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Characterization of Anti-Fungal Inflammasome Responses and the Role of Caspase-8 in Innate Immune Signaling: A Dissertation

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CHARACTERIZATION OF ANTI-FUNGAL INFLAMMASOME RESPONSES AND THE ROLE OF CASPASE-8 IN INNATE IMMUNE SIGNALING

A Dissertation Presented

By

Sandhya Ganesan

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CHARACTERIZATION OF ANTI FUNGAL INFLAMMASOME RESPONSES AND THE ROLE OF CASPASE-8 IN INNATE IMMUNE SIGNALING

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Abstract

The innate immune system is an evolutionarily conserved primary defense system against microbial infections. One of the central components of innate immunity are the pattern recognition receptors which sense infection by detecting various conserved molecular patterns of pathogens and trigger a variety of signaling pathways. In this dissertation, the signaling pathways of several classes of these receptors were dissected. In chapters II and III, the role of two NOD-like receptors, NLRP3 and NLRC4 were investigated in the context of infection with the fungal pathogen, *C. albicans*. *C. albicans* is an opportunistic pathogen that causes diseases mainly in immunocompromised humans and innate immunity is critical to control the infection. In chapters II and III, we demonstrate that a multiprotein-inflammasome complex formed by the NLR protein, NLRP3 and its associated partners, ASC and caspase-1 are critical for triggering the production of mature cytokine IL-1β in response to *C. albicans*. NLRC4, another inflammasome forming NLR that is activated by intracellular bacterial pathogens, was not required for this process in macrophages. Thus, our data indicates that NLRP3 inflammasome responds to fungal infections in addition to its known stimuli such as bacterial and viral infections, toxic, crystalline and metabolic signals.

Interestingly, this NLRP3 dependent inflammasome response was maintained even when the pathogen is not viable, and is either formalin fixed or heat-killed (HK). Hence, in chapter III, we examined β-glucans, a structural cell wall component, as the potential immunostimulatory component of *C. albicans*,

and dissected the inflammasome responses to β-glucans. We observed that NLRP3-ASC-caspase-1 inflammasome was critical for commercially obtained particulate β-glucans similar to the case of *C. albicans*. β-glucan sensing C-lectin receptor dectin-1 and the complement receptor CR3 mediated inflammasome activation, IL-1β production in response to the glucan particles. Interestingly, CR3 which recognizes glucans as well as complement opsonized pathogens was strongly required for HK *C. albicans* induced IL-1β, and partially required for that of live *C. albicans*, while dectin-1 was not required. Consistent with the receptor studies, blocking of β-glucan receptors by pre-incubating cells with non-stimulatory, soluble glucans led to decreased IL-1β production in response to HK *C. albicans* with no effect on IL-1β in response to the live fungus. Dectin-1, CR3 and β-glucan sensing also triggered a moderate dendritic cell death response to β-glucans and HK *C. albicans*. Live *C. albicans* induced cell death requires phagocytosis but not the inflammasome, β-glucan sensing, dectin-1 or CR3.

The Drosophila caspase-8 like molecule DREDD plays an essential, non-apoptotic role in the Drosophila NF-κB pathway called the 'IMD' pathway. Owing to the remarkable evolutionary conservation between Drosophila and mammalian innate immune NF-κB pathways, we explored the potential role of caspase-8 in inflammasomes and in TLR signaling. Using *casp8*$^{-/-}$ *Rip3*$^{-/-}$ macrophages and dendritic cells, we observed that caspase-8, specifically augments β-glucan and HK *C. albicans* induced IL-1β as well as cell death in a caspase-1 independent manner, but not that of live *C. albicans*, in chapter III.

We also found that caspase-8 differentially regulates TLR4 and TLR3 induced cytokine production (chapter IV). Caspase-8 specifically promotes TLR4 induced production of cytokines such as TNF, IL-1β in response to LPS and *E. coli*. On the other hand, caspase-8 negatively regulates TRIF induced IFNβ production in TLR4 and TLR3 signaling in response to LPS and dsRNA. Caspase-8 executed a similar mode of regulation of the cytokine RANTES in MEFs, in part, by collaborating with RIP3. Strikingly, caspase-8 deficiency alone triggers higher macrophage death and IL-1β production in response to TLR ligands, due to the presence of RIP3. Thus, in addition to its conventional roles in apoptosis, caspase-8 modulates TLR4 and TLR3 induced cytokine production and prevents RIP3 mediated hyper inflammation in response to TLR signals.

Together, our findings provide valuable information on fungal pattern recognition and inflammasome pathways and define the contribution of β-glucan sensing to *C. albicans* induced inflammasome responses. In addition, we demonstrate how caspase-8 adds a layer of specificity to inflammasome as well as TLR signaling. Overall, these results also shed light on the cross talk between death signaling components and innate immune pathways to mount a specific and potentially effective innate immune response against microbial pathogens.

List of publications

Ganesan S, Rathinam VAK, Bossaller L, Army K, Kaiser WJ, Mocarski ES, Dillon CP, Green DR, Mayadas TN, Levitz SM, Hise AG, Silverman N, Fitzgerald KA. Caspase-8 modulates Dectin-1 and CR3 driven IL-1β production in responses to β-glucans and *Candida albicans* (*Manuscript submitted to Journal of Immunology. In revision.*)

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Sandhya Ganesan immortalized *Casp8*$^{-/-}$ *Rip3*$^{-/-}$, *Rip3*$^{-/-}$ BMDM and assisted with optimizing genotyping protocols and discussions.

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Sandhya Ganesan performed the *in vitro* experiments to address the role of NLRP3 and NLRC4 inflammasome in *C. albicans* induced IL-1β for the first submission.

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Sandhya Ganesan performed the *in vitro* experiments in *Aim2*$^{+/+}$ and *Aim2*$^{-/-}$ macrophages with Anthrax toxin and *S. typhimurium* in figure panels 3A and 3D.

Hise AG, Tomalka J, **Ganesan S**, Patel K, Hall BA, Brown GD, Fitzgerald KA. An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans*. Cell Host Microbe. 2009 May 8;5(5):487-97.
Sandhya Ganesan performed the *in vitro* experiments (Figures 1A, C-F, S1) with formalin treated *Candida* preps.

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Abbreviations

AIM2	Absent in Melanoma 2
AP-1	Activated Protein-1
ASC	Apoptosis associated Speck like protein containing CARD
ATP	Adenosine Triphosphate
BMDC	Bone marrow derived dendritic cells
BMDM	Bone marrow derived macrophages
CARD9	Caspase Recruitment Domain- protein 9
CD14	Cluster of Differentiation 14
CLEC7A	C-type Lectin domain family 7 member A, aka Dectin-1
CLR	C-Lectin Receptor
CM-Curdlan	Carboxy-methylated curdlan
CR3	Complement Receptor 3, (Mac-1)
Curdlan (I)	curdlan (Invivogen)
Curdlan (S)	curdlan (Sigma)
Curdlan (W)	curdlan (Wako Chemicals)
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
dIAP2	Drosophila Inhibitor of Apoptosis 2
dsRNA	Double stranded RNA
FADD	Fas Associated Death Domain protein
IFN	Interferon
IKK	IκB kinase
IL-1β	Interleukin-1
IMD	Immune deficiency
Itgam	Integrin, Alpha M
IκBα	Inhibitor of κB alpha
LPS	Lipopolysaccharide
LPS	Lipopolysaccharide
MEF	Mouse embryonic fibroblasts
MOI	Multiplicity of Infection
MyD88	Myeloid Differentiation primary response gene 88
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NLR	Nucleotide binding and oligomerization domain (NOD) and Leucine Rich Repeat (LRR) containing protein
NLRC4	NOD like receptor family, CARD domain containing 4
NLRP3	NOD like receptor family, pyrin domain containing 3
PBMC	Peripheral blood mononuclear cells
pdAdT	Poly (deoxyadenylic-deoxythymidylic)
PEC	Peritoneal exudate cells
pIC	Polyinosinic-polycytidylic

PMA	Phorbol Myristate Acetate
PYHIN	PYRIN and HIN domain containing protein
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
RIG-I	Retinoic acid Inducible Gene 1
RIP3	Receptor interacting protein 3
SeV	Sendai virus
ssRNA	Single stranded RNA
Th cells	T helper cells
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
TRADD	Tumor Necrosis Factor alpha Receptor type I Associated Death Domain protein
TRAF3	TNF Receptor Associated Factor 3
TRIF	TIR domain containing adaptor inducing interferon β
WGP agonist	Whole glucan particles (insoluble, high molecular weight)
WGP antagonist	Whole glucan particles (soluble, low molecular weight)
TLR	Toll-Like Receptor
CLR	C-Lectin Receptor
ALR	Aim2-Like Receptor
RLR	RIG-I-Like Receptor
PAMP	Pathogen Associated Molecular Pattern
PRR	Pattern Recognition Receptor
BIR	Bacuolovirus Inhibitor of Apoptosis protein Repeat
IBM	IAP Binding Motif
DAP-type PGN	DiAminoPimelic acid-type Peptidoglycan
Lys-type PGN	Lysine type-Peptidoglycan
OPC	Oropharyngeal candidiasis
PGRP	PeptidoGlycan Recognition Proteins
NK cells	Natural Killer cells
NET	Neutrophil Extracellular Traps
RHIM	RIP Homotypic Interaction Motif
NEMO	NF-κB Essential MOdulator
CYLD	Cylindromatosis
DAI	DNA dependent Activator of IFN regulatory factors
mRNA	Messenger RiboNucleic Acid
DNA	Deoxy RiboNucleic Acid
cFLIP	Cellular FLICE-like Inhibitory Protein
MALT1	Mucosa Associated Lymphoid Tissue lymphoma translocation1
GNBP3	Gram Negative bacteria Binding Protein 3
SCARF1	SCAvenger Receptor class F
CED-1	CEll Death abnormal 1
MAVS	Mitochondrial Antiviral Signaling Protein
TRAM	TRIF Related Adaptor Molecule

Mal	MyD88 adapter like
TANK	TRAF Associated NF-κB activator
TBK1	TANK Binding Kinase 1
IRF	Interferon Regulatory Factor
ISG	Interferon Stimulated Genes

Chapter I:

Introduction

Chapter I: Introduction

Mammalian innate immune response

The innate immune system is the first line of defense of mammals and rapidly responds to infection. The primary goal of the innate immune response is to contain, clear the pathogen and recruit other cells to resolve/eliminate the infection. The innate immune system encompasses many mechanisms such as phagocytosis, microbial killing, and release of soluble inflammatory mediators such as cytokines or chemokines, recruiting other leukocytes and eventually instructing the adaptive immune responses. The past twenty years have witnessed a tremendous burst of research in importance, specificity and efficiency of innate immune responses in various diseases and inflammatory disorders. One of the integral components of host pathogen detection machinery is the Pattern Recognition Receptors (PRRs), which sense the presence of infection by detecting conserved molecular and pathogenic patterns or danger in the environment. Upon sensing of specific molecular signatures, PRRs trigger a variety of inflammatory pathways, which sets off the alarm to induce cell-intrinsic and cell-extrinsic processes to clear the infectious source, recruit other leukocytes, develop more pathogen-specific responses and eventually, to promote tissue repair and develop memory of the attacking pathogen.

