulation with hyphae (Figure 3.2.5).

The adaptor protein CARD9 forms a complex with BCL10 to mediated downstream signaling events. We investigated the effect of PLCγ2 on the association of CARD9 with BCL10, by immunoprecipitating endogenous CARD9 from WT and PLCγ2-deficient macrophages that were stimulated with hyphae. Interestingly we did not observe any difference in CARD9-BCL10 association between WT and PLCγ2-deficient macrophages (Figure 3.2.6). These results suggest that PLCγ2 functions as a critical enzyme downstream of Syk and can regulate the IKK complex independent of CARD9.

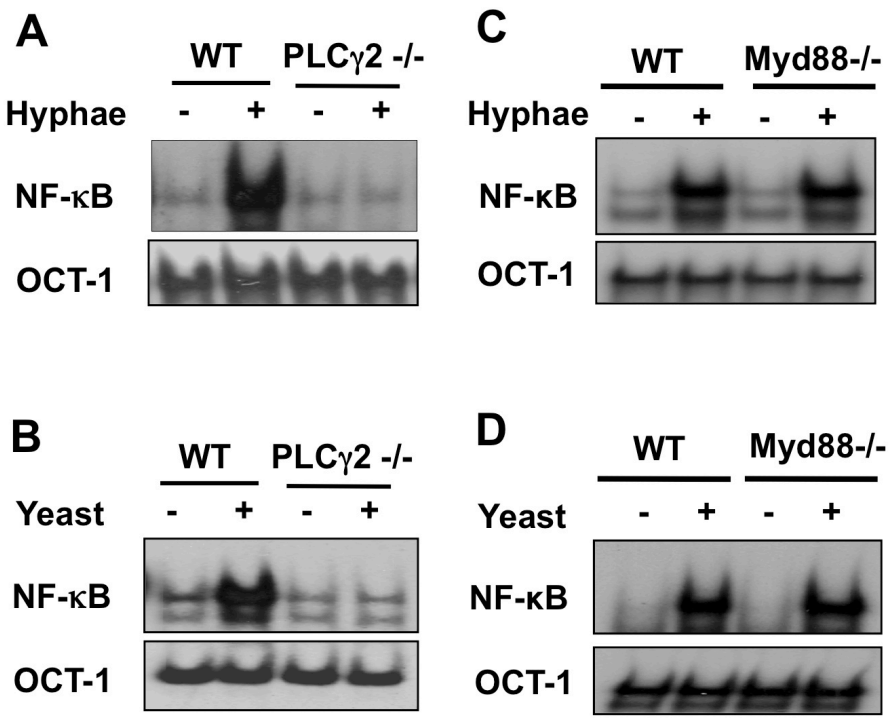

Figure 3.2.3. NF-κB activation induced by *C. albicans* hyphae and yeast is PLCγ2-dependent. *A* and *B*, WT and PLCγ2-deficient BMDMs were either untreated or treated with *C. albicans* hyphae (MOI: 1) (*A*) or heat-inactivated yeast (MOI: 5) (*B*) for 90 min. The nuclear extracts were prepared from these cells and then subjected to the electrophoretic mobility shift assay using 32P-labeled NF-κB or OCT-1 probe. *C* and *D*, WT and Myd88$^{-/-}$ BMDMs were stimulated with *C. albicans* hyphae (MOI: 1) (*C*) or heat-inactivated yeast (MOI: 5) (*D*) for 90 min. The nuclear extracts were subjected to EMSA using 32P-labeled Oct-1 or NF-κB probe.

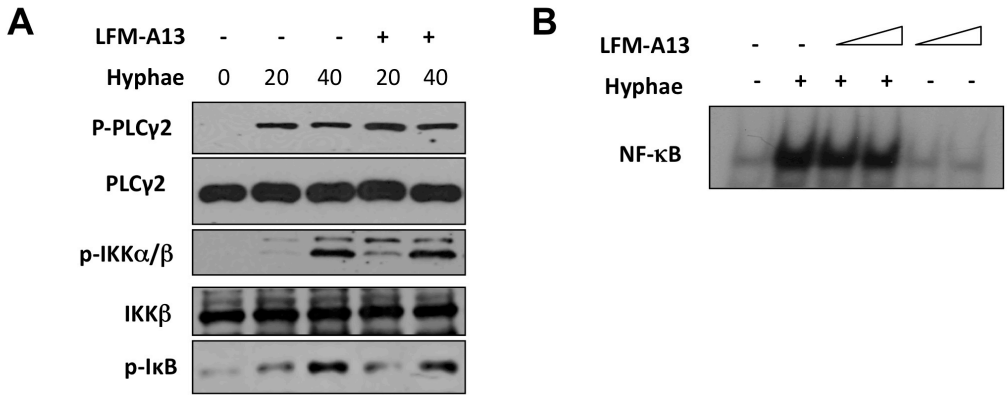

Figure 3.2.4. **NF-κB activation induced by *C. albicans* hyphae is not BTK-dependent.** Wild- type BMDMs were preincubated for 30 min with DMSO or 25 and 50μM BTK inhibitor LFM-A13, followed by stimulation with hyphae for 90 min. and IκB degradation and IKKα/β activation were examined with anti-IκB and anti-phospho IKKα (Ser^{176})/ IKKβ(Ser^{180}) antibodies. WT and PLCγ$2^{-/-}$ BMDMs were either non-treated or treated with *C. albicans* hyphae for 90 min and nuclear extracts were evaluated for NF-κB DNA-binding activity by electrophoretic mobility shift assay. OCT-1 DNA binding was used as a loading control.

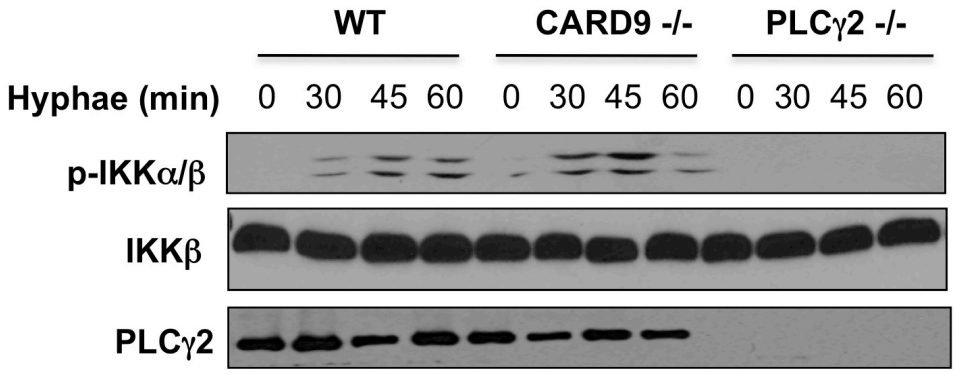

Figure 3.2.5. PLCγ2 but not CARD9 mediates IKKα/β phosphorylation.

WT, *CARD9*$^{-/-}$, and *PLCγ2*$^{-/-}$ BMDMs were stimulated with *C. albicans* hyphae for the indicated time points, and IKKα/β phosphorylation was examined using anti-phospho-IKKα/β (Ser$^{176/180}$) antibody.

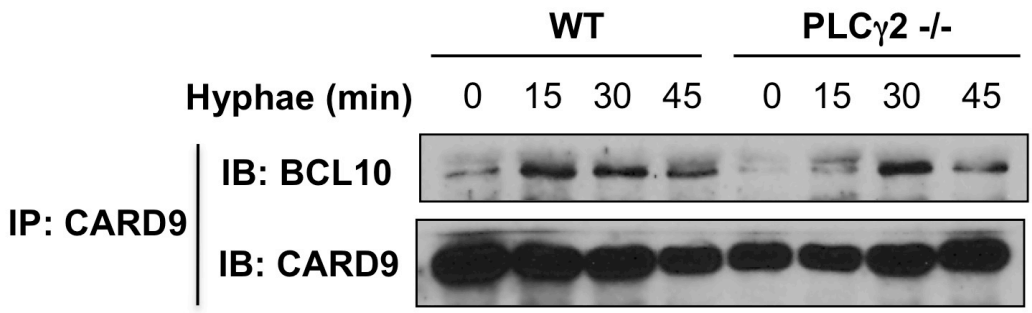

Figure 3.2.6. **PLCγ2 does not mediate CARD9-BCL10 association.** WT and PLCγ2-deficient BMDMs were stimulated with *C. albicans* hyphae (MOI: 1) for the indicated time points, and the cell lysates were immunoprecipitated with CARD9 antibody-conjugated agarose. The immunoprecipitates were probed with BCL10 and CARD9 antibodies. *IP*, immunoprecipitation; *IB*, immunoblot.

3.2.3 PLCγ2 regulates MAPK signaling in response to *C. albicans* yeast and hyphae

Previous studies performed on dendritic cells demonstrate that *C. albicans* induces the activation of MAPKs to induce cytokine production (97). We examined how PLCγ2 regulates MAPK activation in macrophages in response to *C. albicans*. In response to hyphae we found that PLCγ2-deficient macrophages were completely defective in ERK and JNK signaling (Figure 3.2.7). However, p38 and AKT were comparable between WT and PLCγ2$^{-/-}$ macrophages (Figure 3.2.7). In response to yeast JNK activation was significantly decreased in PLCγ2$^{-/-}$ macrophages, whereas ERK activation was only partially defective (Figure 3.2.8). These results suggest that in response to hyphae ERK activation depends solely on PLCγ2 whereas in response to yeast, two pathways mediate ERK activation.

3.2.4. PLCγ1 in macrophages is not required for *C. albicans*-induced MAPK and NF-κB activation

We also investigated the role of PLCγ1, the homolog of PLCγ2 in antifungal immunity. PLCγ1 is also expressed in hematopoietic cells and has been shown to play a major role in TCR signaling (151). The yeast cell wall extracts, curdlan and zymosan, that mainly contain β-glucans have been shown to activate PLCγ1 in dendritic cells (146, 147). Because PLCγ1-deficient mice are embryonic lethal, we used bone marrow derived macrophages from PLCγ1 conditional knockout mice (PLCγ1$^{f/f}$/ Mx1-cre) and its control (PLCγ1$^{f/+/}$ Mx1-cre). We stimulated these macrophages with hyphae, but in contrast to

PLCγ2$^{-/-}$ mice, PLCγ1-deficiency did not affect ERK and IKK activation (Figure 3.2.9). These data ruled out the role of PLCγ1 functioning downstream of the C-type lectin receptors.

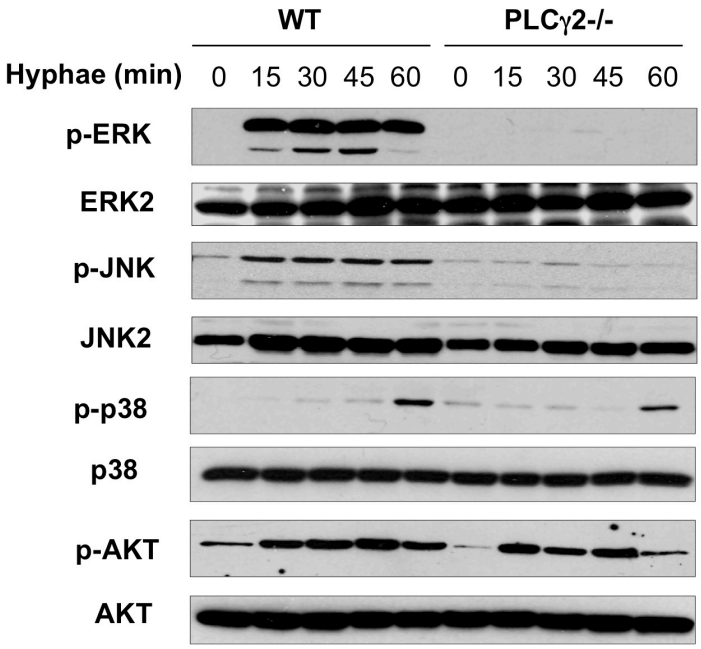

Figure 3.2.7. PLCγ2-deficient BMDMs display defective MAPK activation.

Wild-type and PLCγ2-deficient ($PLC\gamma2^{-/-}$) BMDMs were stimulated with *C. albicans* hyphae (MOI: 1) for the indicated time points. The cell lysates were prepared and subjected to immunoblotting analysis using the indicated antibodies.

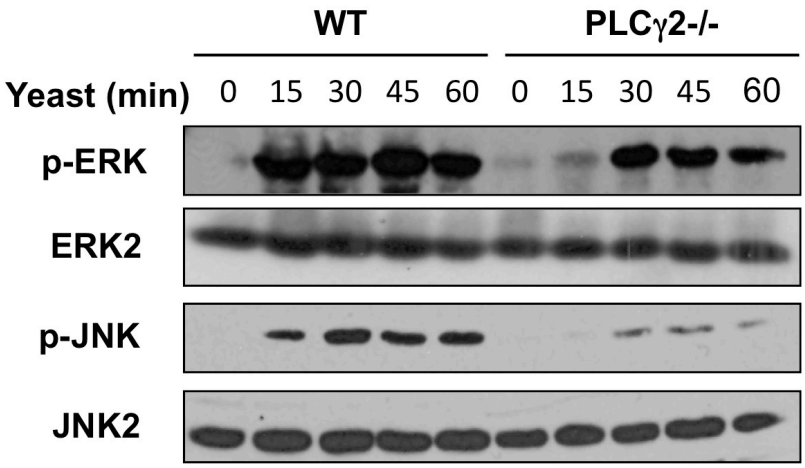

Figure 3.2.8. PLCγ2-deficient BMDMs display defective MAPK activation.

Wild-type and PLCγ2-deficient (PLCγ2$^{-/-}$) BMDMs were stimulated with *C. albicans* yeast (MOI: 5) for the indicated time points. The cell lysates were prepared from these cells and then subjected to immunoblotting analysis using the indicated antibodies.

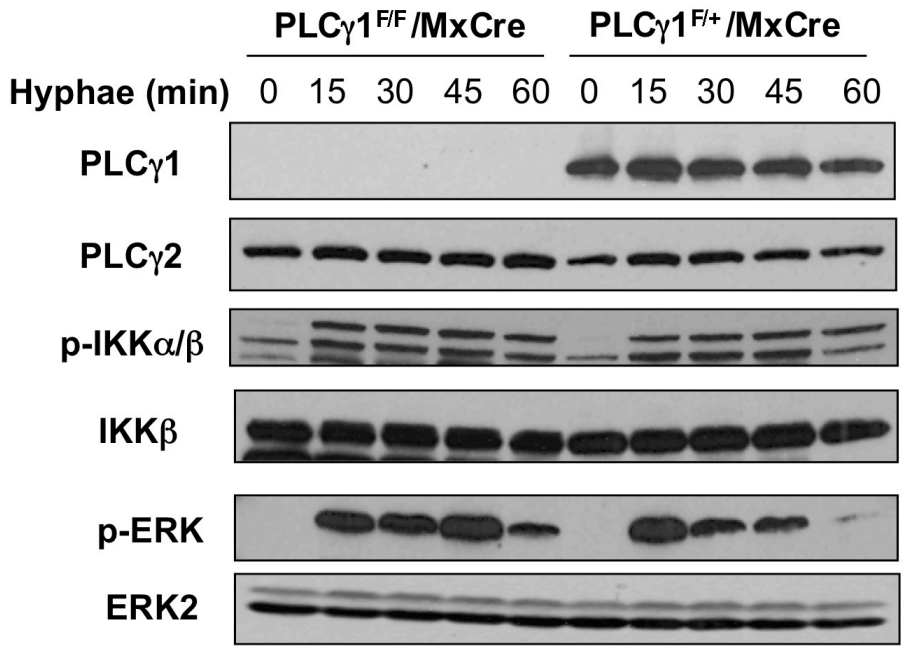

Figure 3.2. 9. PLCγ1-deficient BMDMs are not defective in MAPK activation.

BMDMs from Mx1-cre/PLCγ1$^{fl/-}$ and Mx1-cre/PLCγ1$^{fl/fl}$ mice were stimulated with *C. albicans* hyphae for the indicated time points. Cell lysates were prepared and subjected to Western blotting.

3.2.5 PLCγ2 is the main phospholipase required for *C. albicans* cytokine production

We investigated the requirement of PLCγ homologues to regulate cytokine production in response to *C. albicans*. We compared the levels of cytokine production in response to the hyphal (Figure 3.2.10) and yeast (Figure 3.2.11) form of *C. albicans* in PLCγ2-deficient macrophages. In PLCγ2-deficient BMDMs compared to WT macrophages there was a significant decrease in the levels of TNF-α, IL-10, IL-12p40, IL-6 production both in the yeast and hyphal forms of *C. albicans*.

The homologue of PLCγ2, PLCγ1 is also expressed in macrophages. To determine a possible role for PLCγ1 in macrophage antifungal immunity, we evaluated the role of PLCγ1 in cytokine production in BMDMs in response to stimulation with *C. albicans*. PLCγ1-deficiency causes early embryonic lethality (94), therefore we used conditional Mx1-Cre PLCγ1-deficient mice which lack PLCγ1 only in the hematopoietic system. However, we found lack of PLCγ1 did not alter cytokine production in BMDMs after stimulation with *C. albicans* (Figure 3.2.12). Collectively these data indicate that PLCγ2 is the main phospholipase that is critical in mediating immune responses in macrophages to fungal infections.

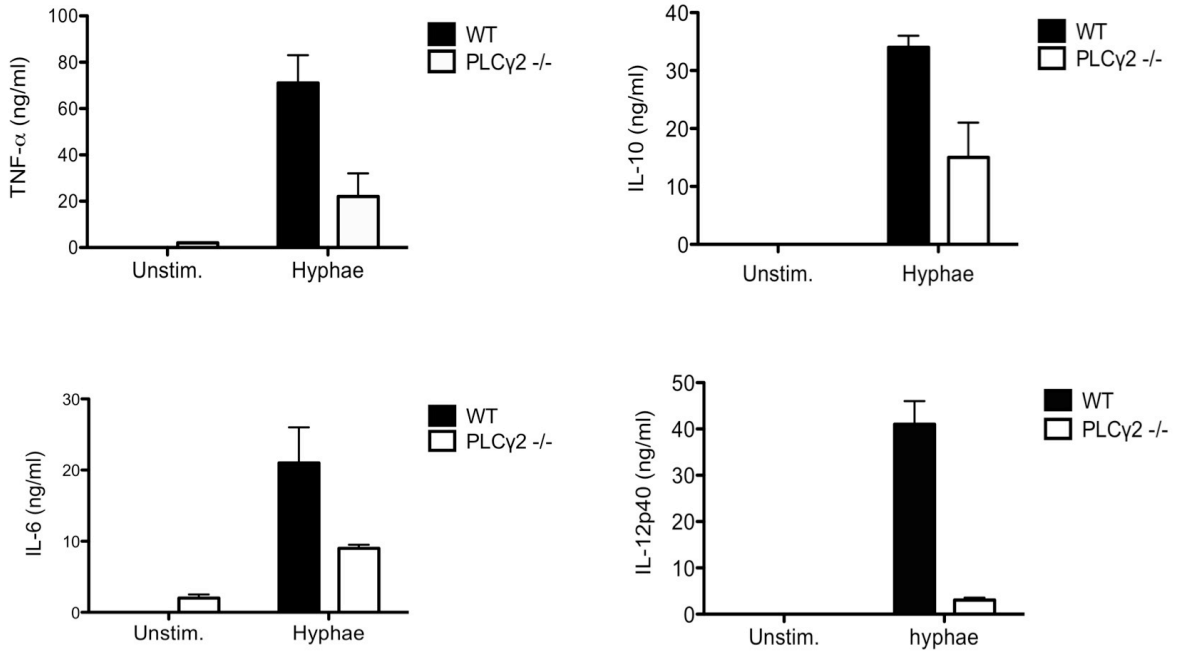

Figure 3.2.10. PLCγ2 contributes to cytokine induction by *C. albicans* stimulation. BMDMs from WT (*black bars*) and PLCγ2-deficient (PLCγ2$^{-/-}$, *white bars*) mice were stimulated overnight with *C. albicans* hyphae (MOI: 1). ELISA was used to measure the level of cytokines in these cultured media. The data are the means ± S.D. of triplicate wells and are representative of three independent experiments.

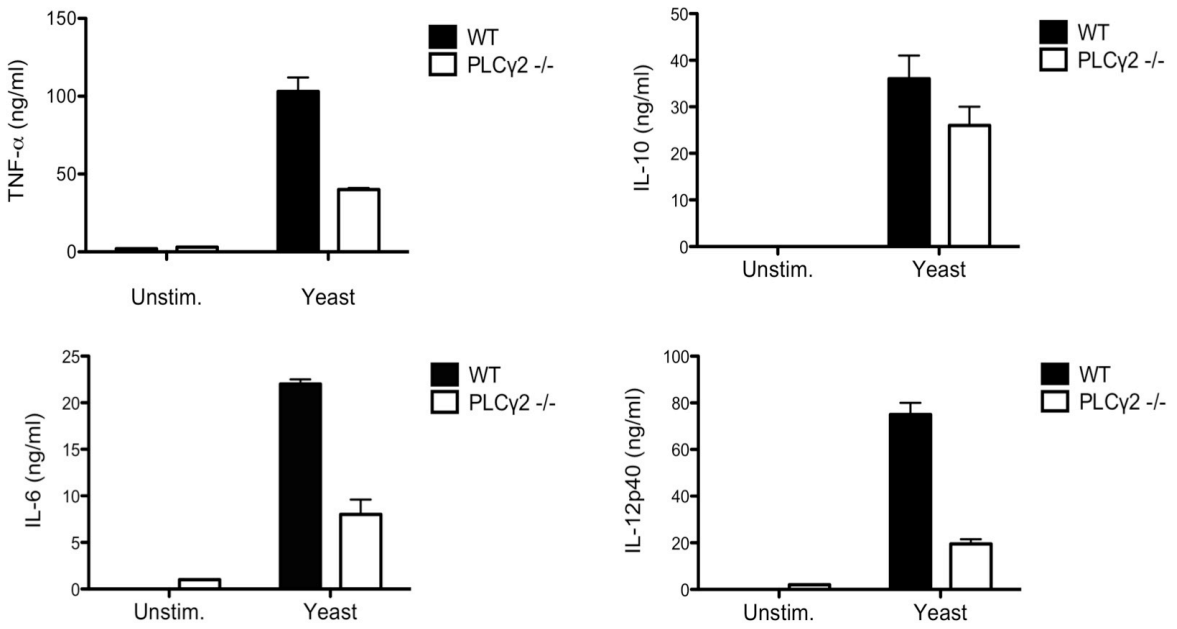

Figure 3.2.11. PLCγ2 contributes to cytokine induction by *C. albicans* stimulation. BMDMs from WT (*black bars*) and PLCγ2-deficient (PLCγ2$^{-/-}$, *white bars*) mice were stimulated overnight with *C. albicans* heat-killed yeast (MOI: 5). ELISA was used to measure the level of cytokines in these cultured media. The data are the means ± S.D. of triplicate wells and are representative of three independent experiments.

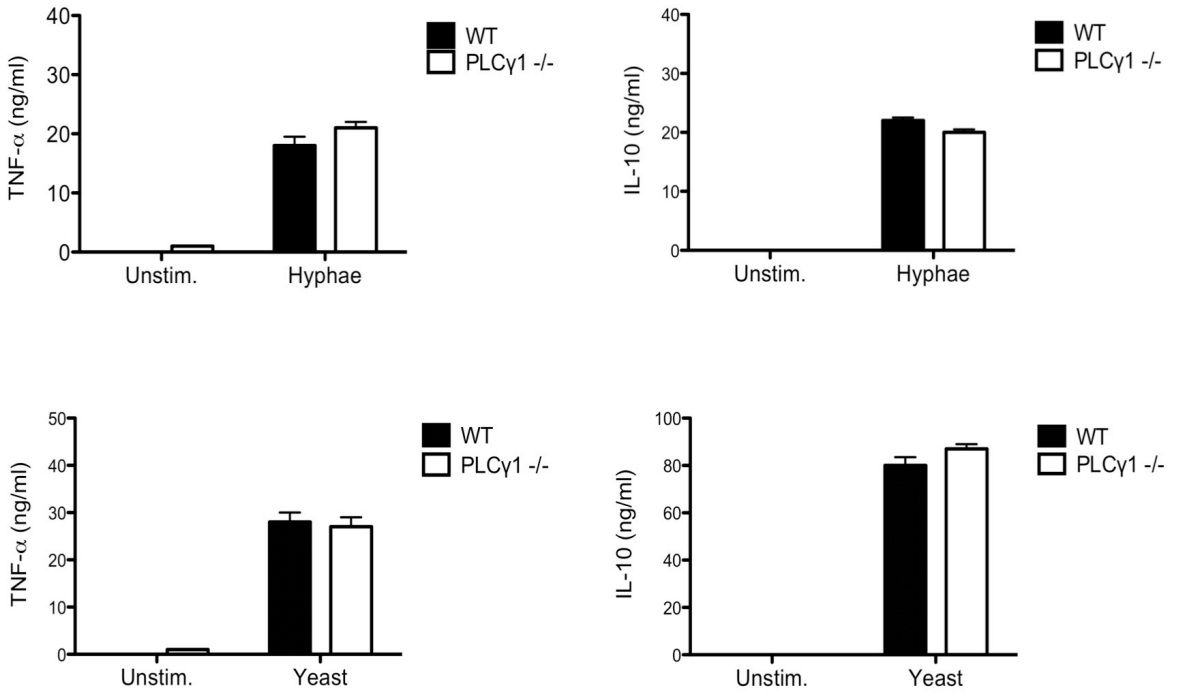

Figure 3.2.12. PLCγ1 is not required for cytokine production by *C. albicans* yeast and hyphae. BMDMs from Mx1-cre/PLCγ1$^{fl/+}$ mice (WT, *black bars*) and Mx1-cre/PLCγ1$^{fl/fl}$ mice (PLCγ1$^{-/-}$, *white bars*) were stimulated overnight with hyphae (MOI: 1) or heat-killed *C. albicans* yeast (MOI: 5) ELISA was used to measure the level of cytokines in these cultured media. The data are the means ± S.D. of triplicate wells and are representative of three independent experiments.

3.2.6. ROS production in macrophages in response to *C. albicans* is controlled by PLCγ2

Reactive oxygen species (ROS) production is an important defense mechanism against fungal infections. Experiments conducted using zymosan have pointed to the role of Syk in mediating ROS production (144). However, downstream of Syk, other signaling components that are involved in ROS production are not clear. PLCγ2 has been shown to mediate NADPH oxidase production in integrin-mediated adhesion in neutrophils (152). Based on these observations, we examined if PLCγ2 is involved in ROS production to *C. albicans* infection. We found that in response to hyphae PLCγ2-deficient macrophages in contrast to WT macrophages were unable to produce ROS (Figure 3.2.13). However, stimulation with phorbol myristate induced robust ROS production in both PLCγ2-deficient and WT macrophages (Figure 3.2.13). In contrast to what we observed in PLCγ2-deficient macrophages, PLCγ1$^{-/-}$ did not show any defect in ROS production compared to WT macrophage (Figure 3.2.13). Collectively, these data indicate that PLCγ2 is critical for initiating ROS production to mount an effective anti-fungal immune response to *C. albicans*.

PLCγ2 induces inositol 1,4,5-triphosphate and diacylglycerol. These components can then increase levels of intracellular calcium and activate PKC. Using a PKC inhibitor GF109203X and a calcium chelator Bapta AM, we examined how PLCγ2 induces ROS generation. We found that induction of ROS production primarily depends on PKC activation, since pretreatment of macrophages with the PKC inhibitor significantly inhibited

ROS production, whereas the calcium chelator was able to only cause a partial decrease in ROS production (Figure 3.2.13).

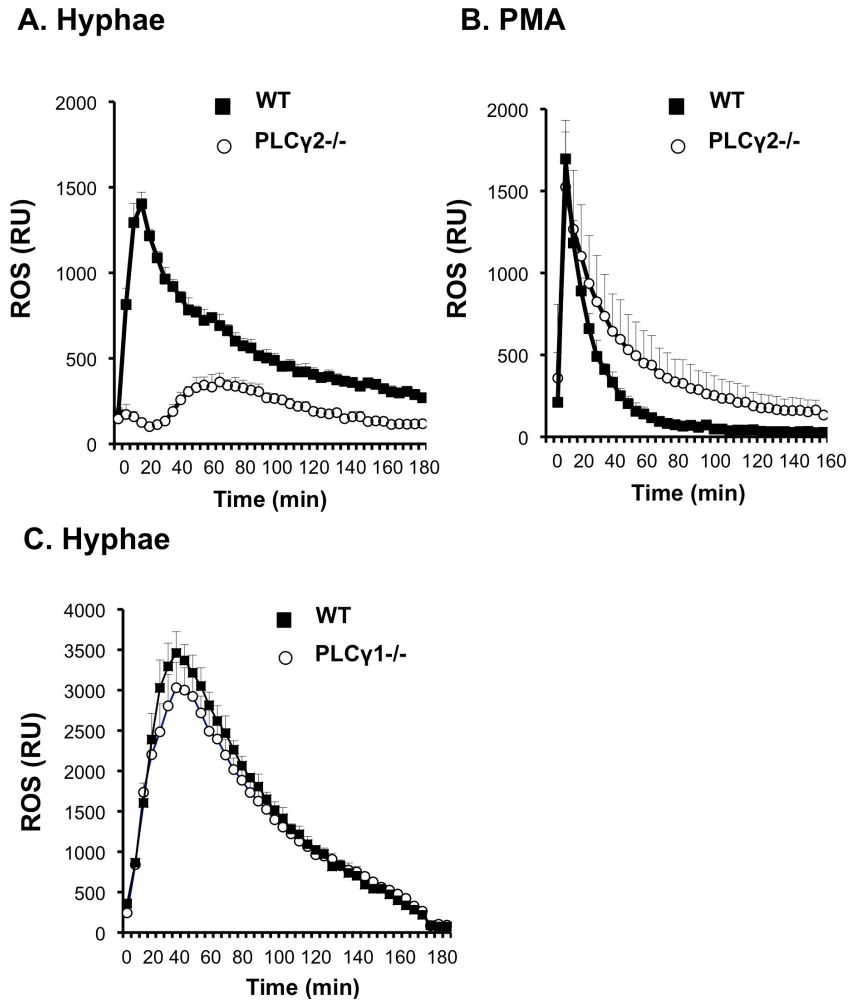

Figure 3.2.13. PLCγ2 is required for ROS production in response to *C. albicans* hyphae stimulation. *A* and *B*, ROS production in WT (*black squares*) or PLCγ2-deficient (*white circles*) BMDMs was measured by using a luminometer following the stimulation with *C. albicans* hyphae (*A*, MOI: 2) or phorbol myristate acetate (*B*, 100μM). *C*, ROS production in Mx1-cre/PLCγ1$^{+/+}$ (WT, *black squares*) and Mx1-cre/PLCγ1 $^{fl/fl}$ (PLCγ1$^{-/-}$, *white circles*) BMDMs was measured following the stimulation with hyphae (MOI: 2). Data are means ± SDs and are representative of at least three independent experiments. *RU*, relative units.

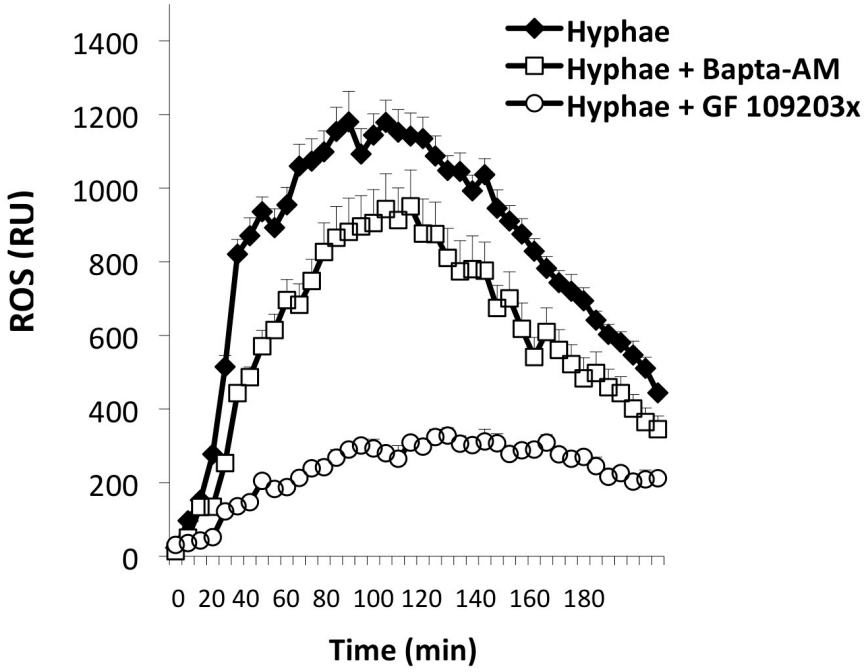

Figure 3.2.14. Hyphae-induced ROS production is dependent on PKC.