Pattern Recognition Receptors

PRRs are germline-encoded receptors, which recognize conserved pathogen associated molecular patterns (PAMPs). These receptors include Toll-like receptors (TLRs), C-lectin Receptors (CLRs), NOD-like Receptors (NLRs), RIG-I-like Receptors (RLRs), AIM2-like receptors (ALRs). They recognize a broad repertoire of microbial signatures from bacterial, fungal, viral and parasitic pathogens. In addition, myeloid cells also express receptors that can bind to pathogens opsonized by complement factors circulating in the serum and include complement receptors and scavenger receptors. In keeping with the context of this dissertation, TLRs, CLRs, NLRs, AIM2 and complement receptors are described in this chapter.

PRRs are highly expressed in innate immune cells such as macrophages, neutrophils, dendritic cells and circulating monocytes. While the expression of some of the PRRs may not be basally high in epithelial cell types, some of them are up regulated upon activation in all cell types [1]. PRRs are found in different cellular compartments, including the cell surface, endosomes or cytosol to recognize extracellular, phagocytosed or intracellular pathogens. PRRs initiate inflammatory responses by inducing the uptake of the microbe, production of reactive oxygen and nitrogen species and soluble inflammatory mediators such as cytokines, chemokines and type-I interferons (type-I-IFN). Cytokines and chemokines serve as the messengers and a way of communication within the

immune system. These molecules act through autocrine and paracrine signals to alert and recruit leukocytes to the sites of infection.

Toll like receptors

TLRs are a highly conserved group of PRRs with the first Toll receptor being initially identified in *Drosophila melanogaster* [2]. TLRs are characterized by the presence of an extracellular Leucine Rich Repeat (LRR) domain that mediates ligand recognition and specificity, a transmembrane domain and an intracellular TIR (Toll/IL-1R) signaling domain. So far, 10 TLRs in humans and 13 TLRs in mice have been identified (reviewed in Thompson. M. R. *et al*.) [3]. TLRs detect various structural signatures of pathogens such as cell-wall components and nucleic acids. Seminal studies have identified the ligand for each of thr TLRs. The first mammalian TLR identified, TLR4 senses lipopolysaccharide (LPS) in association with MD2 and CD14 [4-7]. TLR2 in conjunction with TLR1 or TLR6, recognizes lipoproteins, peptidoglycan and lipoteichoic acid [8,9]. TLR2 and -4 are important for the detection of bacterial cell wall and viral envelope proteins and are critical for host responses against various infections [10,11]. TLR5 recognizes flagellin, a bacterial protein involved in locomotion and sensory perception [12]. Thus, TLR2, -4 and -5 are critical for sensing bacterial and viral cell wall components. Nucleic acid sensing receptors are endosomal, include TLR3, TLR7-9 and are important for the recognition of microbial genomes and replication intermediates of bacteria and viruses. TLR3 senses double stranded RNA (dsRNA) [13], TLR7 and -8 sense single stranded RNA (ssRNA) while TLR9 detects unmethylated CpG motifs in DNA [14-16] [17]. Some of the commonly used

surrogate agonists to mimic natural TLR ligands are Pam2CSK4 (TLR2/TLR6), Pam3CSK4 (TLR2/TLR1), synthetic dsRNA pIC (TLR3), synthetic dsDNA CpG DNA (TLR9), purified flagellin (TLR5) and the imidazoquinoline compound, R848 (TLR7). TLRs signal to activate downstream transcription factors (TFs) such as Nuclear Factor-kappa light chain enhancer of activated B cells (NF-κB), Interferon Response Factors (IRFs) or Activator Protein-1 (AP-1), which induce the transcription of various immune response genes. TLR signaling pathways are described in more detail in the introduction of chapter IV.

C-Lectin Receptors

C-Lectin Receptors are a unique class of proteins with a distinct Carbohydrate Recognition Domain (CRD). CLRs recognize carbohydrate-based structures in microbial cell walls such as mannans, mannosylated proteins, β-glucans. Similar to TLRs signaling through the adaptors MyD88 or TRIF, most CLRs signal through the adaptor molecule Syk kinase (reviewed in Hardison, S. E. *et al*. 2012) [18]. CLRs associate with Syk directly through the immunoreceptor tyrosine based activation motif (ITAM) present in their cytoplasmic tail (e.g.: Dectin-1) or indirectly through other bridging molecules containing ITAM (Dectin-2 uses FcRγ). They are critical for the recognition and phagocytosis of fungal pathogens and polysaccharide microbial patterns. Some of the myeloid CLRs include Mannose receptor, Mincle, DC-SIGN, Dectin-1 and Dectin-2.

NOD like Receptors

The NLR proteins are cytosolic and comprise of a C-terminal LRR domain, central Nucleotide binding Oligomerization domain (NACHT) and a N-terminal CARD/PYRIN/BIR domain. NLRs proteins form multi-protein complexes called inflammasomes, the platform that activates the protease caspase-1 (reviewed in Martinon, F. *et al.* 2007) [19]. Inflammasomes are assembled and activated in the cytosol in response to both microbial and non-microbial danger signals. ASC, an adaptor protein that comprises a PYRIN and a CARD domain bridges the homotypic interaction between NLR/PYHIN proteins and CARD-domain containing caspase-1. Caspase-1 is cleaved in this inflammasome complex into p10 and p20 subunits to form the active caspase-1 complex. This caspase-1 complex is responsible for cleaving the substrates of the pro-inflammatory cytokines pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18 that are released into the extracellular space. Seminal studies have established the importance of NLR and PYHIN proteins such as NLRP3, NLRC4 and AIM2 in the maturation of the IL-1 family of pro-inflammatory cytokines in response to pathogenic and sterile assaults (reviewed in Rathinam, V. A. K. *et al.* 2012) [20].

Complement system

An important arm of pathogen killing and uptake is mediated by the complement system. Complement system is comprised of soluble humoral factors, membrane bound receptors (CR1-CR4) or an array of other regulators (reviewed in Zipfel, P. F. *et al.* 2013)[21]. Complement factors, which circulate in

the serum, can be basally active to a certain extent, sense pathogens directly or through binding to antigen-bound antibodies (opsonization). Their effector functions are induced by the activation of any of the three pathways namely, classical pathway, alternate pathway and the lectin pathway. These pathways proceed through a proteolytic cascade of complement proteins, through a common factor C3, culminating in the direct lysis of pathogens, generating anaphylatoxins and activating the cell-membrane bound complement receptors. These reactions amplify the innate immune anti-microbial mechanisms, recruit leukocytes, promote phagocytosis of pathogens and clear immune complexes (reviewed in Zipfel, P. F. *et al.* 2013)[21]. While un-opsonized pathogens can be sensed and phagocytosed by certain PRRs such as TLRs and CLRs, opsonization by complement provides an effective mechanism of microbial clearance.

TLRs, CLRs, NLRs and complement receptors are critical for the effective first line of defense against several pathogens. This dissertation focuses on two inter-related topics: I. The mechanisms underlying pattern recognition and innate immune response against the fungal pathogen *Candida albicans* (chapters II and III) and

II. Dissecting the evolutionarily conserved role of the protease caspase-8 in inflammasomes, TLR4 and TLR3 pathways (chapter III and IV).

The next sections of this chapter briefly describe some of the known roles of these receptors in anti-microbial defenses, particularly in the context of fungal infections.

Fungal infections and human health

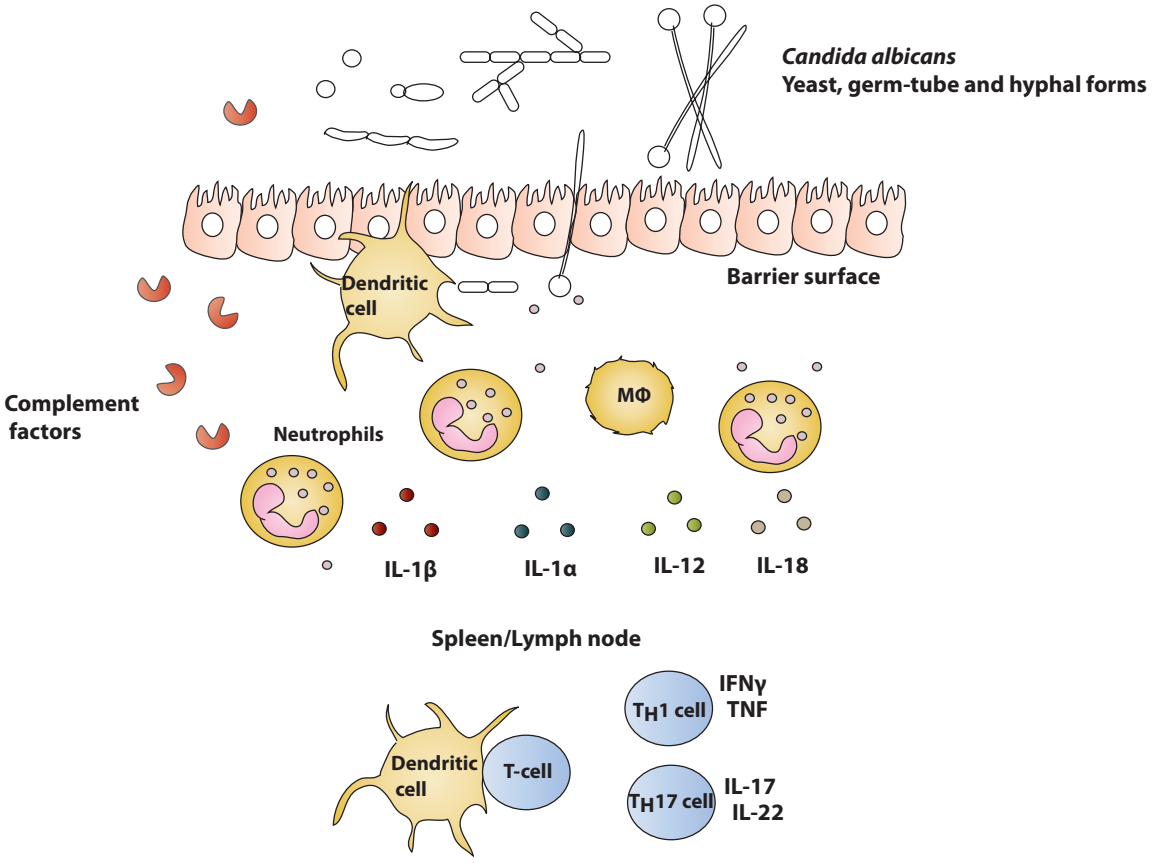
Fungal pathogens are opportunistic pathogens and cause diseases mainly under immunocompromising conditions such as AIDS, tuberculosis or conditions that perturb normal commensal microbiotic balance. Fungal infections are becoming an increasing threat, with mortality rates between 30-50% owing to the rise in immune-debilitating conditions including patients undergoing chemotherapy, antibiotic treatments, and immunosuppressants [22]. The three most common fungal pathogens are *Candida albicans, Aspergillus fumigatus* and *Cryptococcus neoformans* which are the primary cause of candidiasis, aspergillosis and cryptococcosis respectively [23]. *Aspergillus* and *Cryptococcus* can enter through the respiratory tract upon inhalation and can cause severe invasive disease in the lung [23,24]. *Aspergillus* and *Candida* can also be part of the commensal flora in healthy individuals [23,24].

Anti-fungal innate immune response

The host innate immune system maintains a tolerant interaction with commensal microbial flora and can usually, effectively combat invasive fungal infection in healthy, immune-competent individuals. Understanding how these responses are elicited and regulated has important therapeutic implications for the treatment of fungal disease in immune-compromised patients.

Our studies utilized *Candida albicans* to study the host innate immune responses against fungal pathogens. *Candida spp.*, can colonize mucosal and cutaneous surfaces in humans, but certain species, especially *Candida albicans,* can lead to oral, invasive, cutaneous, mucosal, vaginal and systemic infections in an immune-compromised host [25]. The major cells involved in fungal recognition, uptake and effector functions are the professional phagocytes that include circulating monocytes, neutrophils and tissue resident macrophages and dendritic cells (Schematic model 1). The host phagocytic and antigen presenting cells are equipped with a wide variety of pattern recognition receptors such as TLRs, CLRs, complement receptors and NLRs to detect and respond to fungal infection. Phagocytosis of fungal particles, generation of reactive oxygen and nitrogen species, production of antimicrobial peptides, mounting cytokine and chemokine responses to recruit the adaptive immune cells and killing fungi are some of the mechanisms by which macrophages, neutrophils and dendritic cells control fungal infection [26]. Some of the pro-inflammatory cytokine mediators produced by these cells such as IL-1α, IL-1β, IL-12 and IL-18 serve to recruit and activate the anti-microbial mechanisms of other leukocytes and facilitate the differentiation of naïve T cells (Schematic model 1). In addition to innate responses which are particularly important more so in the case of fungal infections, Th17 and Th1 cell responses and their signature cytokines IL-17, IL-12 and IFNγ have also shown to be been strongly required to prevent susceptibility [27-29] (Schematic model 1).

Candida spp., are dimorphic fungi and can switch from unicellular yeast to multicellular filamented hyphal forms, depending on the growth conditions, which further aids progression of the infection (Schematic model 1). The following sections describe about how PRRs sense *Candida albicans* and other fungal infections. Some of these are specialized to differentiate between yeast and hyphal forms, depending on the morphological patterns of the fungi and mount responses accordingly. However, this picture continues to evolve as more receptors, their ligand specificites and signaling pathways are being uncovered.



Schematic model 1. Host immune responses against the fungal pathogen *Candida albicans*. *Candida albicans* is a dimorphic organism that exists as unicellular yeast or can germinate into segmented germ-tubes and filamented multicellular hyphal forms. When *C. albicans* crosses or invades through the host epithelial cell barrier surface, it can be recognized by host phagocytic cells such as macrophages, dendritic cells. These cells detect the fungus through a variety of PRRs, which signal for the production of pro-inflammatory cytokines such as IL-1β, IL-1α, IL-12 or IL-18. These cytokines mediate the recruitment of neutrophils to the site of infection and facilitate the differentiation of naïve T cells into Th17 and Th1 subsets, which are critical for the control of fungal infection. Serum complement factors can bind, opsonize the pathogen and facilitate their uptake by phagocytic cells.

CLRs in anti-fungal innate immune response

Dectin-1

Dectin-1 is the prototypical member of the CLR family of receptors. Dectin-1 is a myeloid cell encoded, type II transmembrane receptor with a C-terminal carbohydrate recognition (lectin) domain, a transmembrane domain and a N-terminal cytoplasmic region and was identified in dendritic cells [30]. Dectin-1 binds to β-glucans of fungal cell walls [31]. 60% of *C. albicans* cell wall are β-glucans and represent an important structural as well as pathogenic pattern [32]. Mannans form the outermost layer of fungal cell wall, however, β-glucans and chitin can be exposed by yeast form at budding junctions during cell division [33]. Mouse dectin-1 is specific towards β(1,3)glucan backbone, and the binding affinities vary according to length, β(1,6)branching and microbial source. Dectin 1 binding to β-glucan and its oligomerizing capacity has been observed with a high-resolution crystal structure of murine dectin-1 [34,35]. Dectin-1 deficient (*Clec7a*$^{-/-}$) macrophages exhibit completely abrogated cytokine production in response to β-glucans [36,37]. *Clec7a*$^{-/-}$mice succumbed to infection with *C. albicans* as well as *A. fumigatus* due to impaired inflammatory cell recruitment and higher fungal burden [38,39]. Importantly, a polymorphism in a highly conserved amino acid (Tyr238C) in dectin-1 was identified in a human family, leading to an early stop codon and loss of 9 amino acids in the CRD region [40]. PBMCs of members with a homozygous mutation showed impaired cytokine production (IL-6, IL-17, TNF-α) in response to β-glucan and *C. albicans* and suffered from recurrent vulvovaginal candidiasis [40].

Mannose Receptor

Mannose Receptor (MR) is cell surface C-lectin receptor identified in human alveolar macrophages and can bind to mannans and neoglycoproteins with highly mannosylated structures of mannose, fucose and N-acetylglucosamine [41-43]. However, MR deficient mice were almost similar compared to WT mice in survival, control of dissemination, inflammatory cell recruitment in response to *C. albicans* [44]. In fact, phagocytosis of *C. albicans* was intact in $MR^{-/-}$ macrophages but could be blocked by β-glucan, suggesting that β-glucan receptors may play a critical role in uptake of the fungus [44].

Dectin-2

Dectin-2 is a transmembrane C-lectin receptor initially identified to be primarily dendritic cell associated [45]. Later found to be myeloid cell associated in general, Dectin-2 is robustly expressed in tissue macrophages and monocytes maturing to macrophages in inflammatory environment and is expressed as many isoforms [45,46]. Interestingly, dectin-2 recognizes and binds to mannans and hyphal forms of *C. albicans* compared to dectin-1 binding β-glucans and yeast forms [47,48]. Even though dectin-2 was primarily identified to respond to hyphal forms, cytokine production to *C. albicans* yeast form was more impaired in the KO compared to the hyphal forms [49]. Dectin-2 knockout (*Clec4n*$^{-/-}$) mice showed that dectin-2 is critical for Th17 differentiation, survival against *C. albicans* infection and cytokine production by mannans [49]. Dectin-2 has a short cytoplasmic tail and hence requires the ITAM containing FcRγ to signal to NF-κB activation [47,49,50]. Dectin-2, dectin-1 and Mincle (described below) signal through

the kinases Syk and PKCδ, CARD domain containing adaptors CARD9 and Bcl10 and the paracaspase MALT1 to activate NF-κB (reviewed in Hardison, S. E. *et al*. 2012) [18]. Blockade of dectin-2 in dectin-1 KO also led to reduced Th17 and Th1 cytokines to *C. albicans*, suggesting an important role for both these receptors in both innate control of infection as well as shaping the T cell responses [51].

Other CLRs

DC-SIGN is a human monocyte and dendritic cell C-type lectin that can bind and induce phagocytosis of *C. albicans* (live as well as heat-killed yeast forms) through recognition of fungal N-linked mannan [52,53] and has been described as antigen uptake receptor not only for fungi but also for viruses [54]. Mincle is another transmembrane receptor for *C. albicans*. Interestingly, Mincle deficient macrophages exhibit reduced cytokine production and knockout (KO) mice harbor higher fungal burden in an intravenous (i.v.) model of infection [55].

TLRs in anti-fungal innate immune response

TLRs are abundantly expressed in monocytes and dendritic cells compared to other cell types [56,57]. The importance of TLRs in innate anti-fungal immune responses is exemplified by the critical requirement of the adaptor molecule MyD88 in controlling infection by the fungal pathogens, *C. albicans* and *A. fumigatus* [58]. Different aspects of innate and adaptive functions such as cytokine production (TNF, IL-12p70, IFNγ), survival, control of fungal growth in organs, internalization, neutrophil mediated candidacidal activity were all severely compromised in *MyD88*$^{-/-}$ mice [58,59]. *MyD88*$^{-/-}$ macrophages showed

impaired cytokine production and fungal killing in response to *C. albicans* yeast as well as hyphae [60].

MyD88 is an adaptor molecule that is engaged by the most TLRs, except TLR3 and the receptors of cytokines such as IL-1 and IL-18 [61]. Consequently, these TLRs and cytokines have also been strongly associated with anti-fungal immune responses. TLR4 has been linked to the recognition of O-linked mannans and GXM (reviewed in Bourgeois et al., 2012) [62]. O-linked mannans, phospholipomannan, and linear β(1,2)oligomannosides are found along with mannans in the fungal cell wall, whereas GXM is a component of *Cryptococcus neoformans* capsule. TLR4 and CD14 have been shown to mediate TNF production in response to fungal mannans in human monocytes [63]. Studies using the TLR4 defective C3H/HeJ mice showed higher kidney fungal burden, reduced chemokines KC, MIP2 and neutrophil recruitment compared to the control C3H/HeN mice. However TLR4 is found to be dispensable by few other studies, potentially due to differences in infection models [58,64,65].

TLR2 senses β -glucan, phospholipomannan, GXM and linear β(1,2)oligomannosides (reviewed in Bourgeois et al., 2012) [62]. *Tlr2*$^{-/-}$ mice were found to be deficient in controlling fungal dissemination and showed higher susceptibility to oral *C. albicans* infection [66]. Interestingly, TLR2 also appears to mediate the production of the anti-inflammatory cytokine, IL-10, in response to *C. albicans* hyphae which can suppress immune response [67]. This is corroborated

by lower *C. albicans* dissemination and higher TNF-α production in the kidneys of *Tlr2*$^{-/-}$ mice [58]. Blocking TLR2 enhanced TNF-α and IL-1 expression in response to *C. albicans* in human peripheral blood mononuclear cells (PBMCs) [64,67]. Although it is not clear what the specific ligand is, TLR1 and TLR6 have also been implicated in the host response against *Candida* and *Aspergillus* based on decreased cytokine levels in mouse models and association of SNPs in patients [68-70]. Thus TLR2 appears positively and negatively regulate anti-fungal responses.

Nucleic acid sensing TLRs such as TLR 7 and 9 have also been implicated in the sensing of fungal RNA and genomic DNA. TLR9 was found to mediate IL-12p40 production in bone marrow dendritic cells (BMDCs) in response to isolated DNA from *C. albicans* and *S. cerevisiae* [71]. Furthermore, *Tlr9*$^{-/-}$ macrophages exhibit lower TNF-α, nitric oxide (NO) production and microbicidal activity to *C. albicans* and *S. cerevisiae* [72]. However, surprisingly, *Tlr9*$^{-/-}$ mice appeared to harbor decreased or similar fungal burden in their kidneys and other organs in response to infection [58,71]. Similar to the case with TLR9, *Tlr7*$^{-/-}$ mice showed lower *C. galbrata* burden in kidneys [73]. TLR7 has been implicated in RNA sensing of *Candida spp*., its uptake and phagosome maturation to mediate IFNβ expression and STAT1 activation [73]. Further studies are required to dissect the precise role of nucleic acid sensing TLRs in infection models.