ROS production in WT BMDMs pretreated for 1 hr with 10μM Bapta-AM (white squares) or 5μM GF109203X (white circles) or DMSO (black squares) and then stimulated with hyphae (MOI: 2). Data are means ± SDs and are representative of at least three independent experiments. RU: relative units.

3.2.7 PLCγ 2 is critical for anti-fungal immunity in an in-vivo model of disseminated candidiasis

Candida causes severe disseminated blood stream infections in patients with compromised immune systems such as patients undergoing chemotherapy or bone marrow transplants. Therefore, we examined whether PLCγ2-deficiency was sufficient to increase a host's susceptibility to *Candida* in vivo. To rule out the influence that lack of PLCγ2 could cause in other organs other than the hematopoietic system, we generated PLCγ2-deficient bone marrow chimera mice by reconstituting γ -irradiated wild-type mice with bone marrow from PLCγ $2^{-/-}$ mice.

To generate an *in vivo* model of disseminated candidiasis we intravenously injected live *C. albicans* into PLCγ$2^{-/-}$ and WT control mice. Initially we injected 1x 10^6 *Candida*, which led to the rapid death (within 24 hrs) of PLCγ$2^{-/-}$ mice but not WT control mice. Next we examined a low dose of *Candida* (2x 10^5) for intravenous injections. With this dose, PLCγ$2^{-/-}$ mice were dead within 48 hrs; however, the WT mice were able to survive for more than 5 days (Figure 3.2.17). We evaluated the level of IL-6 cytokine in the sera from PLCγ$2^{-/-}$ mice at different time points during infection. We found that the PLCγ$2^{-/-}$ chimera mice showed lower levels of IL-6 compared to WT controls (Figure 3.2.15. A). To quantitatively assess the fungal burden in mice, we sacrificed mice 42 hours after *i.v.* infections with *Candida* and collected lungs, livers, spleens and kidneys from these mice. Serial dilutions from the homogenized organs of these mice were plated on yeast extract peptone dextrose agar plates in order to count fungal colonies. Quantitative assessment of the fungal burden

in organs from PLCγ2-deficient mice showed that these mice have a significantly higher level of *Candida* compared to the WT control mice infected (Figure 3.2.15. B). Moreover, tissues section from the organs collected from infected mice stained with periodic acid-Shiff showed a significantly higher level of germinating hyphal *C. albicans* compared to WT mice (Figure 3.2.16). These *in vivo* experimental results demonstrate that PLCγ2 is critical for innate antifungal immune responses.

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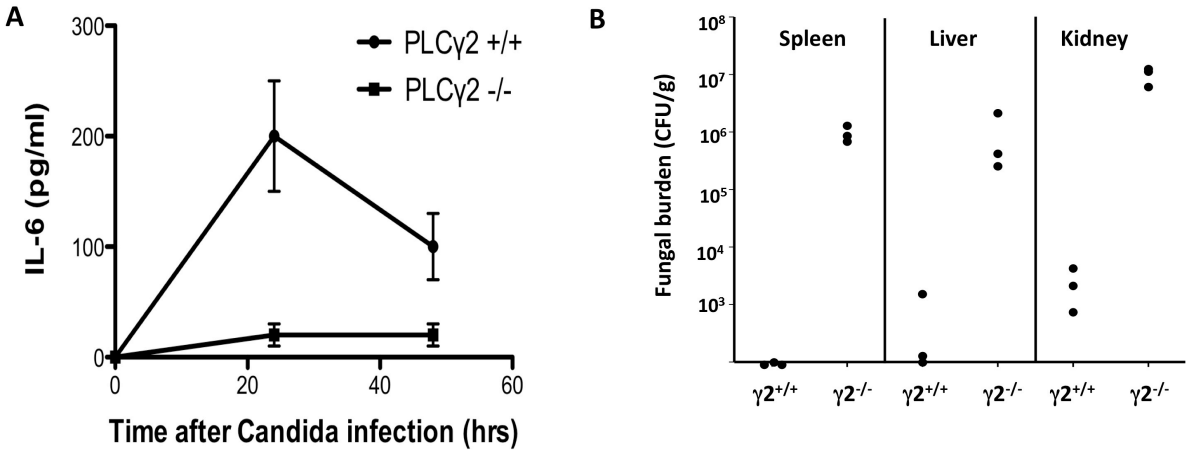

Figure 3.2.15. **PLC**γ2**-deficient mice show higher susceptibility to *C. albicans* infection than WT mice.** *A*, WT and PLCγ2-deficient mice (*n* =3) were challenged with *C. albicans* (2 x 10^5) intravenously, and serum samples from each mouse were tested for IL-6 levels by ELISA. The data are the means ± S.D. from triplicate samples. *B*, 42 h after intravenous infection with *C. albicans* (2 x 10^5), some of the infected mice were sacrificed. The liver, kidney, lung, and spleens from WT and PLCγ2-deficient mice (*n*=3) were homogenized, and serial dilutions of the homogenized organs were plated on yeast extract peptone dextrose agar plates to determine the yeast colony forming unit (*CFU*). The data are colony forming unit/organ weight. Data in panel *A* is generated through collaboration with Mei Yu and Dr. Demin Wang. Data in panel *B* is courtesy of Mei Yu and Dr. Demin Wang.

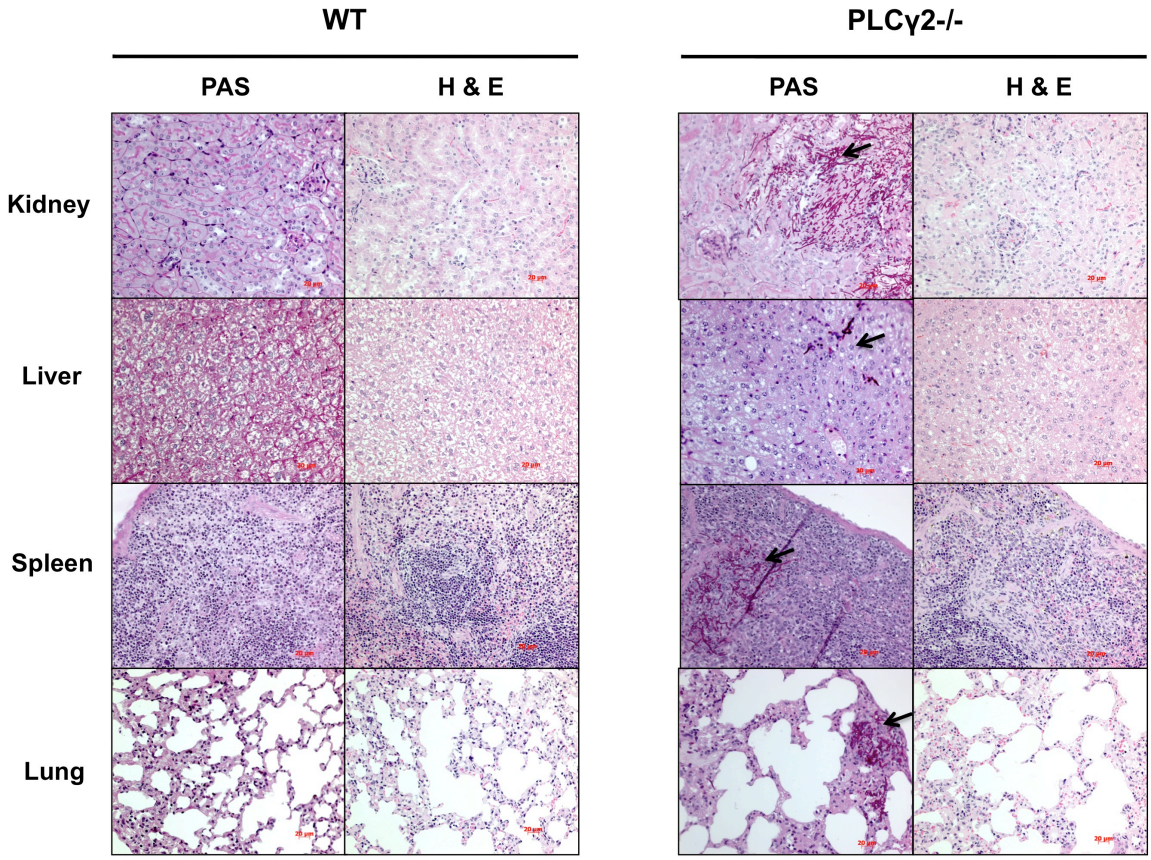

Figure 3.2.16. The histopathology of kidney, lung, spleen, and liver from wild-type and PLCγ2$^{-/-}$ mice after *C. albicans* infection.

The liver, kidney, lung, and spleens of WT and PLCγ2-deficient mice at 42 hours after *i.v.* infection with *C. albicans* ($2x10^5$ cells) were collected. The tissue slides of these organs were stained with hematoxylin and eosin or PAS. Data is generated through collaboration with Mei Yu and Dr. Demin Wang.

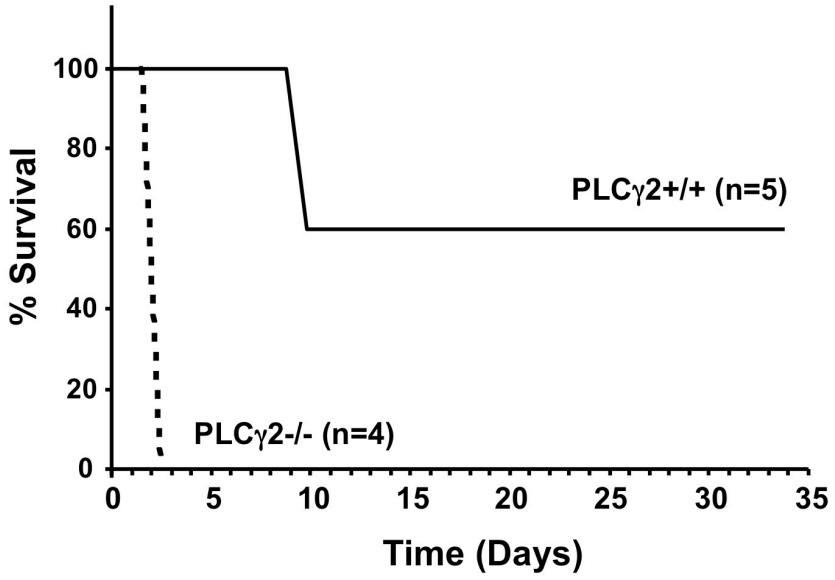

Figure 3.2.17. PLCγ2-deficient mice show higher susceptibility to *C. albicans* infection than WT mice. WT and PLCγ2-deficient mice were challenged with *C. albicans* (2 x10^5) intravenously and monitored every day for lethality. Data is courtesy of Mei Yu and Dr. Demin Wang.

3.3 Discussion

In the C-type lectin receptor signaling pathway, the kinase Syk and the adaptor protein CARD9 are important signaling components for these receptors. But the mechanism of how these proteins link the receptor signaling to NF-κB and MAPK cascades was not defined. In our study, we provide strong evidence that PLCγ2 is the essential PLCγ isoform mediating the signaling cascades downstream of the Dectin-2 receptor. In our study, we demonstrate that NF-κB activation in response to *C. albicans* is not mediated through TLR signaling because we could not detect any difference in NF-κB activation levels between WT and MyD88 deficient macrophages. Previous studies have indicated that TLR signaling collaborated with the Dectin-1 receptor (52, 148). In these studies zymosan, the extract from the yeast cell wall *S. cerevisiae* was used as a ligand for Dectin-1 receptor to measure cytokine production. While NF-κB activation measured by EMSA was similar between MyD88-deficient and WT macrophages, we observed a significant decrease of NF-κB activation in PLCγ2-deficient macrophages stimulated by *C. albicans*. This study indicates differences between using the whole fungal organism versus cell wall extracts. Another interesting observation in our studies is the mechanisms regulating the activation of the IKK complex in response to fungal infections. The IKK complex is activated by the phosphorylation of the IKKα/β subunits alongside the K63 ubiquitination of IKKγ subunit. Studies from our lab have shown that the adaptor protein CARD9 regulates the ubiquitination of IKKγ whereas the kinase Syk is essential for IKKα/β phosphorylation events (62). In this study we show that PLCγ2 regulates the phosphorylation of IKKα/β

whereas we do not observe this decrease in phosphorylation levels in CARD9-deficient macrophages. Furthermore, lack of PLCγ2 did not affect the association of CARD9 with its binding partner BCL10. However, it still remains to be determined if PLCγ2 regulates IKKγ ubiquitination. Collectively these observations indicate that CARD9 and PLCγ2 are activating the IKK complex in an independent manner.

The production of reactive oxygen species is followed by the phagocytosis of *C. albicans* (53). Syk mediates ROS production but not phagocytosis of fungus (144). The adaptor protein CARD9 also regulates ROS production but only partially (137). In our study, we demonstrate that PLCγ2 mediates ROS production in macrophages. Although the exact mechanism that links ROS production to PLCγ2 is not clear, we provide evidence that PKC family members regulate ROS production since ROS production was decreased with the use of a PKC inhibitor but not the calcium chelator. In dendritic cells ROS production is thought to mediate inflammasome activation (153). Future studies are needed to determine if PLCγ2 is required for inflammasome activation.

In our study ERK activation induced by yeast stimulation was only partially reduced in PLCγ2-deficient cells; however, ERK activation induced by hyphae stimulation was undetectable. This finding suggests that in response to yeast, ERK activation is mediated through additional pathways that do not require PLCγ2. The Grb2-SOS-Ras-Raf1 signaling pathway can also mediate ERK activation. In this pathway SOS, which is a Ras guanine nucleotide exchange factor, can be recruited to the membrane by Grb2. These events can lead to the activation of Ras, which then by activating Raf-1 will lead to ERK1 and ERK2 kinase activation (154, 155). Our study points to the possibility that *C. albicans* yeast form

may utilize the Grb2/ Ras/ Raf1 signaling pathway, which in part may help explain the partial reduction that we observe in ERK activation.

For many patients with hematological malignancies, chemotherapy and stem cell transplantation are currently the only treatment options. These treatments damage immune barriers, rendering these patients to infections. Future studies to investigate possible polymorphisms in the PLCγ2 gene in stem cell transplant recipients could improve individualized risk-assessments for fungal infection and aide in new antifungal prophylaxis strategies.

Chapter 4

TAK1 plays an essential role in anti-fungal immunity

Chapter 4: TAK1 plays an essential role in anti-fungal immunity

4.1. Background

Transforming growth factor-β activated kinase-1 (TAK1) is a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family, which plays a pivotal role in adaptive and innate immune signaling (156, 157). TAK1 is activated by a diverse range of stimuli such as ligands for Toll-like receptors, tumor necrosis factor receptor (TNFR) and IL-1 receptor that can lead to the activation of NF-κB and MAPK signaling pathways (158). TAK1 binding protein TAB2 and TAB3 can recruit TAK1 to polyubiquitin chains (159, 160). TAB2 germ-line knockouts are embryonic lethal and deficiency in TAB2 can decrease NF-κB activation (161, 162). Thus, it has been proposed that TAB2 and TAB3 may have some level of redundancy. The *in vivo* tissue specific roles of these proteins remain to be determined.

TAK1 knockout mice are embryonic lethal (163) and deletion of TAK1 in the hematopoietic system leads to uncontrolled apoptosis of hematopoietic stem cells (164). Therefore studies investigating the role of TAK1 in hematopoietic cells have been conducted by using conditional knockouts in a specific lineage. T cell specific TAK1-deficient mice display defects in thymocyte development and in the activation of JNK and NF-κB signaling pathways (165, 166). In the B cell receptor signaling pathway TAK1 only mediates JNK activation (167).