Complement receptors in anti-fungal innate immune response

Complement plays an important role in the resolution of fungal infection, since C3 deficiency leads to increased susceptibility of mice to *C. albicans*, *C. galbrata* and *S. cerevisiae* [74].

Mannose Binding Lectin

MBL binds to carbohydrate structures, e.g.: mannans and can recognize yeast and hyphal forms of *C. albicans* and other *Candida spp*. grown at 37°C [75]. MBL administration enhances the survival of mice infected with *C. albicans* [75]. In line with that, the MBL polymorphism (G→C) in patients was found to be associated with decreased vaginal MBL protein levels and recurrent vulvovaginal candidiasis [76]. Upon binding to the surface of microbes, MBL can activate the complement activation cascade.

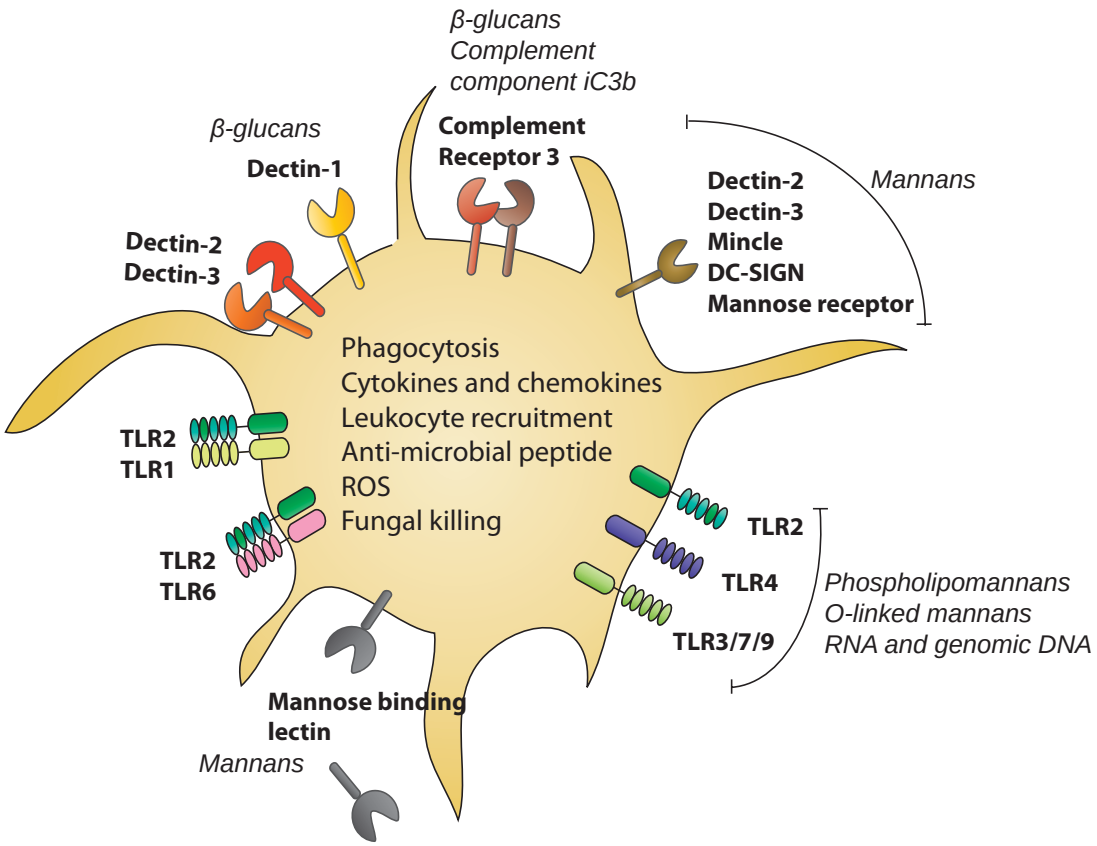
Complement Receptor 3

Another receptor that has been implicated in β -glucan recognition is Complement Receptor 3 (CR3), an $\alpha_M\beta_2$ integrin composed of CD11b and CD18 dimers. It is widely expressed in many immune cells such as monocytes, macrophages, dendritic cells and neutrophils (reviewed in Zipfel, P. F. *et al.* 2013)[21]. In addition to binding the complement component iC3b, CR3 has a distinct lectin site for binding β-glucan [77,78] and is implicated in binding to other carbohydrates as well. CR3 also binds to endogenous ligands such as fibrinogen, coagulation factor X and ICAM-1 which facilitates the extravasation and recruitment of leukocytes to the site of infection. CR3 has been found to mediate phagocytosis of zymosan in transfected CHO cells and mouse

neutrophils in addition to opsonized or complement coated particles [78,79]. Similar to other leukocyte integrins, upon receiving intracellular signals, CR3 undergoes conformational changes required to optimally bind its ligand.

MR, CR3, dectin-1 as well as Fcγ receptors were all required to different extent for the uptake of *C. albicans* yeasts and hyphae in mouse as well as human dendritic cells [80]. Recently, it was shown that dectin-1 activates inside-out signaling of CR3 that in turn promotes CR3 binding of *C. albicans* and subsequent neutrophil anti-fungal effector functions [81]. In a different context, a crude cell wall preparation of the baker's yeast, *Saccharomyces cerevisiae*, zymosan, was shown to activate dectin-1 and TLR2 in a synergistic manner [82]. In addition to TLR2, dectin-1 also synergizes with TLR4 to amplify cytokine production in human monocytes, macrophages and PBMC [83 84].

Thus, CLRs, TLRs and complement receptors co-ordinate and work collectively to effectively recognize, phagocytose and mount adequate responses against fungal pathogens (Schematic model 2). Another recently emerging class of PRRs is the NLRs. Some of the well-characterized NLRs are described below.



Schematic model 2. TLRs, CLRs and complement receptors recognize various fungal PAMPs and induce anti-microbial effector responses. Fungal PAMPs are detected by a variety of PRRs expressed by myeloid cells. TLR -2 and -4 are involved in the recognition of phospholipomannans, O-linked mannans while the endosomal TLRs- TLR -3, -7 and -9 detect the RNA and genomic DNA of Candida. Among the CLRs, while dectin-1 binds to β-glucans, an array of receptors such as dectin-2, dectin-3, Mannose Receptor, Mincle and DC-SIGN have been implicated in the sensing of mannans. Mannose Binding Lectin (MBL) can be secreted and recognizes mannans while Complement Receptor 3 (CR3) can either directly bind β-glucans or the complement iC3b bound β-glucans and *C. albicans*. These PRRs signal to induce anti-fungal effector responses, starting with phagocytosis and extending to cytokine, chemokine, anti-microbial peptide production, leukocyte recruitment, induction of oxidative species and fungal killing mechanisms. Model figure inspired from Cunha, C. *et al.* 2012 [85].

NLRs in anti-microbial responses

In addition to CLRs, TLRs and complement receptors, IL-1 family of cytokines and signaling through their corresponding receptors plays a critical role in the immune response against fungal pathogens. In fact the strong MyD88 dependence that has been observed also signifies its role as an adaptor molecule for the cytokine receptors IL-1R and IL-18R. *Il1r*$^{-/-}$ mice phenocopy the *MyD88*$^{-/-}$ mice and showed a severe impairment in controlling fungal dissemination in an intravenous model of *C. albicans* infection [58]. Importantly, some of these pathways are relevant not only in the context of *C. albicans*, but also *A. fumigatus* and *C. neoformans*. Similar to *C. albicans*, dectin-1, IL-1R and MyD88 play a critical role in controlling infection in a mouse model of keratitis by *A. fumigatus* [86]. Host responses to *Cryptococcus neoformans* also requires the IL-1 family cytokine IL-18 and MyD88 in a pulmonary infection model [87]. Inflammasome complexes formed by NLR and ALR proteins tightly regulate maturation of IL-1 cytokines, specifically IL-1β and IL-18.

NOD1 and NOD2

Cytoplasmic NOD1 and NOD2 are NLR proteins with a CARD domain that sense intracellular bacterial infection. They recognize peptidoglycan fragments of Gram positive and Gram negative bacteria, such as meso diaminopimelic acid (DAP) or muramyl dipeptide (MDP) respectively, and activate the transcription factors, NF-κB, IRF3 or caspase-1 [88-90]. PBMC from patients with NOD2 polymorphisms responded normally to *C. albicans*, in terms of cytokine

production and transfection studies with NOD1 and NOD2 re-emphasised that these receptors may not respond to *C. albicans* [91].

NLRP3

One of the best-studied and characterized inflammasomes is the NLRP3. NLRP3 is found to sense gram positive as well as gram negative bacteria (e.g.: Group B Streptococcus, *E. coli*)[92], viruses (e.g. influenza) [93,94], pore-forming toxins (e.g. nigericin)[95], beta-amyloid and sterile crystalline particles (e.g. silica, asbestos). In addition to responding to microbial/foreign ligands, NLRP3 also senses endogenous host danger signals released likely by dying cells such as ATP [95], monosodium urate crystals [96](Schematic model 3) . It is unlikely that NLRP3 directly binds to this broad repertoire of ligands and hence some secondary activators have been proposed to trigger NLRP3 activation. These mechanisms include phagosomal/lysosomal de-stabilization [97], potassium efflux [98,99], generation of mitochondrial ROS, release of mitochondrial DNA or host and pathogenic enzymes such as cathepsins [100] and molecular patterns [101]. NLRP3 has also been found to respond to nucleic acid signals such as dsRNA, viral and bacterial RNA and DNA [92,102,103].

AIM2

AIM2 is PYHIN family protein comprising of HIN-200 domains that recognize DNA and a PYRIN domain that bridge its interaction with caspase-1 through the adaptor ASC. Previous studies from our lab and others' have elucidated the critical role of AIM2 inflammasome in recognizing cytosolic dsDNA (double stranded DNA) via its HIN200 domain to potentiate IL-1β and IL-18

maturation [104-106]. The AIM2 inflammasome is important for IL-1β, IL-18 and IFNγ production in response to viruses such as Vaccinia, mouse cytomegalovirus as well as bacteria such as Francisella [104,105] (Schematic model 3). Importantly, AIM2 may also respond to mitochondrial DNA or DNA from damaged cells [107]. While adenoviral DNA activates the NLRP3-ASC inflammasome, other viral and bacterial, and host DNA appear to induce IL-1β maturation in an ASC dependent manner, potentially through AIM2 [108]. Interestingly, a broad class of HIN domain containing proteins in the mouse and house genome has been identified (reviewed in Schattgen, S. *et al*. 2011) [109]. Their potential roles as DNA sensors in inducing type I IFN induction and inflammasome (called 'ALRs') are under active investigatio [110].

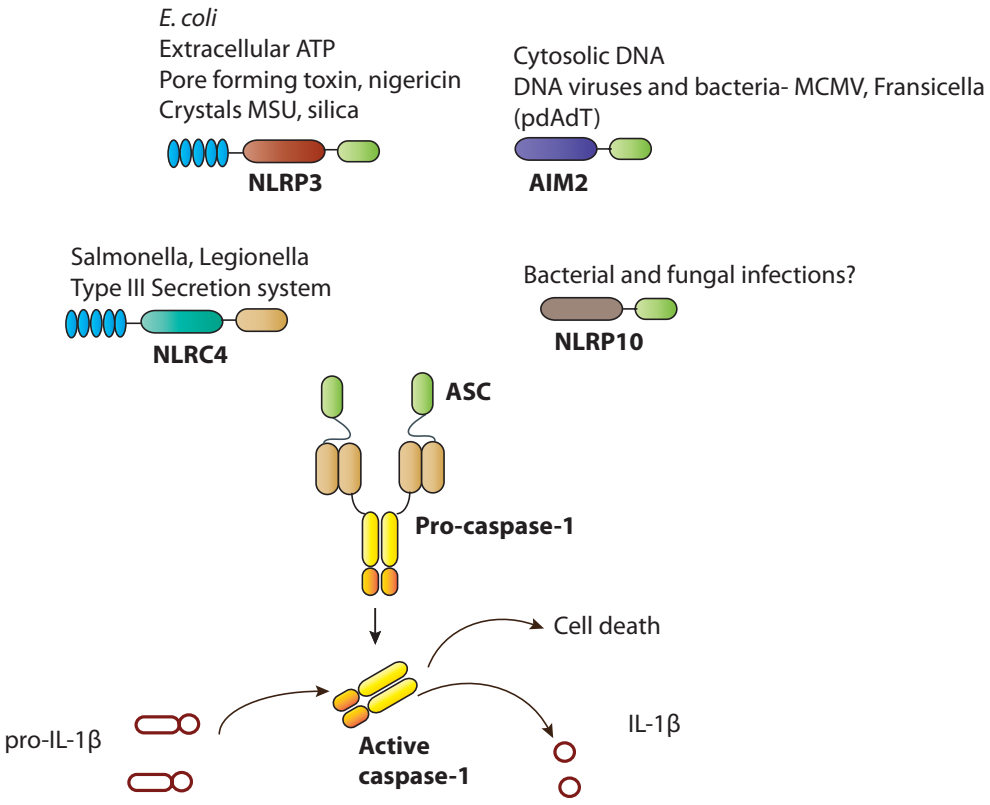
NLRC4

NLRC4 responds to flagellin or PrgJ rod proteins that are translocated into the host cells through the Salmonella Pathogenecity Island I (SPI) type III secretion system (TTSS) or Legionella type IV secretion system [111]. NLRC4 is critical for defense against intracellular bacteria such as *Salmonella typhimurium* [112]*, Pseudomonas aeroginosa, Legionella pneumophila* [113] (Schematic model 3). NAIP5, NLR apoptosis inhibitory protein 5 contains bacuoloviral IAP repeat (BIR) domain instead of CARD domain, is known to form heterodimers with IPAF and is in part required for the inflammasome functions of IPAF [113]. A conserved C-terminal 35aa in the flagellin has been mapped to be critical for IPAF and NAIP5 dependent responses [113]. Although NLCR4 has a CARD domain and can potentially interact with caspase-1 directly, ASC appears to be critical for

caspase-1 activation, IL-1β production and to an extent cell-death responses to Salmonella [112]. Although its function in bacterial infection has been established, its role in fungal infection is not clear.

NLRP10

NLRP10 does not have a LRR domain like other NLR proteins, but contains PYRIN and NOD domain, binds to ASC and has been perceived to be a negative regulator of ASC and caspase-1 mediated inflammasome activation [114,115]. NLRP10 aids DC mediated development of T helper cell immunity in the context of adjuvants and fungal infection [116,117]. It also appears to associate with another NLR protein, NOD1 to augment anti-bacterial responses [118]. However, how NLRP10 detects various stimuli and exerts its function is largely unclear (Schematic model 3).



Schematic model 3. Inflammasomes respond to infectious, sterile and danger signals to induce IL-1β maturation and cell death. Bacterial, viral infections, crystals, toxins, sterile, and host endogenous danger signals can trigger the formation of cytosolic multiprotein complexes called “inflammasomes”. Depicted here are some of the inflammasomes relevant to context of this dissertation. While NLRP3 responds to a broad category of triggers, NLRC4 detects flagellin and rod proteins of certain intracellular pathogens. AIM2 recognizes cytosolic DNA whereas NLRP10 has been implicated in bacterial and fungal infections. ASC bridges the interaction between the protease caspase-1 and the NLRs and AIM2. Assembly of inflammasomes leads to the activation and processing of pro-caspase-1 into the mature enzyme, which then cleaves pro-IL-1β into its active, mature form that is released from the cell. Inflammasome activation is mostly accompanied by the death of the activated cell.

While NLR and ALRs sense a variety of bacterial and viral infections through direct or indirect mechanisms, their roles in anti-fungal responses are not clear. Further, how interact with TLRs, CLRs and complement receptors to potentiate their responses also needs to be investigated in detail.

Chapter II:

NLRP3 and NLRC4 inflammasomes in anti-fungal innate immune responses

Chapter II: Copyright information

Most of the data and some of the subject content in this chapter have appeared in the following publications:

Hise AG, Tomalka J, **Ganesan S**, Patel K, Hall BA, Brown GD, Fitzgerald KA. An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans*. Cell Host Microbe. 2009 May 8;5(5):487-97.

Tomalka J, **Ganesan S**, Azodi E, Patel K, Majmudar P, Hall BA, Fitzgerald KA, Hise AG. A novel role for the NLRC4 inflammasome in mucosal defenses against the fungal pathogen *Candida albicans*. PLoS Pathog. 2011 Dec;7(12):e1002379.

Chapter II: NLRP3 and NLRC4 inflammasomes in anti-fungal innate immune responses

Abstract

Candida albicans is an opportunistic human fungal pathogen that primarily affects patients with immunocompromising conditions such as AIDS and patients undergoing immunosuppressive antibiotic treatments. It is critical to understand the host immune response to *C. albicans* to combat these opportunistic infections. The innate immune system plays an important role in detection, control and clearance of fungal pathogens. In this study, we have addressed the role of the NOD-like Receptors NLRP3 and NLRC4 and the pro-inflammatory cytokine IL-1β in host defense against *C. albicans*. To investigate the role of IL-1 and inflammasomes in a common opportunistic infection caused by *C. albicans*, called 'oropharyngeal candidiasis' (OPC), we used a clinical strain (GDH2346) isolated from a patient with OPC. Our studies using formalin fixed prep of this strain showed robust processing and release of IL-1β in human as well as mouse immune cells. Being a dimorphic pathogen that occurs in different morphological forms such as yeast, germ-tube or hyphal stages, we observed the germ-tube forms of *C. albicans* to be more immunostimulatory. Importantly, we demonstrate that *C. albicans* induced IL-1β production is strictly dependent on the phagocytosis of the fungus and the NLRP3 inflammasome and its associated components, ASC and caspase-1. In addition to NLRP3, NLRC4/IPAF also forms an inflammasome capable of activating caspase-1 and IL-1β cleavage in response to intracellular pathogens. We found that *C. albicans* induced IL-1β

maturation and release from macrophages and dendritic cells do not require the NLRC4 inflammasome. Further, the relevance of our findings is highlighted by the suppression of *C. albicans* induced IL-1β inhibition by inhibiting caspase-1 in human peripheral blood monocytic cells. Our findings illustrate the robust, inducible production of a critical pro-inflammatory cytokine IL-1β by innate immune cells in response to the fungal pathogen *C. albicans* and the non-redundant role of the cytosolic NLRP3 inflammasome complex in this pathway.

Introduction

Oral candidiasis caused by *Candida spp.*, is one of the most common opportunistic infections that is found in HIV patients [119]. *Candida spp.*, are also the predominant causes of fungal nosocomial infections, with *C. albicans* being the most frequent cause [120]. As described in chapter I, the innate immune system plays a crucial role in the control of fungal infection. The important factors that contribute to effective fungal clearance and host protection are production of pro-inflammatory cytokines, sufficient neutrophil recruitment to the site of infection and development of Th1 and Th17 immunity. In this context, IL-18, IL-1α, IL-12 and IL-1β are all important for *C. albicans* triggered IFNγ production [121]. In particular, inflammasome associated cytokines, IL-1β, IL-18 and IL-1α play a protective role against disseminated candidiasis, both prophylactically and administration post-infection, as it increases the survival and decreases kidney fungal burden in an IFNγ dependent manner [122,123] [123-126]. This set the stage for further work in understanding the role and molecular mechanisms of *C. albicans* induced inflammasome activation and in particular, IL-1β responses.

IL-1β is a highly pro-inflammatory cytokine produced by many cell types and is critical for promoting inflammation. IL-1β mediates the recruitment of leukocytes to the site of infection, first among those being neutrophils. Synthesis and maturation of IL-1β is a two-step process and is tightly regulated. In most cell types, secretion of mature, bioactive IL-1β requires at least two signals: synthesis of the precursor pro-IL-1β (and sometimes the NLR, NLRP3) through NF-κB activation (signal 1) and the subsequent activation of caspase-1 through the formation of an inflammasome (signal 2). Circulating monocytes and tissue resident macrophages, dendritic cells are the first cells to encounter and sample the fungus in an actual infection. Hence, we used the human PBMCs, human monocytic cell line (THP1), thioglycollate elicited mouse peritoneal exudate cells and bone marrow derived macrophages and dendritic cells to examine IL-1β production in response to *C. albicans*. We also wanted to understand the role of the dimorphic nature of the fungus in inducing IL-1β. Hence, we used formalin fixed preparations of *C. albicans* in different morphological forms to capture the cytokine response at each identified stage.

As described in the previous chapter, the roles of TLRs, CLRs and complement receptors in anti-fungal innate immune responses and the specific PAMPs they recognize have been characterized to a significant extent. However, the role of the inflammasome forming NLR or PYHIN proteins in anti-fungal immune response is not well understood. Hence, in this study, we

investigated the role of the NLR proteins, NLRP3 and NLRC4 in particular, in the production of IL-1β in response to *C. albicans*. NLRP3 responds to a variety of stimuli including bacteria, viruses, crystalline or host generated alarmins. In contrast, NLRC4 exercises more specificity and responds to flagellin and rod proteins translocated through the type III or type IV secretion system (T3SS) of intracellular bacterial pathogens. Using cells from mice deficient in NLRP3, NLRC4 and the other core components of inflammasomes- ASC and caspase-1, we demonstrate a critical role for the NLRP3, but not NLRC4 inflammasome, in *C. albicans* induced IL-1β production. While the results presented here represent the *in vitro* data, the laboratory of Dr. Hise investigated the relevance of our findings in *in vivo* model of oropharyngeal candidiasis (OPC) [84]. Mice model of OPC revealed that NLRP3, ASC, caspase-1 and IL-1R are critical for inhibiting fungal dissemination and mortality [84] indicating the important role of innate immune responses, especially inflammasomes and IL-1 signaling in anti-fungal immunity.