In non-innate immune cells such as embryonic fibroblast and B cells, TAK1 can mediate NF-κB and AP-1 activation upon TLR stimulation (167, 168). Recently the role of

TAK1 in TLR signaling in myeloid cells was investigated. The generation of myeloid-specific TAK1-deficient mice has produced somewhat contradictory phenotypes. In one report myeloid-specific TAK1-deficiency did not affect the ability of myeloid precursors to differentiate into macrophages and granulocytes. In addition TAK1-deficiency in macrophages caused impairment in JNK and NF-κB activation in response to LPS stimulation. Furthermore, LPS stimulation caused an increase in IL-1β, TNF-α and IL-6 in TAK1-deficient macrophages compared to WT (169). In a separate mouse model of myeloid-specific TAK1-deficiency, BMDMs from TAK1-deficient mice died after culturing for 3 days in conditional media containing M-CSF due to apoptosis (170). Compared to WT controls, TAK1-deficient peritoneal macrophages in this mouse model did not show a defect in JNK and NF-κB activation in response to LPS stimulation.

In TLR signaling the adaptor molecule MyD88 activates the Tumor necrosis factor receptor-associated factor 6 (TRAF6), which contains E3 ubiquitin ligase activity. In response to various stimuli including TLR ligands, IL-1 and RANK ligand, TRAF6 interacts with TAK1 through the TAK1-associating protein TAB2 (171-173). In the TCR pathway it has been implicated that TRAF6 and TAK1 can mediate IKK activation through BCL10 and MALT1 (95). However, TRAF6 knockout mice do not show any defects in T cell activation (174, 175). Therefore, it has been speculated that TRAF2 may compensate for the lack of TRAF6 in T cells. A recent study using B cell specific TRAF6 knockout mice showed a role for TRAF6 in mediating classical NF-κB and MAPK activation in response to TLR and CD40 receptor stimulation (176). Similar to previous reports (170), we observed that myeloid-specific TAK1-deficient mice could not develop macrophages from bone marrow.

In collaboration with Dr. Bryant Darnay in the Department of Experimental Therapeutics at The University of Texas MD Anderson Cancer Center in Houston, we used myeloid-specific TAK1-deficient mice crossed with TNFR1-deficient mice, which were generated in Dr. Darnay's laboratory, to rescue the BMDM developmental defect observed in myeloid TAK1-deficient mice. In this study we were interested in understanding the role of TAK1 and TRAF6 in mediating immunity against the opportunistic fungus *Candida albicans*. The mechanisms controlling C-type lectin receptor signaling in response to fungal infections are not well understood. In this work, we report that in macrophages TAK1 and TRAF6 are essential for mediating anti-fungal immunity by regulating NF-κB and MAPK signaling and regulating proinflammatory cytokine production.

4.2. RESULTS

4.2.1 TAK1 is essential for NF-κB, JNK and p38 activation in response to *Candida albicans*

Our previous study demonstrated that phospholipase γ2 (PLCγ2) was an essential component for NF-κB and MAPK activation in response to *C. albicans* (177). NF-κB activation induced by *C. albicans* is not mediated through MyD88 because we could not detect any difference in NF-κB activation in MyD88-deficient macrophages compared to WT in response to *C. albicans* stimulation.

To examine the possible role of TAK1 in mediating anti-fungal immunity, we investigated the activation of the NF-κB pathway in TAK1-deficient mice. By evaluating the DNA-binding activity of NF-κB transcription factor (Figure 4.2.1), we found that in response to both the yeast and hyphal form of *C. albicans*, NF-κB activation was defective in TAK1-deficient mice compared to TNFR1-deficient or WT mice. Furthermore, in TAK1-deficient mice we demonstrate that the defect in NF-κB activity is due to the absence of phosphorylation of IKKα/β subunits of the IKK complex (Figure 4.2.1). Since TAK1 is suggested to regulate the MAPKs we determined the role of TAK1 in mediating JNK and p38 activation. TAK1-deficient mice compared to TNFR1- deficient or WT mice showed a decrease in JNK and p38 activation in response to *Candida* hyphae (Figure 4.2.2).

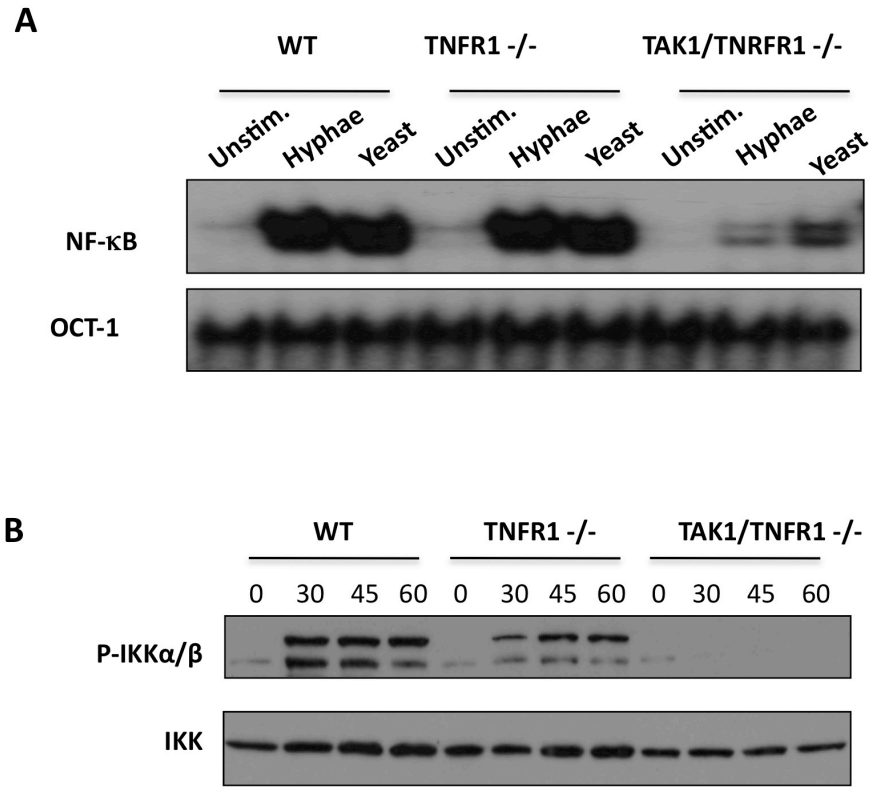

Figure 4.2.1. NF-κB activation induced by *C. albicans* hyphae and yeast is TAK1-dependent. *(A)* WT, TNFR1-deficient and TNFR1$^{-/-}$TAK1$^{-/-}$ BMDMs were either untreated or treated with *C. albicans* hyphae (MOI: 1) or heat-inactivated yeast (MOI: 5) for 90 min. The nuclear extracts were prepared from these cells and then subjected to the electrophoretic mobility shift assay using 32P-labeled NF-κB or OCT-1 probe. (B) WT, TNFR1-deficient and TNFR1$^{-/-}$TAK1$^{-/-}$ BMDMs were stimulated with *C. albicans* hyphae for the indicated time points, and IKKα/β phosphorylation was examined using anti-phospho-IKKα (Ser-176)/IKK-β (Ser-180) antibody.

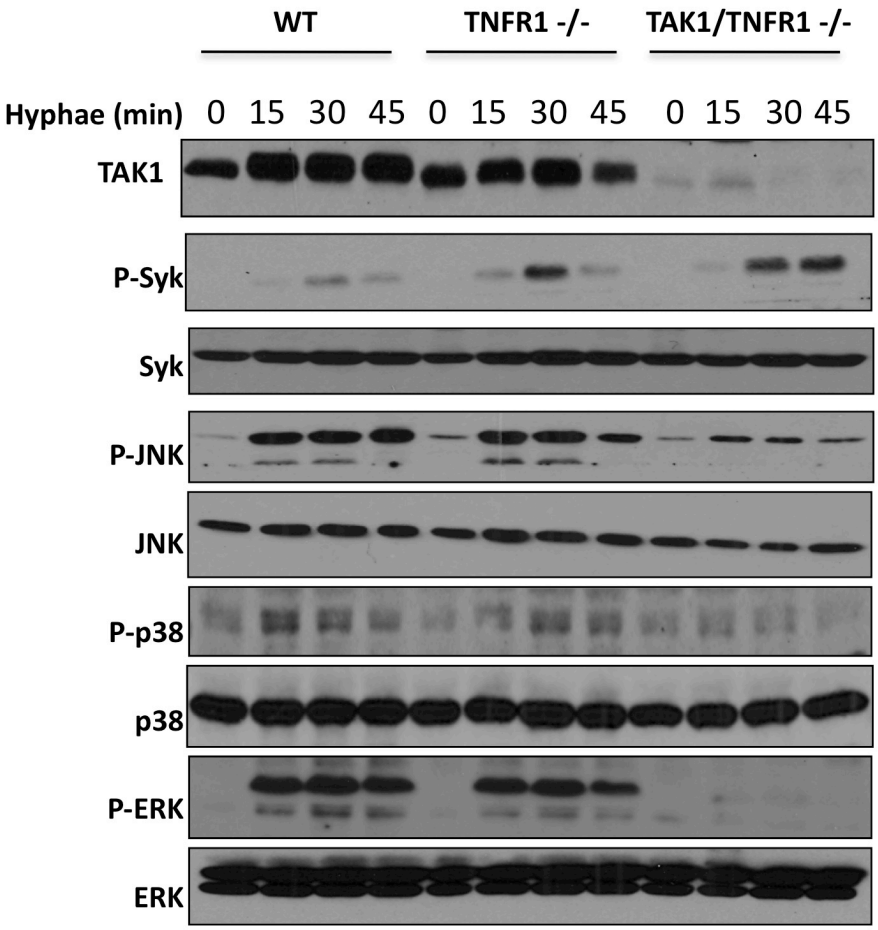

Figure 4.2.2. TAK1-deficient BMDMs display defective MAPK activation.

WT, TNFR1$^{-/-}$, TNFR1$^{-/-}$TAK1$^{-/-}$ BMDMs were stimulated with *C. albicans* hyphae for the indicated time points. Cell lysates were prepared from these cells and then subjected to Western blotting.

4.2.2. TAK1 regulates cytokines necessary for T helper differentiation in response to *Candida albicans*

To determine if TAK1-deficiency can regulate the production of cytokines important in inducing an adaptive immune response to fungal infections, we evaluated the levels of IL-6, IL-12 and TNF-α in TAK1/TNFR1-deficient mice compared to $TNFR1^{-/-}$ and WT macrophages. As shown in (Figure 4.2.3) TAK1-deficiency caused a decrease in proinflammatory cytokine production compared to $TNFR1^{-/-}$ and WT mice. The cytokine IL-1β plays an important role in the differentiation of Th17 cells. In contrast to dendritic cells IL-1β production in macrophages is a two-step process. Stimulation of macrophages with hyphae alone cannot induce detectible levels of IL-1β (Figure 4.2.4). Priming of cells with zymosan prior to stimulation with hyphae is a necessary step to induce IL-1β production (Figure 4.2.4). We investigated the levels of IL-1β production in TAK1/ TNFR1-deficient cells compared to TNFR1 and WT macrophages. Compared to WT macrophages, TNFR1-deficient macrophages showed lower levels of IL-1β production (Figure 4.2.4). However, TAK1/TNFR1-deficient macrophages demonstrated a lower level of IL-1β production compared to TNFR1-deficient (Figure 4.2.4), which demonstrates an essential role for TAK1 in IL-1β production.

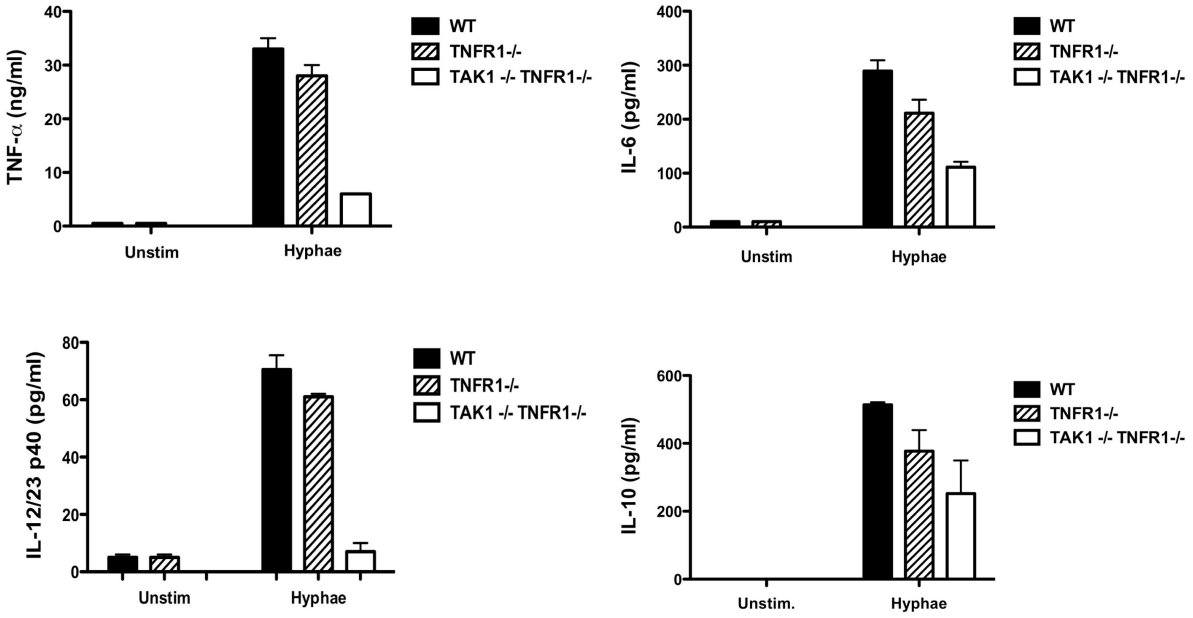

Figure 4.2.3. TAK1 contributes to cytokine induction by *C. albicans* stimulation. BMDMs from WT (*black bars*), TNFR1-deficient *(lined bars)*, TNFR1$^{-/-}$TAK1$^{-/-}$ (*white bars*) mice were stimulated overnight with *C. albicans* hyphae (MOI: 1). ELISA was used to measure the level of cytokines in these cultured media. The data are the means ± S.D. of triplicate wells and are representative of three independent experiments.

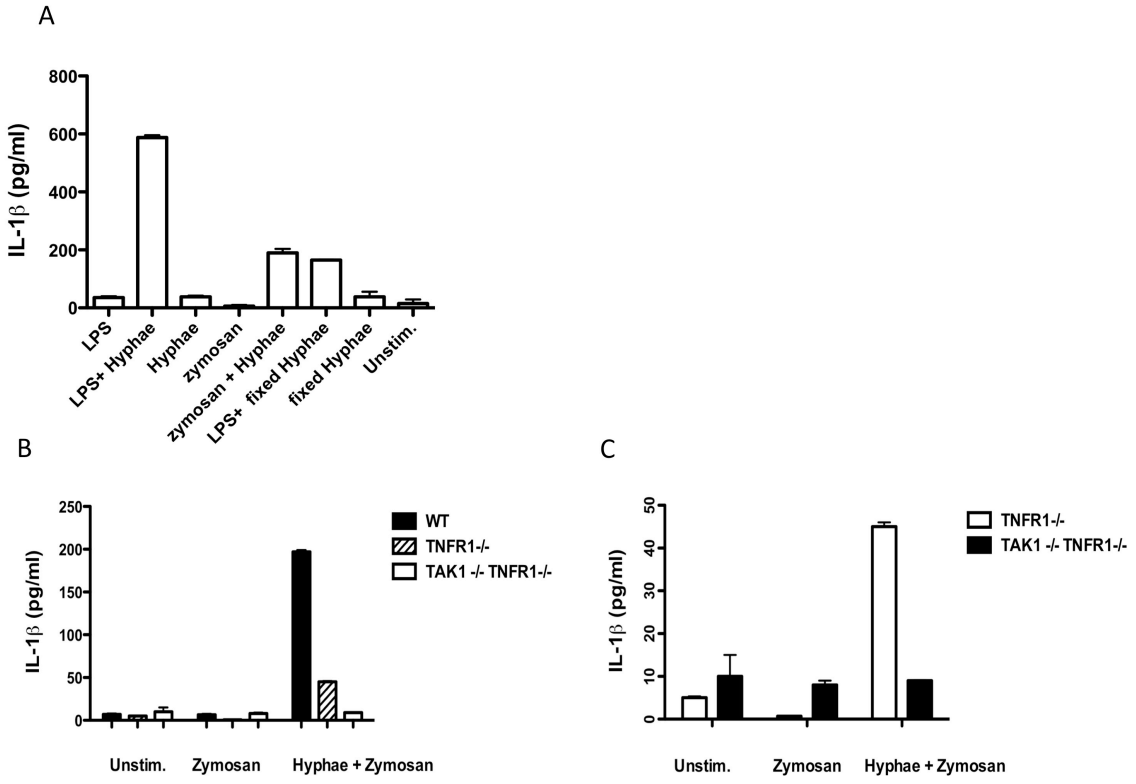

Figure 4.2.4. TAK1 contributes to cytokine induction by *C. albicans* stimulation.