Results

First, we set out to examine if innate immune cells produce IL-1β in response to *C. albicans.* We measured IL-1β production by different cell types including: human PBMCs (Figure 1A), human monocytic cell line (THP1-Figure 1B), mouse bone marrow derived macrophages or dendritic cells (BMDM or BMDC- Figure 1C, D), thioglycollate elicited mouse peritoneal exudate cells (PEC- Figure 1E) in response to *C. albicans*. Cells were primed with the TLR4

agonist LPS which activates NF-κB and up regulates pro-IL-1β and NLR proteins themselves since basal levels of NLRs, such as NLRP3, is quite low in BMDM compared to dendritic cells. Cells were then stimulated with formalin fixed preps of *C. albicans.* Formalin fixed preps of *C. albicans* were used to maintain the intact morphological structure of fungus, while blocking its filamentation and other virulence strategies. Cells were also treated with the ATP (a danger associated molecular pattern- DAMP), pore-forming toxin nigericin or crystalline particles such as silica as positive controls, since these stimuli have been shown to trigger IL-1β production in macrophages [95,127]. We observed that *C. albicans* triggers IL-1β production in both human as well as mouse cell types.

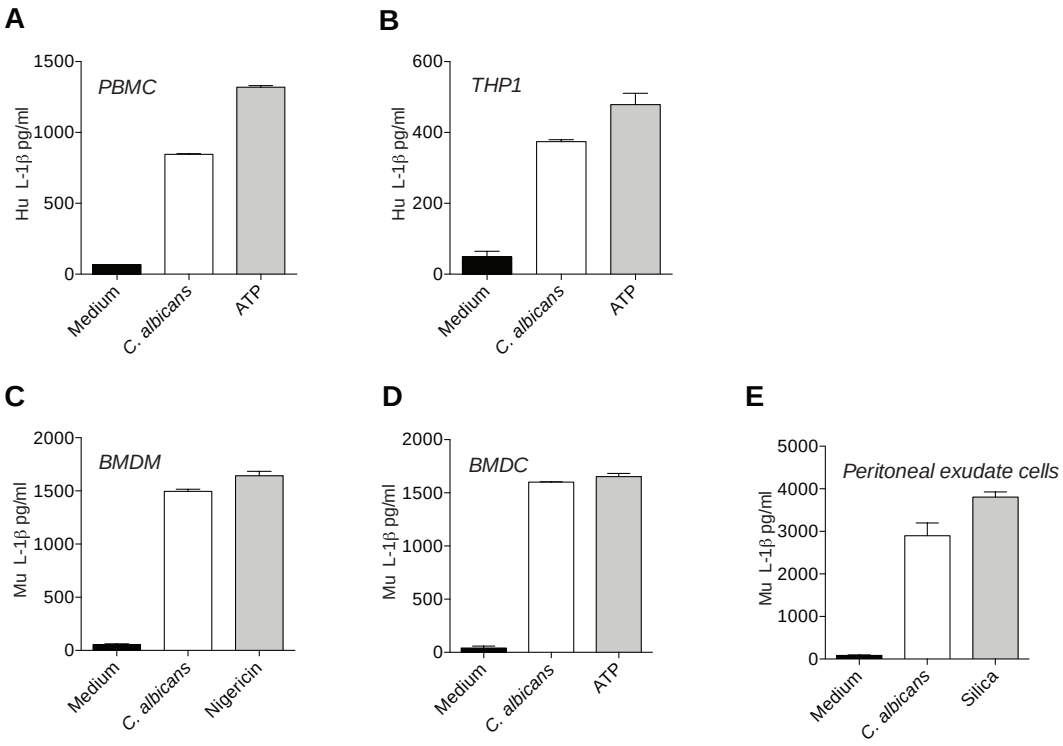


Figure 1 *C. albicans* induces the production of IL-1β in various immune cell types. Peripheral blood mononuclear cells (PBMC-A), human monocytic cell line (THP1-B), bone marrow derived macrophages (BMDM-C), bone marrow derived dendritic cells (BMDC-D) or thioglycollate elicited peritoneal exudate cells (E) were primed with LPS for 3h and stimulated with formalin fixed germ tube prep of *C. albicans* or silica for 6h, ATP or nigericin for 1h. Amount of IL-1β released in the supernatants was measured by Enzyme Linked ImmunoSorbent Assay (ELISA) in panels A-E.

C. albicans is a dimorphic fungus and can transition from unicellular yeast to filamentous, multicellular hyphal phase depending on the growth conditions and environment. To investigate the immunostimulatory capacity of each of these forms, *C. albicans* was formalin fixed at different morphological stages of culture such as yeast, germ tube or pseudohyphal (early-2h, mid-4h, late-6h) and fully hyphal phase (24h). LPS primed BMDM and peritoneal exudate cells were treated with some of these distinct stages. As shown in figure 2A and B, we observed high IL-1β production in response to the germ tube forms (Figure 2A and B). Processed IL-1β (p17) was observed in the supernatants of stimulated bone marrow and peritoneal macrophages (Figure 2C and D). Further, caspase-1 processing, an indicator of inflammasome and caspase-1 activation was also noticed in the supernatants of *C. albicans* stimulated peritoneal macrophages (Figure 2D). These results showed that *C. albicans,* germ-tube forms in particular, triggers inflammasome activation, caspase-1 and IL-1β processing and release in macrophages.

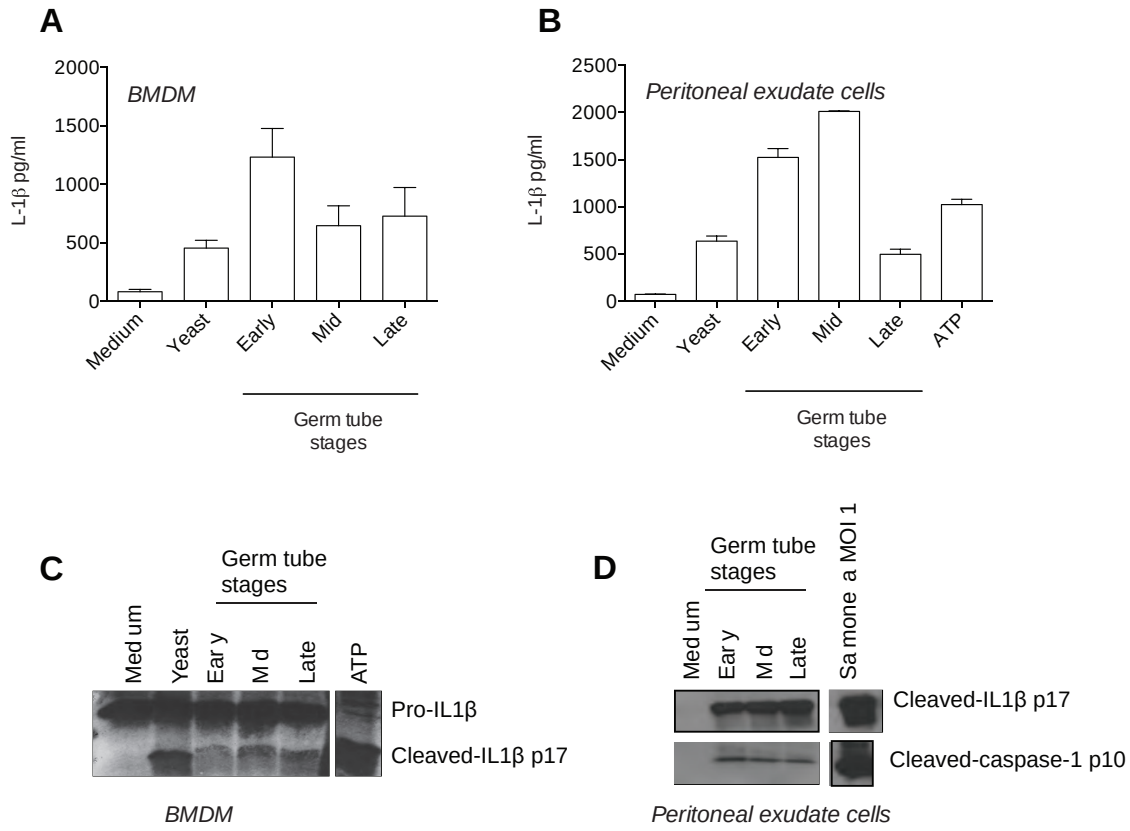


Figure 2. *C. albicans* induces caspase-1 activation, processing and production of IL-1β in bone marrow and peritoneal macrophages. Bone marrow derived macrophages (A, C) or peritoneal exudate cells (B, D) were primed with LPS for 3h and stimulated with formalin fixed preps of *C. albicans* in different morphological stages for 6h, ATP for 1h or *S. typhimurium* MOI 1 for 2h. Early, mid and late represent different stages of germ tube formation. Amount of IL-1β released in the supernatants was measured by ELISA (A, B) and precipitated supernatants were probed for the processing of IL-1β and caspase-1 (C, D).

To identify the specific NLR that forms the inflammasome and mediates IL-1β production in response to *C. albicans*, we first tested the NLRP3 inflammasome since it responds to a variety of stimuli in contrast to NLRC4 (Type III secretion system of intracellular bacteria) or AIM2 (cytosolic DNA). NLRP3 inflammasome is composed of the NOD like receptor NLRP3, the PYRIN-CARD containing adaptor protein ASC and the protease caspase-1. LPS primed bone marrow derived macrophages from *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ and *Casp1*$^{-/-}$ or WT mice were stimulated with germ tube or hyphal stages of *C. albicans.* Cells were also treated with nigericin or *S. typhimurium* at MOI 1 as controls. While nigericin is a classical activator of the NLRP3 inflammasome, *S. typhimurium* is sensed by the NLRC4 inflammasome, as described earlier. As seen in figure 3A, *C. albicans* induced IL-1β production is completely abrogated in all the KOs that are deficient in components of the NLRP3 inflammasome. The *Casp1*$^{-/-}$ mice have been noted to carry the *Casp11* passenger mutation associated with the 129 mouse strain in which they were created [128,129]. To test if the deficient IL-1β production noticed in *Casp1*$^{-/-}$ cells was due to the deficiency in caspase-1 or capase-11, BMDM from 129X1/SvJ mice that lack caspase-11 expression were used. As seen in figure 3B, *C. albicans* induced IL-1β production was not affected in the caspase-11 deficient cells pointing to a specific role for caspase-1 in this process.