A) BMDMs from WT (*white bars*) were primed with either LPS or zymosan then left untreated or stimulated with live hyphae or paraformaldehyde fixed hyphae for overnight. ELISA was used to measure the level of cytokines in these cultured media. B) WT (*black bars*) TNFR1-deficient *(lined bars)* and TNFR1$^{-/-}$TAK1$^{-/-}$ (*white bars*) mice were primed with zymosan with or without live *C. albicans* hyphae (MOI: 1) for overnight. ELISA was used to measure the level of cytokines in these cultured media. C) TNFR1-deficient *(white bars)* and TNFR1$^{-/-}$TAK1$^{-/-}$ (*black bars*) were primed with zymosan with or without live *C. albicans* hyphae (MOI: 1) for overnight. ELISA was used to measure the level of cytokines in the media. The data are the means ± S.D. of triplicate wells and are representative of three independent experiments.

4.2.3 TRAF6 is crucial for *C. albicans* mediated NF-κB and JNK activation

Tumor necrosis factor receptor-associated factor 6 (TRAF6) can form a complex with TAK1 in response to receptor activator of NF-κB ligand (RANKL) stimulation (171). In the interleukin-1 signaling pathways TRAF6 and TAK1 can form a complex that is mediated by TAB2 (172), leading to IKK and MAPK activation. To investigate if TRAF6 is mediating proinflammatory responses to *Candida* infections we evaluated NF-κB activation in TRAF6-deficient macrophages in response to hyphae. TRAF6-deficient macrophages showed a decrease in the DNA-binding activity of NF-κB transcription factor and were defective in the degradation of IκB compared to WT mice (Figure 4.2.5). However, in contrast to what we showed in TAK1-deficient macrophages, we could not detect any difference in the levels of IKKα/β phosphorylation between TRAF6-deficient wild-type mice (Figure 4.2.6). We next examined how TRAF6-deficiency would impact MAPK activation. In response to hyphae, $TRAF6^{-/-}$ macrophages showed a decrease in JNK but not ERK activation when compared to WT macrophages (Figure 4.2.6). To examine the role of TRAF6 in antifungal immunity we evaluated the levels of proinflammatory cytokines IL-6 and IL-12 in response to hyphae. Indeed, $TRAF6^{-/-}$ macrophages were defective in the production of IL-6 and IL-12 cytokines (Figure 4.2.7), indicating a role for TRAF6 in mediating anti-fungal immune responses. To determine how TRAF6 is regulating NF-κB activation we found that upon hyphae stimulation TRAF6 becomes ubiquitinated (Figure 4.2.8). In addition we find that in BMDMs TRAF6 binds to BCL10 and TAK1 (Figure 4.2.8). Although TRAF6 binds BCL10 constitutively it appears that the complex formation

of TAK1 and TRAF6 is inducible. Therefore, we conclude that Traf6 mediates antifungal immunity by regulating NF-κB and JNK signaling pathways that are crucial for proinflammatory cytokine production.

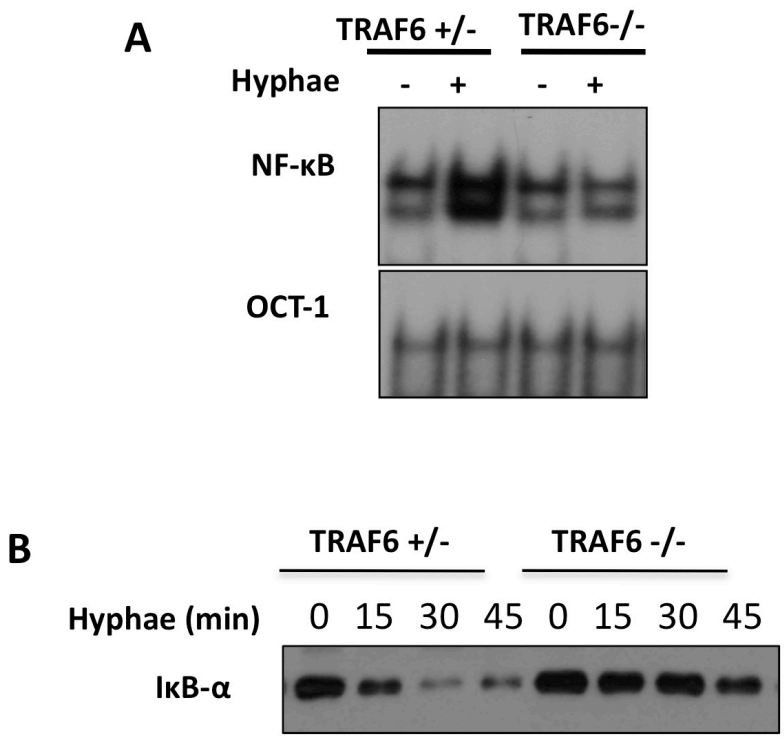

Figure 4.2. 5. **NF-κB activation induced by *C. albicans* hyphae is TRAF6-dependent.**

(A) WT and TRAF6-deficient BMDMs were either untreated or treated with *C. albicans* hyphae (MOI: 1) for 90 min. The nuclear extracts were prepared from these cells and then subjected to the electrophoretic mobility shift assay using 32P-labeled NF-κB or OCT-1 probe. *(B)* WT and TRAF6-deficient BMDMs were stimulated with *C. albicans* hyphae for the indicated time points, and IκB-α was examined using the indicated antibody.

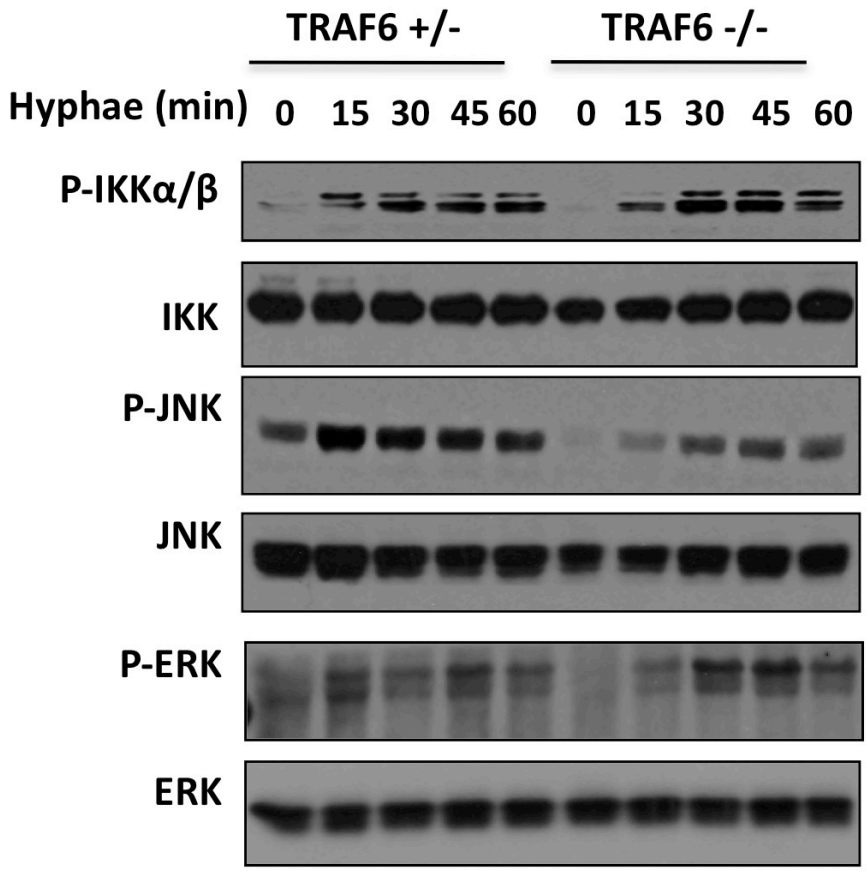

Figure 4.2.6. TRAF6 contributes to JNK but not IKKα/β phosphorylation.

WT and TRAF6-deficient BMDMs were either untreated or treated with *C. albicans* hyphae (MOI: 1) for the indicated time points, and phosphorylation was examined using the indicated antibodies.

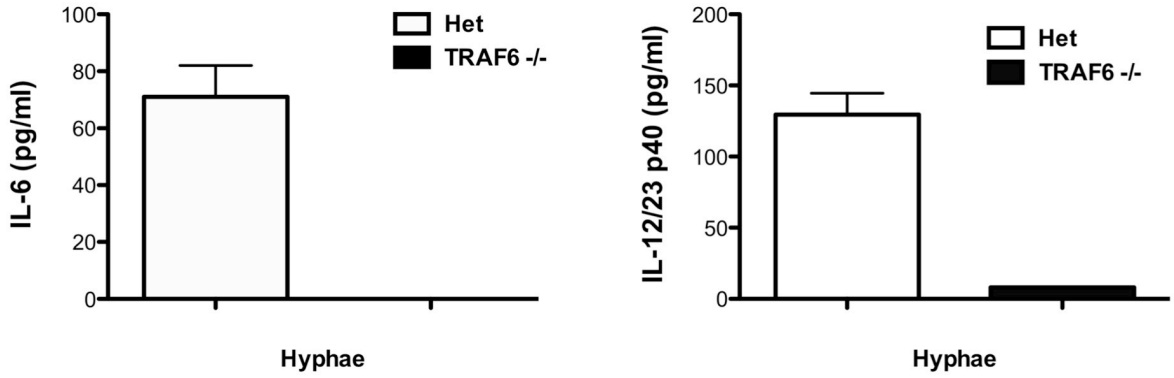

Figure 4.2.7. TRAF6 contributes to cytokine induction by *C. albicans* stimulation.

BMDMs from Heterozygous (*white bars*) and TRAF6-deficient *(black bars)* mice were stimulated overnight with *C. albicans* hyphae (MOI: 1). ELISA was used to measure the level of cytokines in these cultured media. The data are the means ± S.D. of triplicate wells and are representative of three independent experiments.

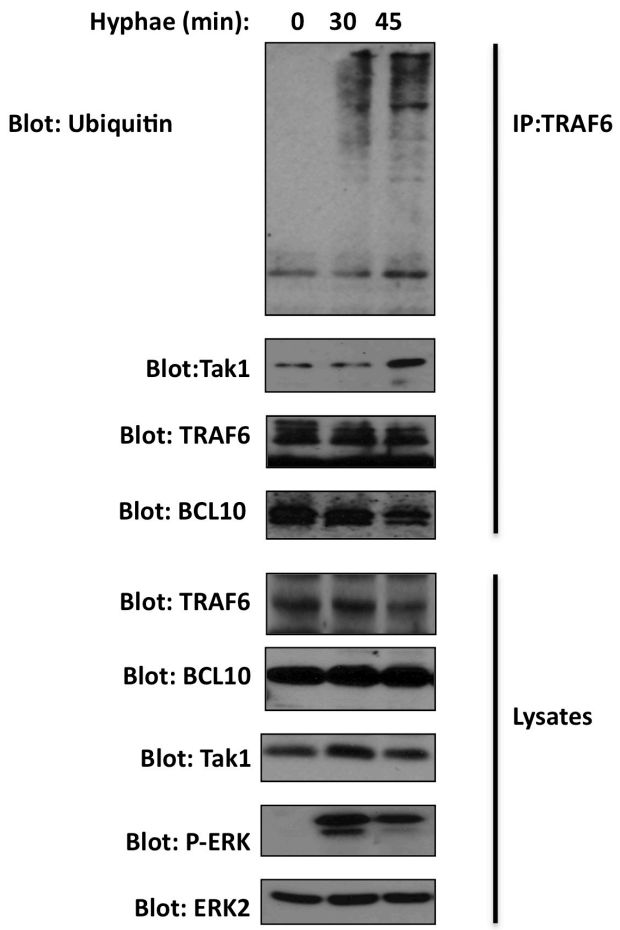

Figure 4.2.8. TRAF6 is ubiquitinated upon *C. albicans* stimulation and forms a complex with BCL10 and TAK1.

WT BMDMs were stimulated with *C. albicans* hyphae (MOI: 1) for the indicated time points, and the cell lysates were immunoprecipitated with TRAF6 antibody-conjugated agarose. The immunoprecipitates were probed with the indicated antibodies. *IP*, immunoprecipitation; *WCL,* whole cell lysate.

4.2.4. TRAF6 is not required for hyphae induced reactive oxygen species production.

Reactive oxygen species (ROS) are a critical component of the immune system to fight against pathogens. Previous studies in bone marrow derived monocytes show that RANK ligand stimulation can generate reactive oxygen species production. The ROS production in these cells was dependent on TRAF6 since TRAF6-deficient osteoclast precursors had significantly reduced levels of ROS production compared to WT (178). To examine the importance of TRAF6 in *C. albicans* hyphae induced ROS production, we compared ROS production in TRAF6-deficient BMDMs and wild-type cells. The level of ROS production by hyphae was similar in TRAF6- null BMDMs compared to wild-type (Figure 4.2.9), which indicates that *C. albicans* induced ROS production in macrophages is not dependent on TRAF6.

4.2.5. CYLD is not involved in antifungal immunity

The deubiquitase CYLD can regulate TAK1 activation by inhibiting autoactivation and ubiquitination by physically interacting with TAK1 in T cells (179). Therefore we hypothesized that CYLD may regulate antifungal immunity by regulating TAK1. We examined the DNA-binding activity of NF-κB transcription factor in response to *C. albicans* yeast and hyphae in WT and CYLD-deficient macrophages. However, we could not detect any difference in NF-κB activation or in TNF-α cytokine production between CYLD-deficient and WT macrophages (Figure 10). In addition $CYLD^{-/-}$ and WT macrophages were

comparable in the levels of JNK and ERK activation in response to *C. albicans* yeast and hyphae (Figure 4.2.11). These results suggest that CYLD does not regulate C-type lectin receptor signaling in macrophages.

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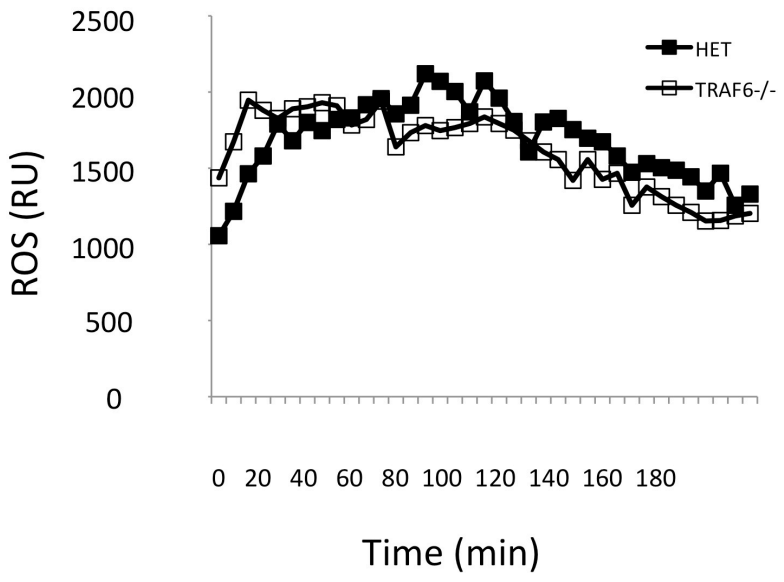

Figure 4.2.9. TRAF6 is not required for ROS production in response to *C. albicans* hyphae stimulation. ROS production in heterozygous (*black squares*) or TRAF6-deficient (*white squares*) BMDMs was measured by using a luminometer following the stimulation with *C. albicans* hyphae (MOI: 2). Data are means and are representative of at least three independent experiments. *RU*, relative units.