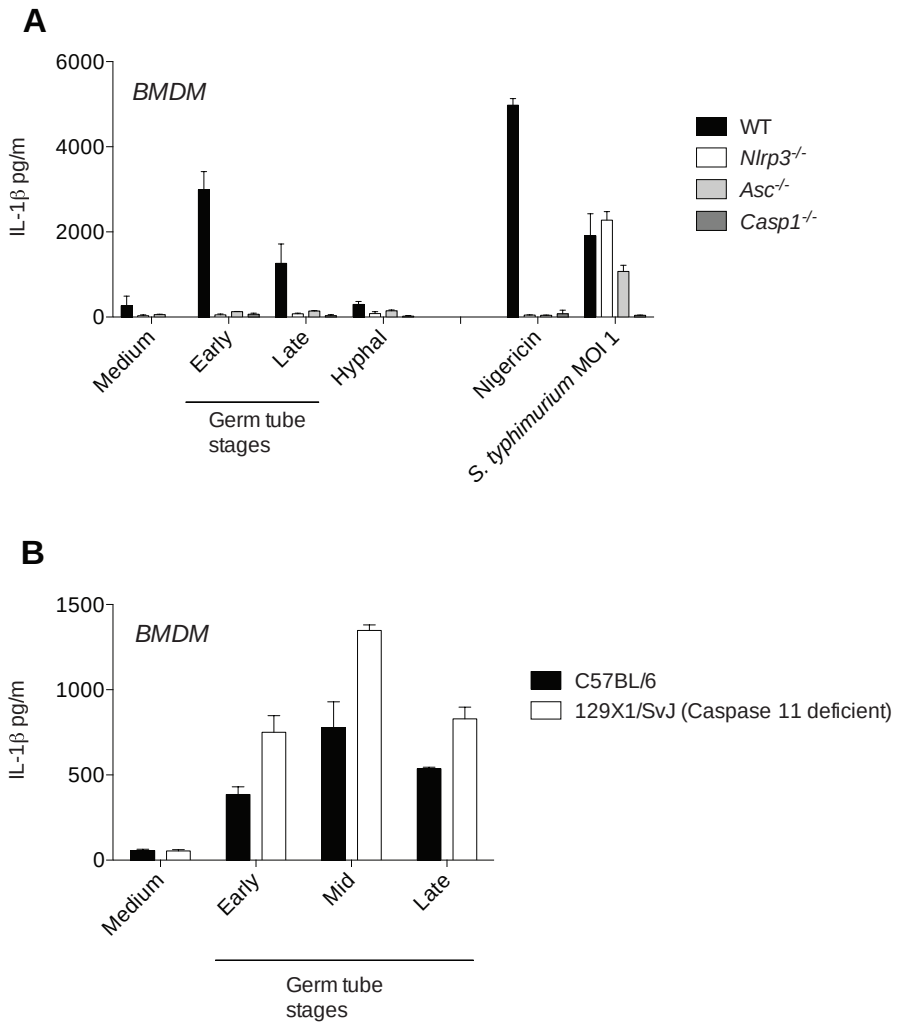


Figure 3. *C. albicans* induced IL-1β production requires the NLRP3 inflammasome.

Bone marrow derived macrophages from *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *Casp1*$^{-/-}$ (A) or 129/SvJ (B) and C57BL/6 mice were primed with LPS for 3h and stimulated with formalin fixed preps of *C. albicans* in different morphological stages for 6h, *S. typhimurium* at MOI 1 or 10 for 2h, ATP or nigericin for 1h. Amount of IL-1β released in the supernatants was measured by ELISA.

The lab of Dr. Amy Hise developed a mouse model of oropharyngeal candidiasis (thrush) similar to that of the human disease with respect to epithelial ulceration, which allows mucosal colonization and dissemination [130]. Since WT immune-competent mice do not develop an infection with *C. albicans* when injected [28], mice were antibiotic treated prior to infection as described [84]. In a mouse model of oropharyngeal candidiasis, *Nlrp3*$^{-/-}$ mice showed reduced serum and mucosal IL-1β, reduced neutrophil recruitment to the mucosal epithelium, higher mortality, fungal dissemination and kidney burden compared to WT controls[84,131]. Remarkably, *Asc*$^{-/-}$, *Casp1*$^{-/-}$ and *Il1r*$^{-/-}$ mice phenocopied the *Nlrp3*$^{-/-}$ mice in this model, indicating that these molecules are part of one pathway [66].

Surprisingly, NLRC4 deficient mice also showed a similar phenotype with higher mortality, fungal dissemination and kidney burden in the *in vivo* infection model [84,131]. To probe this further and to identify the cellular compartment that contributes to NLRC4 dependent response, *in vitro* experiments in *Nlrc4*$^{-/-}$ cells were performed. Bone marrow derived macrophages (BMDM), dendritic cells (BMDC) or thioglycollate elicited peritoneal exudate cells (PEC) were stimulated with germ tube or hyphal forms of *C. albicans*, along with ATP, nigericin or Salmonella as controls (Figure 4A-C). Of note, BMDC produced high levels of IL-1β irrespective of the morphological form of the fungus (Figure 4C). These results indicated that *Nlrc4*$^{-/-}$ hematopoietic immune cells are fully competent in inflammasome activation and IL-1β production even though *Nlrc4*$^{-/-}$ mice exhibit a severe defect in protection against candidiasis [131]. This emphasized the specific

role of NLRP3 inflammasome in *C. albicans* triggered IL-1β production in these cells. Indeed, bone marrow chimera experiments as depicted in figure 7 in Tomalka, J. et al. 2011 demonstrated that NLRC4 is in fact important in the non-hematopoietic/stromal compartment for controlling the oral growth of the fungus while the NLRP3 and ASC are critically required in both hematopoietic and stromal compartments to control mucosal infection as well as dissemination into the kidneys [131].

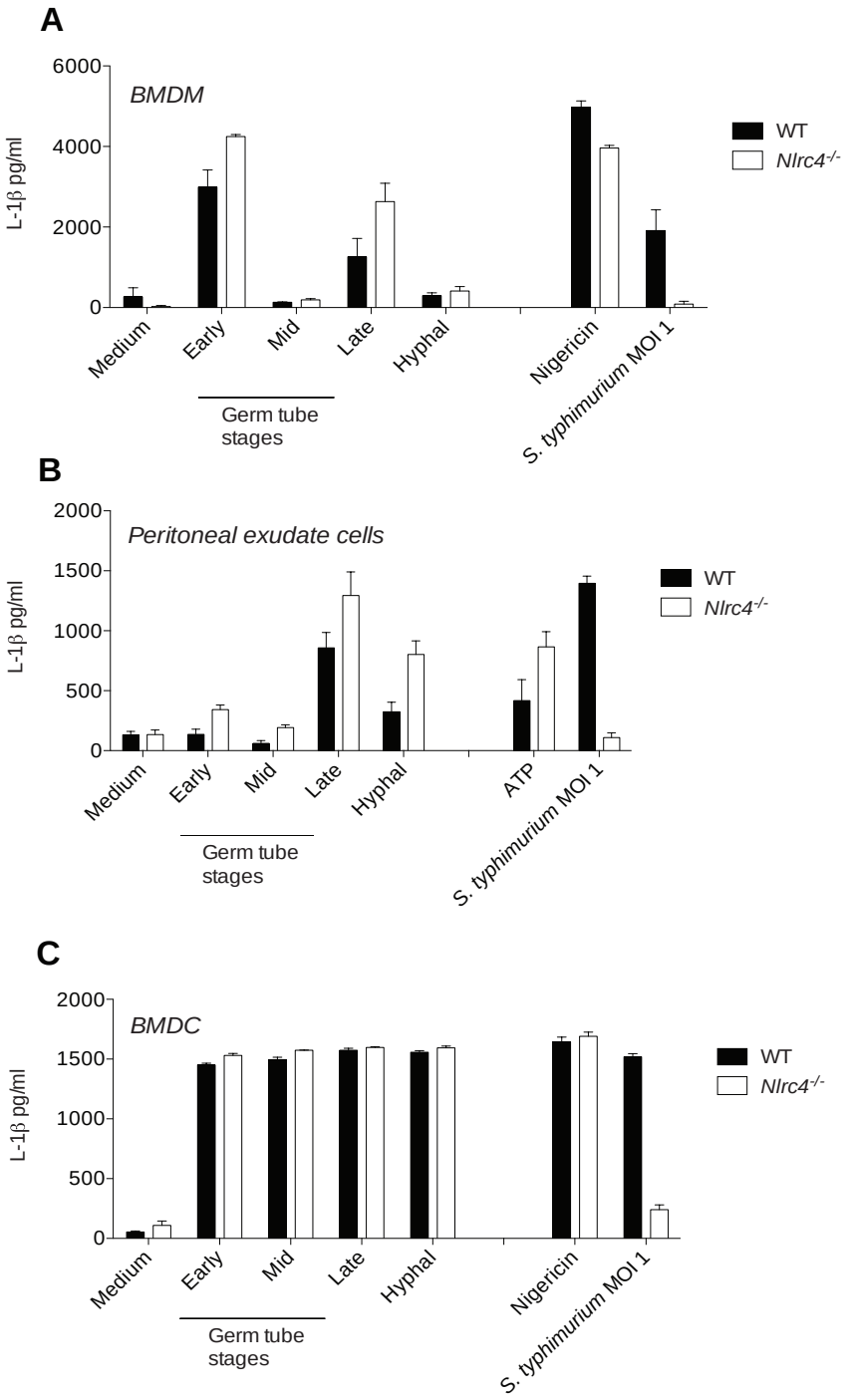


Figure 4. *C. albicans* induced IL-1β production does not require NLRC4 inflammasome in innate immune cells. Bone marrow macrophages (A), thioglycollate elicited peritoneal exudate cells (B) or dendritic cells (C) from *Nlrc4*$^{-/-}$ or C57BL/6 mice were primed with LPS for 3h and stimulated with formalin fixed preps of *C. albicans* in different morphological stages for 6h, *S. typhimurium* at MOI 1 for 2h, ATP or nigericin for 1h. Amount of IL-1β released in the supernatants was measured by ELISA (A-C).

In order to examine the effect of *C. albicans* morphology on IL-1β production in human cells, human peripheral blood mononuclear cells (PBMC) or the monocytic cell line (THP1) were treated with germ-tube or hyphal forms of *C. albicans*. We found that higher IL-1β production was observed in LPS primed PBMC or THP1 cells in response to germ tube forms (Figure 5A and B). Correspondingly, IL-1β processing into its mature form was observed in the supernatants of LPS primed PBMC stimulated with *C. albicans* (Figure 5C). LPS primed cells were also separately stimulated with ATP or HSV as positive controls. To investigate if the IL-1β production in human cells also requires the effector protease caspase-1, PBMC were pre-treated with the caspase-1 inhibitor, z-YVAD-fmk before stimulating them with germ-tube or hyphal forms of *C. albicans*. Results depicted in figure 5D indicate that similar to mouse macrophages, human cells also require caspase-1 and its activity for producing IL-1β in response to both germ-tube as well as hyphal forms of *C. albicans*.