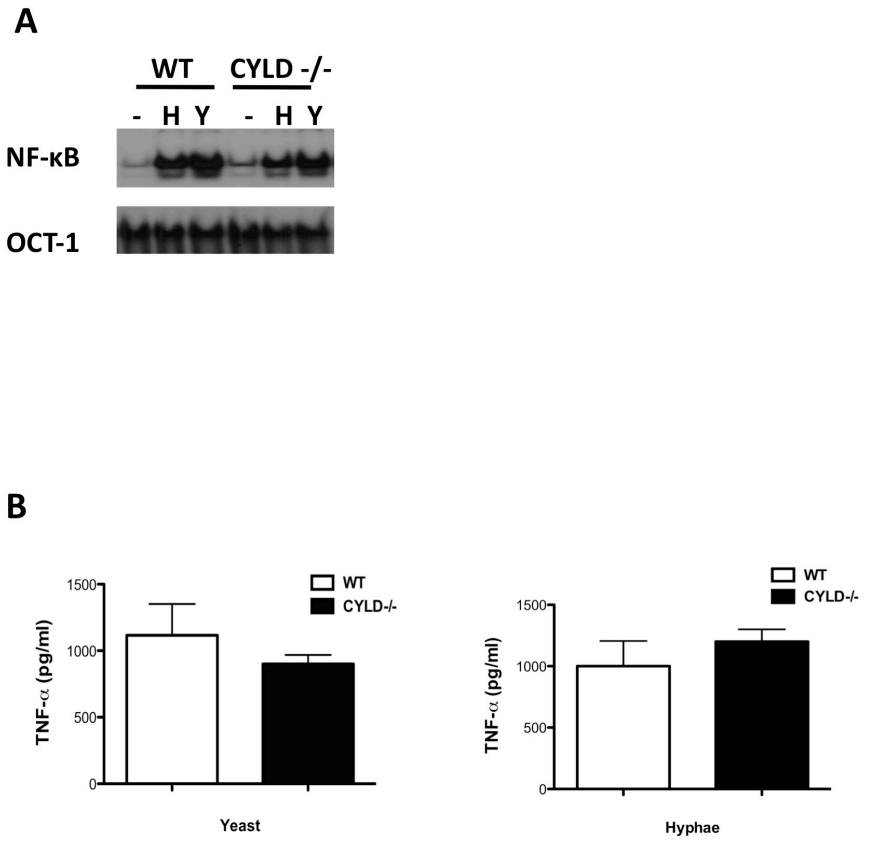

Figure 4.2.10. CYLD does not contribute to NF-κB activation or TNF cytokine induction by *C. albicans* stimulation. *A)* WT and CYLD-deficient BMDMs were either untreated or treated with *C. albicans* hyphae (MOI: 1) for 90 min. The nuclear extracts were prepared from these cells and then subjected to the electrophoretic mobility shift assay using 32P-labeled NF-κB or OCT-1 probe. *B)* Enzyme-linked immunosorbent assay of TNF in the supernatants from BMDMs of WT (*white bars*) and CYLD-deficient *(black bars)* mice that were stimulated overnight with *C. albicans* hyphae (MOI: 1) or heat- killed yeast (MOI: 5).

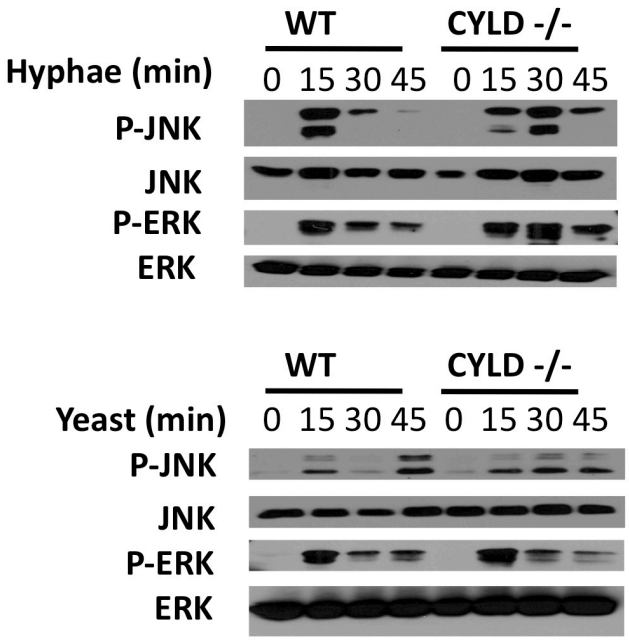

Figure 4.2.11. CYLD-deficient BMDMs are not defective in MAPK activation in response to *C. albicans.* Wild-type and CYLD-deficient ($CYLD^{-/-}$) BMDMs were stimulated with *C.albicans* hyphae (MOI: 1) or heat- killed yeast (MOI: 5) for the indicated time points. The cell lysates were prepared from these cells and then subjected to immunoblotting analysis using the indicated antibodies.

4. 3. Discussion

In this study we demonstrate an important role for TAK1 in mediating antifungal immunity. We report that in response to both the yeast and the hyphal form of *C. albicans* TAK1/TNFR1$^{-/-}$ macrophages are defective in NF-κB activation compared to TNFR1$^{-/-}$ and WT macrophages. Furthermore, we show that TAK1-deficiency causes a decrease in JNK and p38 activation. Collectively, this reduction in NF-κB and MAPK signaling cascades is leading to decreased levels of TNF, IL-6, IL-12 and IL-1β cytokine production in TAK1$^{-/-}$ /TNFR1$^{-/-}$ macrophages compared to WT and TNFR1$^{-/-}$ macrophages. Studies using mice with specific deletion of TAK1 in the myeloid lineage demonstrate that loss of TAK1 promotes macrophage apoptosis and increases the proliferation of neutrophils (170). Experiments performed with peritoneal macrophages from these mice demonstrated that TAK1-deficiency did not affect NF-κB or MAPK activation in response to LPS stimulation. However, in neutrophils loss of TAK1 caused an increase in NF-κB and MAPK activation in response to LPS stimulation (170). Collectively, these results demonstrate the cell type-specificity of TAK1 in regulating the innate immune response to bacterial versus fungal pathogens. In addition, we find that in macrophages TRAF6-deficiency causes a decrease in NF-κB and JNK activation. We demonstrate the mechanism that TAK1 and TRAF6 use to activate the IKK complex in response to *C. albicans* stimulation differs.

Previous observations show a critical role for TRAF6 in TLR and CD40 receptor signaling (180). Our findings together with previous studies using TRAF6-deficient cells indicate that TRAF6 in an important signaling component in myeloid cells that converges

signals both from TLR and C-type lectin receptors. How TAK1 is activated in response to fungal stimuli remains to be elucidated. Previous studies suggest that TAK1 undergoes autophosphorylation (181, 182). One possibility is that the complex of TRAF6-TAB2-TAK1 may cause a conformational change in TAK1, thus promoting TAK1 kinase activity. Although it is proposed that TAK1 is the upstream kinase that phosphorylates the IKK complex, the mechanistic details of IKK activation is not fully elucidated. We show that TAK1-deficiency causes a defect in the phosphorylation of the IKK complex. Recently it was shown that TAK1 phosphorylation is mediated by CARD9 (183). However, we did not observe any difference in the phosphorylation of IKKα/β in CARD9-deficient mice compared to WT mice. This observation is similar to what has been reported in CARMA1-deficient T cells, which do not show any defect in IKKα/β phosphorylation in response to TCR stimulation compared to wild-type T cells (150). Based on our experimental results we propose that TRAF6 is important in linking the CARD9-BCL10 complex to the ubiquitination of IKKγ. Furthermore, TRAF6 polyubiquitination recruits TAK1 leading to its possible autophosphorylation and consequently the phosphorylation of IKKα/β subunits in response to *C. albicans*.

Pharmacological inhibition or gene mutations that cause the inactivation of NF-κB is reported to sensitize cells to TNF-α-induced apoptosis (184). However, adoptive transfer of NF-κB-deficient fetal liver cells does not affect the survival of hematopoietic stem cells or myeloid lineage cells (185) suggesting that NF-κB signaling alone is not responsible for myeloid cell survival. Reactive oxygen species levels in cells can also affect cell survival. Although TRAF6-deficiency has been shown to promote TNF-induced cell death and

accumulation of reactive oxygen species (186), we did not observe a defect in macrophage differentiation or hyphae-induced reactive oxygen species production using TRAF6-deficient mice. In our studies we did not determine reactive oxygen species levels in TAK1-deficient macrophages. One possibility is that TAK1-deficient macrophage precursors are highly sensitive to TNF-induced ROS accumulation. TAK1-deficient neutrophils are reported to produce higher levels of ROS compared to WT cells in response to LPS treatment (170). Quantitative differences in signaling molecules expression levels may affect cell type specific development. For example, in myeloid precursors a high level of c-Src expression affects osteoclast, but not macrophage or dendritic cell development (187). Microarray analysis of TAK1-deficient macrophages compared to TNFR1-deficient macrophages may help in identifying key components for macrophage cell survival.

The TAK1-binding proteins (TAB), TAB2 and TAB3 are required for TAK1 activation (159). In the IL-1, RANKL, TNF signaling pathways the functional interaction between TAK1 and TAB2/ TAB3 is crucial for mediating IKK and MAPK signaling (188). Further studies will be needed to investigate if TAB2 and TAB3 mediate C-type lectin receptor signaling and whether or not TAB2 and TAB3 play redundant functions in immune signaling.

Previous studies report that in response to bacteria TRAF6-depleted macrophages showed a decrease in the levels of mitochondrial ROS production, which impaired the ability of these mice to effectively kill intracellular bacteria (189). In our studies we could not detect any difference in NADPH mediated reactive oxygen species production upon stimulation with *C. albicans* hyphae between TRAF6-deficient and WT BMDMs. Whether

in macrophages *C. albicans* stimulation can trigger mitochondrial ROS production in a TRAF6 dependent manner is a topic of future investigation.

In this study we investigated the role of CYLD in mediating macrophages immune response to *C. albicans*. However, we could not detect any difference between CYLD-deficient macrophages and WT in the activation of MAPKs, NF-κB or in cytokine production upon *C. albicans* stimulation. In lymphocytes, CYLD has an important role in regulating TCR signaling (179, 190) and negatively regulating NF-κB signaling in B cells (191). However, studies using CYLD-deficient macrophages show undetectable to moderate changes in NF-κB activation in response to TNFR or TLR signaling (190, 192). Our findings together with previous studies indicate that CYLD is not critical in regulating pattern recognition receptor signaling in macrophages. The ubiquitin-modifying enzyme A20 is crucial in terminating TLR responses and can be inducibly expressed by these cells (193). Therefore, it is possible that in pattern recognition receptor signaling, CYLD plays a redundant role with A20.

Chapter 5

Identification of CARD9 binding partners

Chapter 5: Identification of CARD9 binding partners

5.1. Background

The adaptor protein CARD is an important signaling component downstream of C-type lectin receptors and is necessary to mediate anti-fungal immunity (62, 68, 100, 194). CARD9 was identified by a database search for CARD-containing proteins and can form a complex with BCL10 and MALT1 to mediate downstream NF-κB activation (98). CARD9 protein structure consists of a C-terminus coiled-coil domain and a N-terminus CARD domain and is primarily expressed in myeloid lineage cells (98, 100). CARD9 does not contain a PDZ domain. The PDZ domain in CARD-containing membrane-associated guanylate kinase (CARMA) family members is critical for these proteins to associate to the plasma membrane (98). CARD9 regulates NF-κB activation by regulating the IKK complex (62). Both Syk and CARD9 are required for the activation of the IKK complex. Syk is required for the phosphorylation of IKKα/β subunits, whereas CARD9 regulates the ubiquitination of IKKγ (62). The signaling components that link Syk to CARD9 activation are not well understood. In this study, we were interested in indentifying novel protein binding partners for CARD9 to help elucidate the C-type lectin receptor signaling pathways.

5.2. Results

To identify CARD9 binding partners we chose to use the SILAC method (12). SILAC is a method for metabolic labeling in which the quantitative tags used for measurements in mass spectrometry are incorporated into the cells through cell culturing. In

this method we can minimize experimental artifacts and variations that are often encountered by processing each sample individually (16-18). To minimize non-specific proteins that will immunoprecipitate will our protein of interest we constructed a double-tagged vector for CARD9 containing the FLAG and HA vectors. We overexpressed FLAG-CARD9-HA vector in 293T cells and performed a tandem immunoprecipitation. We first immunoprecipitated our 293T cell lysate with FLAG beads. Following an elution with FLAG peptide we performed a second IP using HA beads. We ran the denatured immunoprecipitates on an SDS PAGE gel and stained the gel by silver-staining method. As shown in Figure 5.2.1 we found that tandem immunoprecipitation greatly reduced the background of non-specific bands visualized in our initial single immunoprecipitations experiments (data not shown) and that in this overexpression system the strong CARD9 band corresponded to the correct size of CARD9.

C-type lectin receptors and CARD9 are primarily expressed in myeloid cells therefore we stably transfected the RAW 264.7 cell line to express FLAG-CARD9-HA or the empty vector. The RAW 264.7 cell line expressing FLAG-CARD9-HA was divided into two portions. One group was cultured in DMEM media depleted of normal lysine and supplemented with lysine D4 whereas the other portion was cultured in DMEM media depleted of normal lysine and supplemented with lysine $^{13}C_6{}^{15}N_2$. The RAW 264.7 cell line stably expressing the emptor vector was cultured in lysine depleted DMEM supplemented with lysine. These cells were passaged at least five times to efficiently label the cells. We used the RAW 264.7 cell line expressing FLAG-CARD9-HA labeled with lysine $^{13}C_6{}^{15}N_2$ and stimulated this cell line with *C. albicans*. Using the same cell number we made cell

lysates from all three RAW 264.7 cell lines cultured in different labeling conditions. We then combined these lysates and immunoprecipitaed using FLAG beads overnight. After several washes we eluted CARD9 from the beads by incubating the FLAG beads with elution buffer for thirty minutes then collecting the flow-through from the columns. We combined the eluted proteins and performed a second immunoprecipitation using HA beads. After several washes we boiled the beads and ran the immunoprecipitate on a SDS-PAGE gel and stained the gel with sypro ruby staining. We observed various bands on the gel and we cut the gel into ten different sections (Figure 5.2.4 A). Each band was analyzed by mass spectrometry to determine the immunoprecipitated proteins and to compare the ratio for different isotopes of lysine. Although we did successfully detect CARD9 as one of the proteins identified in the mass spectrometry results (Table 5.2.2) we could not detect any specific potential binding partner since the ratio for the normal lysine was higher as shown for example in the two proteins Myh9 and Actb (Table 5.2.2). In an independent experiment, when comparing unstimulated Raw 264.7 cell line expressing FLAG-CARD9-HA to the Raw 264.7 cell line expressing the empty vector, we identified CARMA1 as a potential binding partner. CARMA1 received a high peptide hit, which validated the protein identification in mass spectrometry (Table 5.2.3). We further validated the binding of CARMA1 to CARD9 by western blots (Figure 5.2.4. B). However, CARMA1 has no known role in ITAM-associated receptor signaling in myeloid cells and CARMA1-deficient macrophages do not show any defect in NF-κB activation in response to both the yeast and hyphal form of *C. albicans* (Figure 5.2.5).

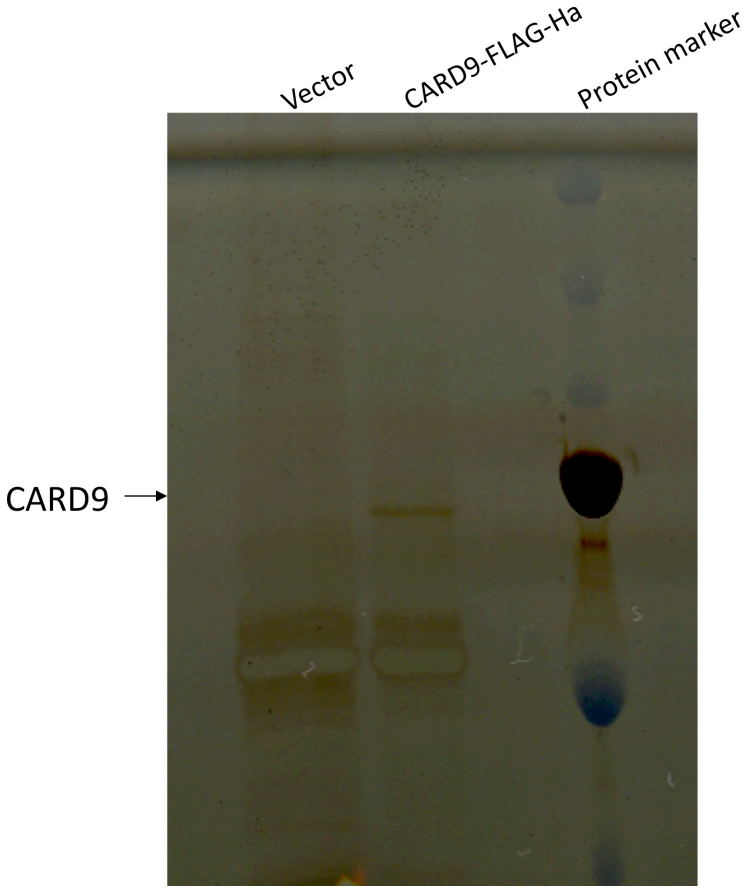

Figure 5.2.1. Silver staining of CARD9-FLAG-HA in 293Tcells. 293T cells were transiently transfected with a FLAG-CARD9-HA construct or an empty vector. The cell lysates were tandem immunoprecipitated by FLAG-conjugated and HA-conjugated beads respectively. The immunoprecipitates were loaded onto a SDS-PAGE gel and silver stained.

Hit	Accession	M/L	H/L	Description
1	IPI00123181	0.9379	0.6643	**Myh9 Myosin-9**
2	IPI00762625	0.001867	0.001927	Gm1499 Putative uncharacterized protein
3	IPI00850020	0.005502	0.001716	Igk-C If kappa light chain (Fragment)
4	IPI00110850	0.6452	0.5325	**Actb Actin, cytoplasmic 1**
5	IPI00473320	0.6454	0.5375	**Actb Putative uncharacterized protein**
6	IPI00471444	0.005502	0.002012	Igk Igk protein
7	IPI00785509	0.005502	0.002012	ENSMUSG00000076577 ENSMUSG00000076577 protein
8	IPI00808222	0.005502	0.002012	Igk-V21-4 Igk-V21-4 protein
9	IPI00556888	0.005502	0.002012	Igk-C Igk-C protein
10	IPI00761808			LOC637260 ScFv B8E5 protein (Fragment)
11	IPI00348883	82.58	78.07	**Card9 Caspase recruitment domain family, member 9**
13	IPI00308213			Ighg1 Ig gamma-1 chain C region, membrane-bound form
14	IPI00408534	0.000633	0.001845	Gm1524 Monoclonal anti-idiotypic Schistosoma japonicum antibody NP30 immunoglobulin light chain
15	IPI00915071			heavy chain variable region (Fragment)
16	IPI00461840			LOC641088 Ig heavy chain V region RF
17	IPI00828887		0.001464	Igh-V7183 B9-scFv
18	IPI00886297			Hspa8 Hspa8 protein
19	IPI00221528	0.6241	0.5348	Actbl2 Beta-actin-like protein 2
20	IPI00900442			Myh14 Nonmuscle myosin II-C2
21	IPI00625729			Krt1 Keratin, type II cytoskeletal 1
22	IPI00462140			Krt77 Keratin, type II cytoskeletal 1b
23	IPI00322209			Krt8 Keratin, type II cytoskeletal 8
24	IPI00347110			Krt73 Keratin, type II cytoskeletal 73
25	IPI00468956			Krt71 Keratin, type II cytoskeletal 71
26	IPI00227299		2.846	Vim Vimentin
27	IPI00117352			Tubb5 Tubulin beta-5 chain
28	IPI00461427			Igh-6 Igh-6 protein
29	IPI00124499			Krt79 Keratin, type II cytoskeletal 79
30	IPI00230044	0.905	0.5707	Tpm3 Isoform 2 of Tropomyosin alpha-3 chain
31	IPI00755181			Krt10 keratin complex 1, acidic, gene 10
32	IPI00885270			Card11 Isoform 1 of Caspase recruitment domain-containing protein 11
33	IPI00515398			Myh10 Myosin-10
34	IPI00109044	1.01	0.6069	2900073G15Rik myosin light chain, regulatory B-like
35	IPI00319992			Tax_Id=10090 Gene_Symbol=Hspa5 78 kDa glucose-regulated protein
36	IPI00754649	1.271	0.7457	Myo1c myosin IC isoform a
37	IPI00623776	1.246	6.058	LOC674678 similar to histone H4
38	IPI00133208			Hspa1a;Hspa1l;ENSMUSG00000073415 Heat shock 70 kDa protein 1L
39	IPI00308885			Hspd1 Isoform 1 of 60 kDa heat shock protein, mitochondrial
40	IPI00139301			Krt5 Keratin, type II cytoskeletal 5
41	IPI00169500			Atxn2l Isoform 1 of Ataxin-2-like protein
42	IPI00474225			Tax_Id=10090 Gene_Symbol=Igh-1b;LOC675759;LOC634206;Igh-1a Igh-1a protein
43	IPI00117348	1.172	1.107	Tax_Id=10090 Gene_Symbol=Tuba1b Tubulin alpha-1B chain
44	IPI00137931			Tax_Id=10090 Gene_Symbol=- Ig kappa chain V-II region 7S34.1
45	IPI00831151			Tax_Id=10090 Gene_Symbol=LOC100043977 similar to Igh protein
46	IPI00109910			Tax_Id=10090 Gene_Symbol=Ighg Ighg protein
47	IPI00664150			Tax_Id=10090 Gene_Symbol=LOC380804 Ig heavy chain V region 914
48	IPI00133916	0.7798	0.8602	Tax_Id=10090 Gene_Symbol=Hnrnph1 Heterogeneous nuclear ribonucleoprotein H
49	IPI00831140			Tax_Id=10090 Gene_Symbol=- Vk protein (Fragment)
50	IPI00307837			Tax_Id=10090 Gene_Symbol=Eef1a1 Elongation factor 1-alpha 1

Table 5.2.2. Mass spectrometry analysis of CARD9 binding partners.

Hit	Accession	M/L	H/L	Description
51	IPI00131695			Tax_Id=10090 Gene_Symbol=Alb Serum albumin
52	IPI00457756			Tax_Id=10090 Gene_Symbol=2810405J04Rik Protein FAM98A
53	IPI00624663			Tax_Id=10090 Gene_Symbol=Pzp Alpha-2-macroglobulin
54	IPI00831050			Tax_Id=10090 Gene_Symbol=EG667683 Anti-VIPase light chain variable region (Fragment)
55	IPI00666636			Tax_Id=10090 Gene_Symbol=- 68 kDa protein
56	IPI00309214	0.006449		Tax_Id=10090 Gene_Symbol=Apcs Serum amyloid P-component
57	IPI00339468	0.7022	2.042	Tax_Id=10090 Gene_Symbol=Dhx9 Isoform 2 of ATP-dependent RNA helicase A
58	IPI00757645			Tax_Id=10090 Gene_Symbol=LOC676175 similar to kappa-tnp V-J
59	IPI00762051			Tax_Id=10090 Gene_Symbol=Rpl24 60S ribosomal protein L24
60	IPI00406030			Tax_Id=10090 Gene_Symbol=Mprip Rho interacting protein 3
61	IPI00831423	0.7468	0.7255	Tax_Id=10090 Gene_Symbol=Tpm1 29 kDa protein
62	IPI00131209	0.5174		Krt16 Keratin intermediate filament 16a
63	IPI00127841			Slc25a5 ADP/ATP translocase 2
64	IPI00109293			Lactb;LOC677144 Serine beta-lactamase-like protein LACTB, mitochondrial
65	IPI00272681			Gnal guanine nucleotide binding protein, alpha stimulating, olfactory type isoform 2
66	IPI00652902			Gnai2 Putative uncharacterized protein
67	IPI00138860			Golga4 Golgin subfamily A member 4
68	IPI00380436			Actn1 Alpha-actinin-1
69	IPI00109061			Tubb2b Tubulin beta-2B chain
70	IPI00128818			TDhx15 Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15
71	IPI00353672			Xirp2 Isoform 1 of Xin actin-binding repeat-containing protein 2
72	IPI00676959			Krt36 Keratin 36
73	IPI00396797			Ddx17 Isoform 1 of Probable ATP-dependent RNA helicase DDX17
74	IPI00118899			Actn4 Alpha-actinin-4
75	IPI00387557			Actn2 actinin alpha 2
76	IPI00331664			Actn2 Alpha-actinin-2
77	IPI00136701			10090 Gene_Symbol=Actn3 Alpha-actinin-3
78	IPI00223750	0.8372	2.694	Efna5 Putative uncharacterized protein
79	IPI00329843			Ankfy1 Isoform 1 of Ankyrin repeat and FYVE domain-containing protein 1
80	IPI00659000			OTTMUSG00000007855 predicted gene, OTTMUSG00000007855
81	IPI00408207	0.7883	0.8001	Myo1d Isoform 1 of Myosin-Id
82	IPI00553537		0.048	LOC100047053 similar to monoclonal antibody kappa light chain
83	IPI00420363			Ddx5 Probable ATP-dependent RNA helicase DDX5
84	IPI00130095			G3bp1 Ras GTPase-activating protein-binding protein 1
85	IPI00856379			Aldoa Fructose-bisphosphate aldolase
86	IPI00221402			Aldoa Fructose-bisphosphate aldolase A
87	IPI00648100			Mprip Rho interacting protein 3
88	IPI00133708			D1Pas1 Putative ATP-dependent RNA helicase Pl10
89	IPI00227140			Krt14 Keratin, type I cytoskeletal 14
90	IPI00468696			Krt42 Keratin, type I cytoskeletal 42
91	IPI00124287			Pabpc1 Polyadenylate-binding protein 1
92	IPI00169916			Cltc Clathrin heavy chain 1
93	IPI00346834			Krt76 Putative uncharacterized protein
94	IPI00135646			Abcd3 ATP-binding cassette sub-family D member 3
95	IPI00403328			Pip5k1c Isoform 3 of Phosphatidylinositol-4-phosphate 5-kinase type-1 gamma
96	IPI00223243			Prdm16 transcription factor MEL1
97	IPI00226073	0.7798	0.8602	Hnrnpf Isoform 1 of Heterogeneous nuclear ribonucleoprotein F
98	IPI00351932			Fam101a Protein FAM101A

Table 5.2.2. Continued

Table 5.2.3. Protein identification of CARD9 binding partners by mass spectrometry analysis.

Reference	Accession	Peptide (Hits)	avg Ratio
Caspase recruitment domain family, member 11	IPI00348883.6	207	4.24:3
Card9 Caspase recruitment domain family, member 9	IPI00348883.4	205	4.24:1
Myh9 Myosin-10	IPI00123181.5	383	1.16:2
Gm189 Anti-VlPase light chain variable region	IPI00169755.5	208	1:3.33
Gm189 Anti-VlPase light chain variable region	IPI00169755.6	209	1:3.34
Myh9 Myosin-12	IPI00123181.7	385	1.16:4
Gm189 Anti-VlPase light chain variable region	IPI00169755.5	208	1:3.33

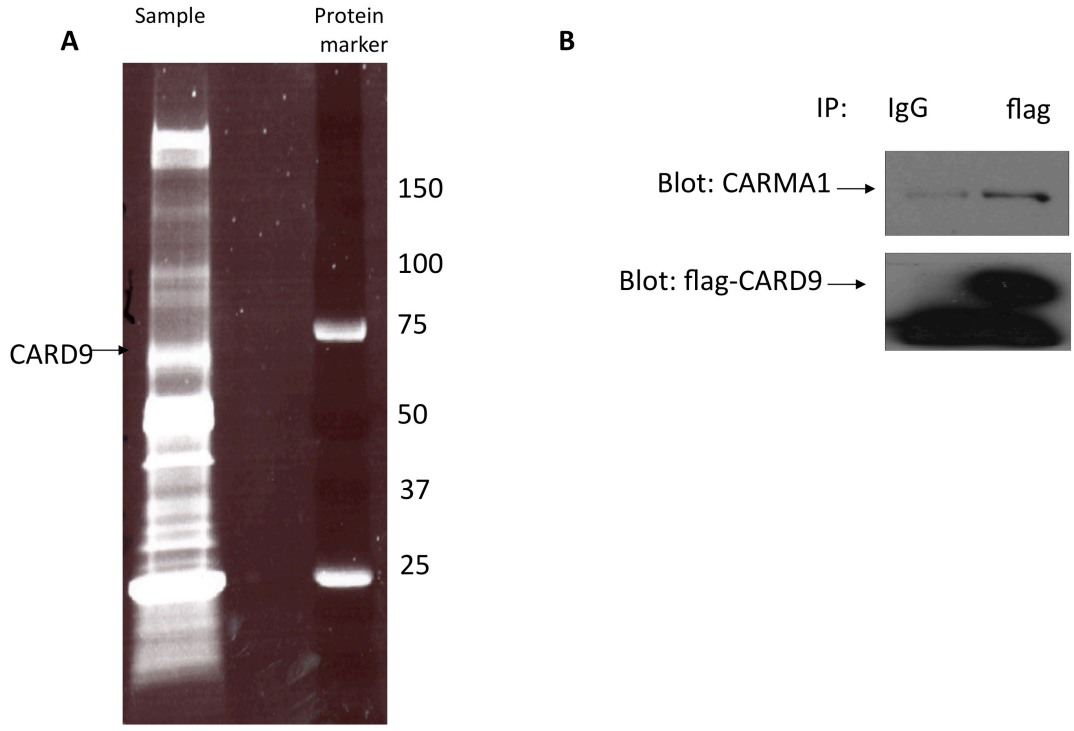

Figure 5.2.4. CARMA1 constitutively binds to CARD9

A**.** Cell lysates from RAW 264.7 cell line stably expressing a FLAG-CARD9-HA (stimulated with *C. albicans* or left untreated) was mixed with an equal protein quantity from cell lysates of RAW 264.7 stably expressing the empty vector. The cell lysates were tandem immunoprecipitated with FLAG and HA conjugated beads respectively. The immunoprecipitates were run on a SDS-PAGE gel and stained by Sypro Ruby. B. Cell lysates from RAW 264.7 cell line stably expressing a FLAG-CARD9-HA construct was immunoprecipitated with Flag-conjugated or IgG-conjugated agarose. The immunoprecipitates were probed with CARMA1 or FLAG antibodies. *IP*, immunoprecipitation; *IB*, immunoblot.

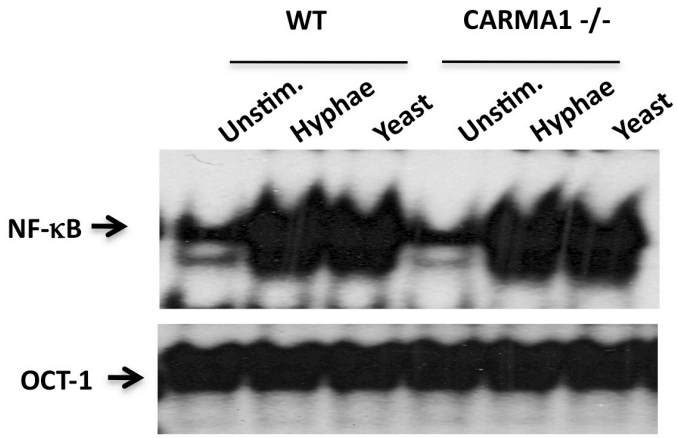

Figure 5.2.5. NF-κB activation induced by *C. albicans* hyphae and yeast is not CARMA1 dependent.

WT and CARMA1-deficient BMDMs were either untreated or treated with *C. albicans* hyphae (MOI: 1) or heat-inactivated yeast (MOI: 5) for 90 min. The nuclear extracts were prepared from these cells and then subjected to the electrophoretic mobility shift assay using 32P-labeled NF-κB or OCT-1 probe.

5.3. Discussion

Most cell signaling cascades involve the regulation of multiprotein complexes. The multiplex labeling method enables us to compare different cell populations using a single experiment, improving the efficiency and accuracy in quantitative proteomics. Protein functions such as catalytic activity or specificity to substrates are regulated by polypeptides in a holoenzyme complex. In this scenario protein binding partners might be present in amounts that are not stoichiometric (195, 196). This property of proteins poses a problem in indentifying specific binding partners that are expressed at low levels from a vast number of highly expressed but low affinity non-specific binding proteins that are identified in immunoprecipitation techniques. One simple solution for this problem would be to increase the stringency level of purification methods. The caveat of this route is that the probability of still being able to detect specific binding partners with low affinity will most likely be reduced. In this study, we chose to use the stable isotope labeling with amino acids in cell culture (SILAC) quantitative proteomics as a method to identity CARD9 binding partners. In our experimental design we had the opportunity to incorporate a negative control in our mass spectrometry analysis which would enable use to distinguish specific binding partners from non-specific background proteins without using harsh washing conditions. Our mass spectrometry results have identified a large number of proteins; however, when comparing the ratios of specific to non-specific binding proteins, CARD9 was the only specific protein detected. In a separate experiment we detected CARMA1 as a binding partner for CARD9 but in this case the signal-to-background ratio was still close to background levels.

CARMA1 is known to regulate ITAM- associated receptor induced NF-κB activation in lymphoid lineage cells (85). Currently, there is no known ITAM-associated receptor in myeloid lineage cells that utilizes CARMA1. Thus, the question remains why in myeloid lineage cells, CARD9 but not CARMA1 functions downstream of ITAM-associated receptors and if there is redundancy between CARMA1 and CARD9 in myeloid lineage cells.

Immunoprecipitation and mass spectrometry has been the classical method for identifying protein-protein interactions and is proven to be quite successful in identifying the core interacting proteins. We speculate that CARD9-BCL10 is one of these core complexes. However, this does not rule out the possibility of other proteins interacting with CARD9 but with a detection threshold too low for mass spectrometry analysis. Aside from BCL10, CARD9 is shown to bind to Rac-GDI protein using the yellow fluorescent protein (YFP)-based protein complementation assay (PCA/bimolecular fluorescent complementation (BiFC) (137). In this method protein-protein interactions will bring two fragments of YFP that have been tagged to two separate proteins into close proximity of each other, allowing for them to fold into a fluorescent protein (197).

One of the challenges of immunoprecipitation coupled to mass spectrometry is the large numbers of false-positive proteins that are identified in the sample as well as in negative controls. Although tandem affinity purification has helped to reduce the level of background contaminants it also involves multiple purification steps that may cause the lost of transient or weak associations. Although the comparison of the experimental sample to the negative control is the first step into filtering the mass spectrometry data, it is still

possible that a real high-abundance interacting protein for the given target protein is detected at low levels in negative control samples. Therefore, in the field of proteomics designing novel methods for quantitative approaches is an active field of investigation. One of these new methods to help in quantification of mass spectrometry data is termed Significance Analysis of INTeractome (SAINT). This computational approach utilizes label-free quantitative data (i.e. Spectral counts) to assign each individual protein-protein complex a confidence value (198). To derive the probability of a true protein-protein interaction the SAINT algorithm will analyze data from control purifications and generates separate distributions for false and true interacting proteins. Although the power of this method of statistical analysis of the distributions of spectral counts has not yet been fully tested in experiments testing the biological function of specific protein-protein interactions, it may prove to enhance current mass spectrometry analysis of novel protein binding partners from the unavoidable background.

Chapter 6: Summary and Future Perspectives

The results reported in this thesis elucidate a novel cell signaling pathway that our innate immune cells utilize, to recognize and combat fungal infections by *Candida albicans*. In our studies, phospholipase γ2, was identified as the main phospholipase activated in response to *C. albicans*. In macrophages, PLCγ2 is crucial for regulating JNK and ERK but not p38 pathways in response to *C. albicans* hyphae. In response to the yeast form of C. *albicans,* PLCγ2-deficient macrophages showed significantly decreased JNK activation whereas ERK activation was only partially defective. These results suggest that in response to hyphae ERK activation depends solely on PLCγ2 whereas in response to yeast, ERK activation is mediated by an additional pathway, possibly the Grb2/ Ras/ Raf1 signaling pathway. NF-κB activation in response to *C*. *albicans* is not mediated through MyD88. Our biochemical analysis revealed that PLCγ2 is required for NF-κB activation in response to both the yeast and hyphal form of *C. albicans.* The activation of the IKK complex downstream of the Dectin-2 receptor independently requires both CARD9 and PLCγ2. PLCγ2 is required for the production of proinflammatory cytokine and reactive oxygen species in innate anti-fungal immune responses. The defective immune response in PLCγ2-deficient mice in comparison to WT mice was confirmed *in vivo* by challenging these mice with *C. albicans*. In our *in vivo C. albicans* infections, PLCγ2-deficient mice showed a higher fungal burden and decreased survival rates compared to WT mice.

Recent studies using myeloid specific TAK1-deficient mice, reported that peritoneal macrophages from TAK1-deficient mice did not show any obvious defects in NF-κB and

MAPK activation in response to TLR stimulation compared to WT mice. This finding was somewhat unexpected since it had been assumed that TAK1 regulates pattern recognition receptor signaling in innate immune cells. In a separate study, utilizing myeloid specific TAK1-deficient mice it is reported that these mice have impaired differentiation of bone marrow derived macrophages. These macrophages are defective in NF-κB and MAPK activation in response to TLR stimulation. In our studies, bone marrow from myeloid specific TAK1-deficient mice could not differentiate into macrophages. However, in mice that were both TAK1 and TNFR1-deficient, macrophages could be differentiated from their bone marrow. In bone marrow derived macrophages TAK1 is critical for NF-κB and MAPK activation. TAK1-deficiency decreased the amount of proinflammatory cytokine production, which could possibly dampen the adaptive immune system to fight *Candida* infections and increase the susceptibility of these mice to fungal infections. Upon *C. albicans* stimulation, TRAF6 is necessary to mediate NF-κB and MAPK activation. However, the mechanism which TAK1 and TRAF6 use to activate the IKK complex in response to *C. albicans* is different. Our results demonstrate that TAK1 regulates the phosphoylation of IKKα/β subunits whereas TRAF6 does not.

Given the complex network of the immune system, the activation of this system is strictly regulated. Chronic inflammation caused by stimuli such as fungi has been linked to autoimmune diseases such as rheumatoid arthritis and psoriasis (199, 200). A recent study of candidemia incidence in unselected hematologic cancer patients at the University of Texas M.D. Anderson Cancer Center from 2001 to 2007 reports that the use of older and newer prophylaxis antifungal agents did not result in decreased incidence of candidemia (201).

Furthermore, despite the use of new antifungal agents attributable and crude mortality rates in these patients are still similar to those reported in the older studies (202) and the use of combination antifungal treatments did not associate with significant improvements in mortality rates (201). We hope that by better understanding the molecular mechanisms that regulate innate immune cell signaling we will provide a conceptual framework that will enable us to manipulate these responses to provide new therapies for human infection and disease.

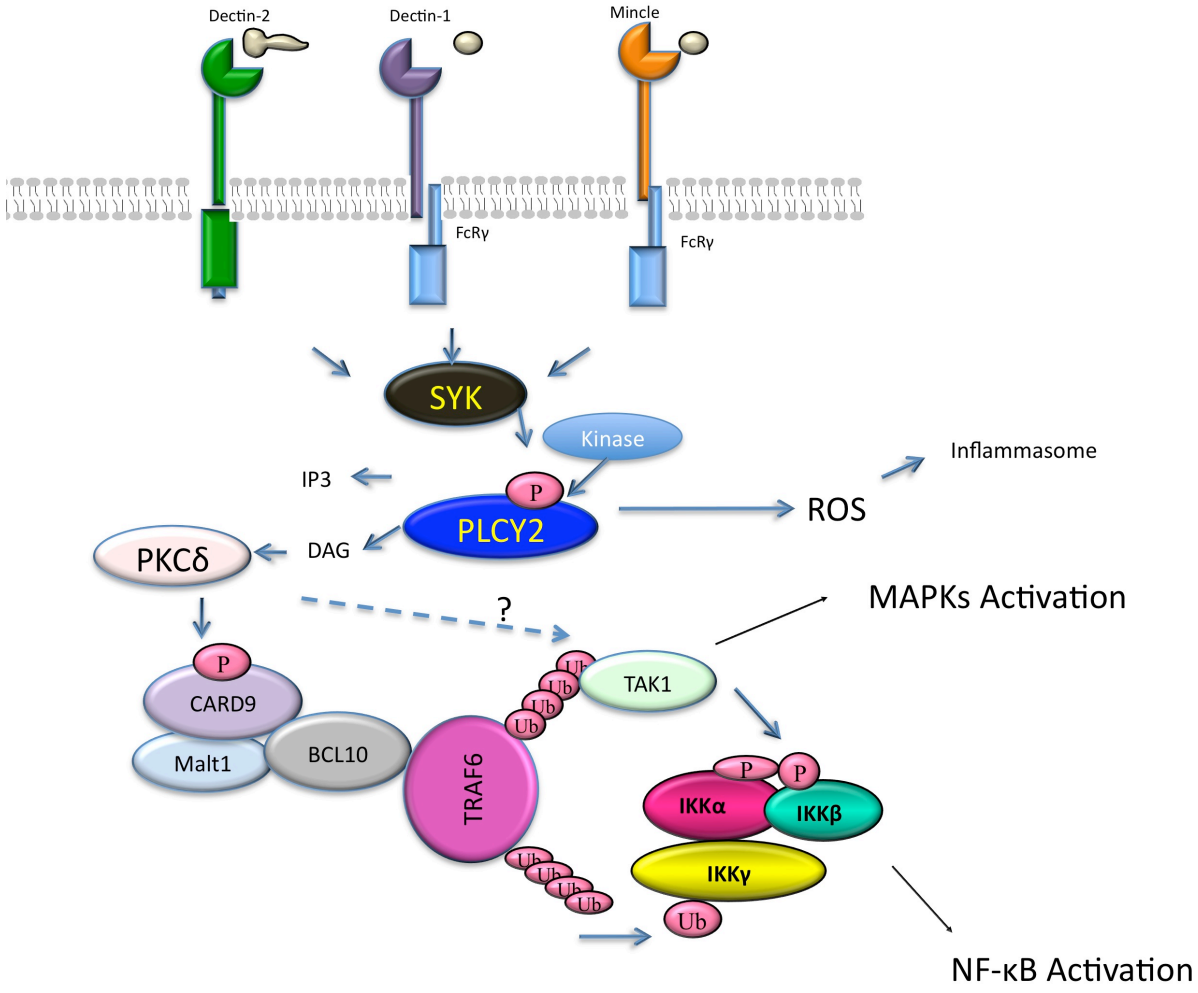

Figure 6. Summary of the proposed model for C-type lectin receptor signaling in macrophages.

Future Perspectives

The studies presented here delineate critical signaling pathways downstream of the C-type lectin receptors, which are required for innate host defense against fungal infections with *C. albicans*. These studies raise new questions that should be the topic of future investigations. We show that the activation of PLCγ2 is dependent on Syk. However, BTK inhibitors could not inhibit the phosphorylation of PLCγ2 or the activation of NF-κB in macrophages stimulated by *C. albicans* hyphae. BTK has a well-established role in BCR signaling. Following BCR activation, BTK translocates to the plasma membrane where it is phosphorylated by Src family kinases (203). BTK, using the adaptor protein BLNK, can phosphorylate PLCγ2, resulting in downstream BCR signaling. The genetic discorder, X-linked agammaglobulinemia (XLA), which is characterized by loss-of-function mutations in the gene that encodes BTK, is characterized by recurrent bacterial and enteroviral infections but not fungal infections (204). This suggests that BTK is dispensable in anti-fungal immunity. Whether Syk directly phosphorylates PLCγ2 and if BLNK is required for PLCγ2 activation are questions that need further investigation.

The negative regulators of C-type lectin receptor signaling have yet to be described. In our studies, CYLD-deficient macrophages were not defective in cytokine production or NF-κB activation upon *C. albicans* stimulation. Further studies using A20-knockout macrophages will help to clarify the negative regulators of CLR signaling.

C. albicans can trigger the activation of the cytosolic sensor NLRP3 inflammasome (153, 205). The NLRP3 inflammasome controls the activation of caspase-1, which cleaves

pro-IL-1β into mature IL-1β in the innate immune cells (206). Studies using Syk inhibitors and Syk-deficient dendritic cells show impaired pro-IL-1β production and inflammasome activation upon fungal stimulation (153). Furthermore, *C. albicans* induced inflammasome activation depends on Syk mediated ROS production (153). Our studies demonstrate that PLCγ2 can mediate hyphae-induced ROS production; Further studies are needed to determine if PLCγ2 is mediating inflammasome activation and caspase -1 processing. Aside from caspase-1, pro-IL-1β is processed by noncanonical inflammasomes in response to TLR4 and TLR3 ligands (207, 208). In DCs stimulated with curdlan, a noncanonical caspase-8-MALT1 inflammasome can become activated in a Syk-dependent manner. Similar to Syk inhibition, silencing of CARD9, BCL10 or MALT1 abrogated caspase-8 activity in DCs stimulated with curdlan. Future studies using PLCγ2-deficient macrophages to investigate caspase-8 activity will provide insight into the newly discovered noncanonical inflammasome activation pathway.

In addition to the established role of C-type lectin receptor signaling in innate host defense, the role of these signaling pathways in the pathogenesis of autoimmune diseases remains largely unknown. The majority of studies on commensal microbiota and its link to autoimmune diseases have focused on the intestinal bacteria. The interaction between the mammalian gut fungal community with innate immune receptor, Dectin-1 was shown to influence chemically induced colitis (209). Treatment with the anti-fungal drug Fluconazole ameliorated colitis in Dectin-1-deficient mice. A polymorphism in the Dectin-1 gene (*CLEC7A*) is strongly linked with a severe form of ulcerative colitis (209). In the gastrointestinal niche, whether other C-type lectin receptors such as Dectin-2 or Mincle

influence susceptibility to colitis remains to be investigated. The possibility exisits that commensal fungal organisms residing in the intestines not only affect immune responses locally, but also can alter immune responses in organs that are not in direct contact with intestinal microbiota.

In the K/BxN T cell receptor transgenic mouse model of inflammatory arthritis, mice display attenuated arthritis when housed in germ-free conditions (210). The question remains if the K/BxN mouse model would be selectively colonized with specific strains of commensal fungi (gnotobiotic), how would this impact arthritis development? Furthermore, we could test the impact of C-type lectin receptor signaling in the development of arthritis by using the K/BxN serum transfer arthritis model on Dectin-1 or PLCγ2-deficient bone marrow chimera mice. It will also be interesting to investigate if Fluconazole treatment in the aforementioned experimental setting would ameliorate arthritis symptoms? It is tempting to speculate that if the K/BxN serum transfer arthritis model would be conducted under germ-free conditions mice will not develop autoimmune arthritis both in PLCγ2-deficient and WT mice. However if we reconstitute the mice intestines with one or more commensal fungal organisms this will increase arthritis symptoms in WT mice but not in PLCγ2-deficient mice.

Type-1 diabetes development in the NOD mouse strain can increase with cleaner housing facilities (211). How commensal fungi recognition influences type-1 diabetes penetrance has not been investigated. Generating NOD mice crossed with Dectin-1 or Dectin-2-deficient mice can be used to determine the role of commensal fungi in the pathogenesis of type-1 diabetes. The influence of commensal organisms on autoimmune

disease progression cannot be generalized. In the Aire-deficient mouse model of human disease, Autoimmune Polyendocrinopathy Syndrome type 1, germ-free facilities did not have any significant impact on the severity or progression of this multi-organ autoimmune disease (212). The pleiotropic effect of commensal organisms on autoimmune disease pathogenesis may in part be explained by the role that certain T helper subsets have in each disease. In autoimmune disease mouse models of colitis and rheumatoid arthritis, autoreactive Th17 cells have an important role in pathogenicity (210, 213). There is no published data demonstrating that Aire- deficiency affects Th17 cell populations, which may in part explain why germ-free housing does not have any significant impact on the progression of this disease. Dectin-1, Dectin-2 and CARD9 have been implicated in the differentiation of Th17 cells (214). Understanding the immune response to mycobiota and the affect of these signaling pathways on autoimmune diseases may directly be linked to how certain commensal organisms promote differentiation of certain subsets of T helper cells, such as Th17 cells in the small-intestinal lamina propria. Future investigations on the host innate immune response to commensal fungi may identify better therapeutic strategies for fighting invasive fungal infections and provide an opportunity to investigate the underlying mechanisms of autoimmune diseases that are influenced by inflammation.

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Vita

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