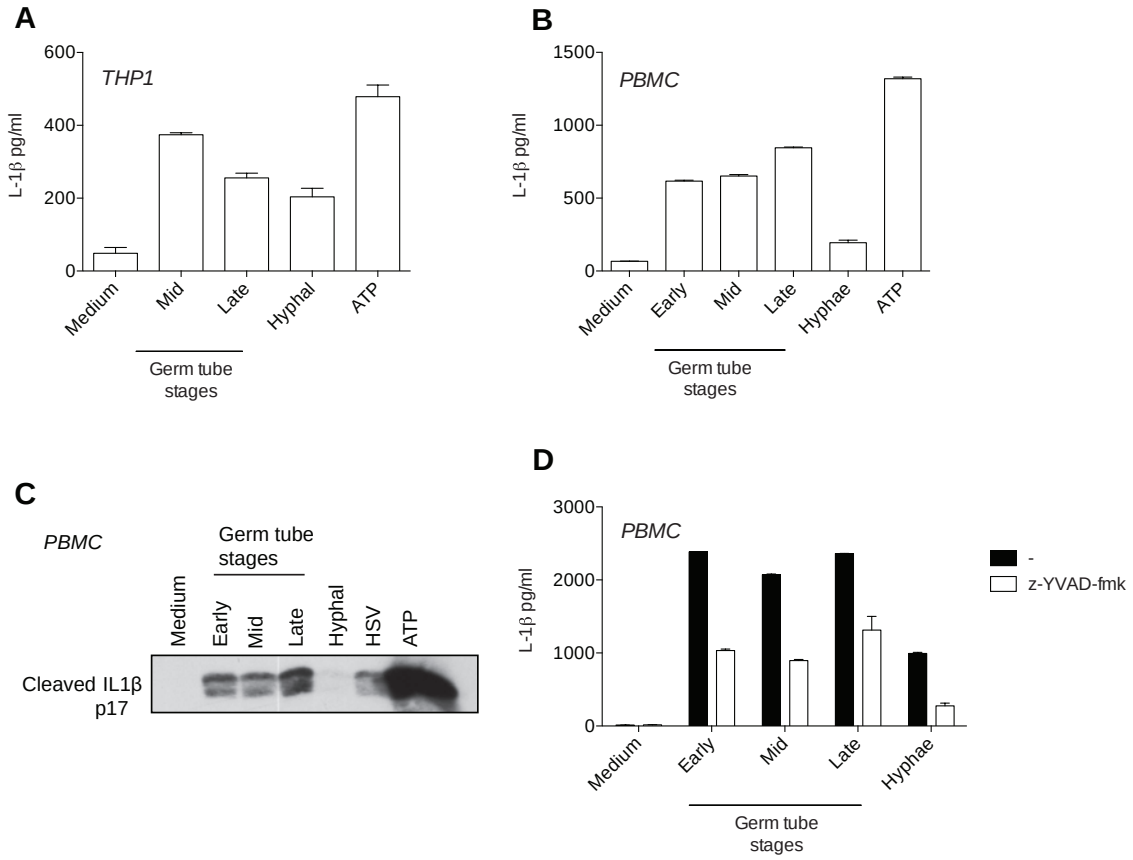


Figure 5. *C. albicans* induces the production and processing of IL-1β in human cells in a caspase-1 dependent manner. Human monocytic cell line THP1 (A) or human PBMCs (B-D) were primed with LPS for 3h and stimulated with formalin fixed preps of *C. albicans* in different morphological stages for 6h or ATP for 1h. LPS primed cells were pretreated with 10μM z-YVAD-fmk, the caspase-1 inhibitor for 30mins prior to stimulation with *C albicans* (D). Amount of IL-1β released in the supernatants was measured by ELISA (A, B and D) and precipitated supernatants were probed for the processing of IL-1β (C).

One of the characterized mechanisms of inflammasome activation is the phagosomal uptake, phagosomal-lysosomal fusion leading to lysosomal destabilization and release of cathepsins that in turn activate the inflammasome in the case of silica or mono-sodium urate crystals. Macrophages, dendritic cells and neutrophils are efficient in the uptake of *C. albicans* through C-lectin receptors suggesting that phagocytosis and inflammasome activation might be linked in the case of *C. albicans* induced IL-1β production. To begin understanding the mechanism by which *C. albicans* triggers inflammasome activation and IL-1β production, the requirement of phagocytosis in this process was first examined. To address this, an inhibitor of actin polymerization, cytochalasin D was used to block phagocytosis. As seen in figure 6A, cytochalasin D effectively blocked *C. albicans* induced IL-1β production, similar to silica [127].

Dying cells may release uric acid crystals that in turn can trigger IL-1β production by other cells, as observed with the experimental stimulus MSU [96,132]. In order to check whether *C. albicans* triggered IL-1β is direct, or through the uric acid DAMP released by dying cells, LPS primed cells were treated with *C. albicans* in the presence of the enzyme uricase to digest uric acid. However, *C. albicans*, but not MSU, triggered IL-1β was unaffected, as observed in figure 6B. Further efforts in dissecting the underlying mechanisms of *C. albicans* triggered inflammasome activation and IL-1β production are described in chapter II.

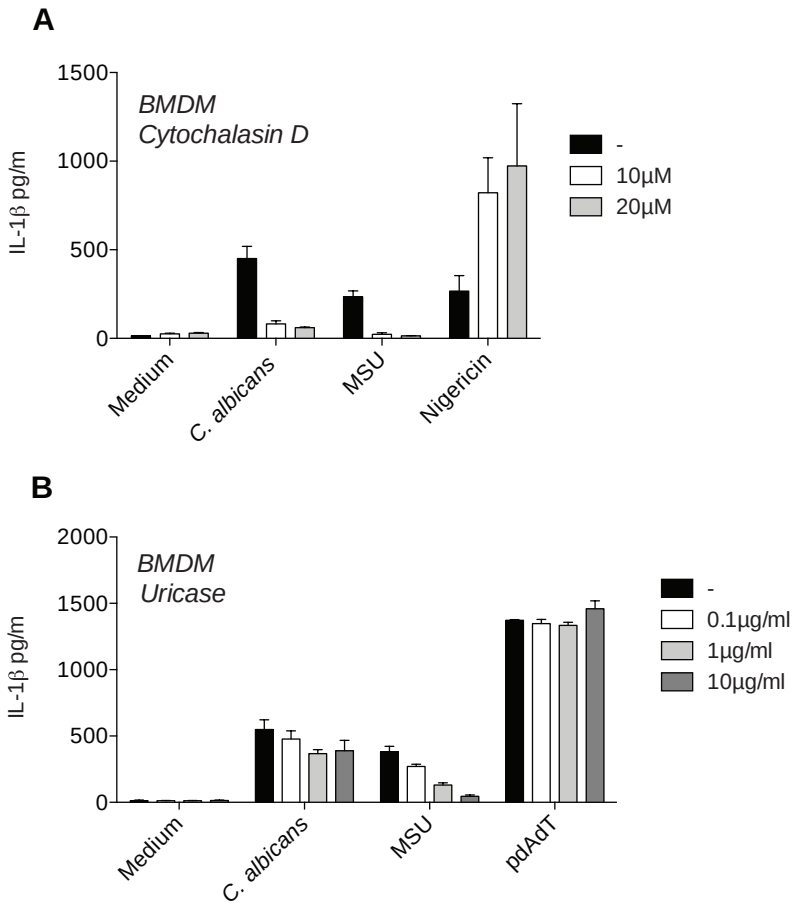
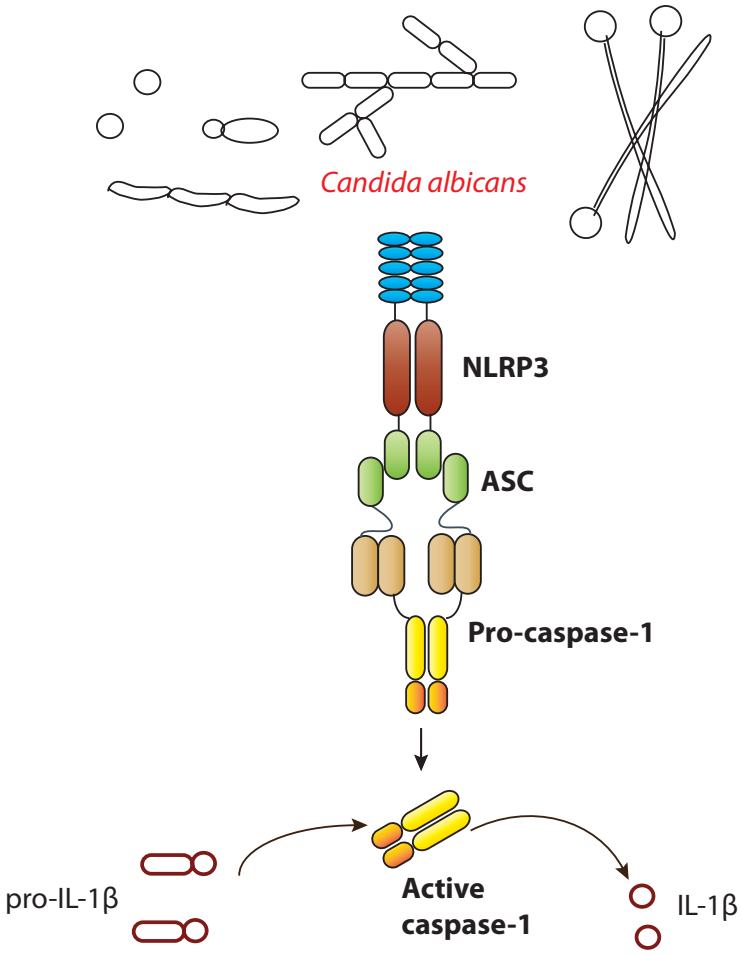


Figure 6. *C. albicans* induced IL-1β production is mediated by phagocytosis but not uric acid released by dying cells. BMDM were primed with LPS for 3h, pretreated with increasing concentrations of cytochalasin D (A) or uricase (B) and then stimulated with formalin fixed germ tube prep of *C. albicans*, pdAdT or MSU for 6h or nigericin for 1h. Amount of IL-1β released in the supernatants was measured by ELISA (A-B).



Schematic model 4. NLRP3 inflammasome in *C. albicans* mediated IL-1β maturation. The inflammasome forming NLR protein NLRP3, along with the adaptor ASC and protease caspase-1 mediate pro-IL-1β processing and IL-1β production in response to various morphological forms of the fungal pathogen *C. albicans.*

Discussion

The results described in this chapter demonstrate that *C. albicans* induces IL-1β production in different mouse and human hematopoietic immune cells. This process is mediated by the activation of the NLRP3-ASC-caspase-1 inflammasome in mouse macrophages and is suppressed by caspase-1 inhibition in human PBMCs. *In vivo* mouse model of oral candidiasis (developed by Dr. Hise lab) concurred with the *in vitro* data and showed a critical role for NLRP3 inflammasome components in systemic IL-1β production, control of fungal dissemination and survival [84]. Circulating IL-1β levels were completely absent in *C. albicans* infected *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice and consistent with that, *Il1r*$^{-/-}$ mice phenocopied the *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *Casp1*$^{-/-}$ mice indicating that the impaired anti-fungal responses in these mice is likely due to the deficient IL-1β production [84].

NLRC4 was found to not be required for *C. albicans* induced IL-1β production in peritoneal exudate cells, bone marrow derived macrophages or dendritic cells, and these particular results have also been confirmed by other groups [133,134]. However, the *in vivo* model of infection revealed an interesting role for the NLRC4 inflammasome with an overall phenotype similar to that of the NLRP3 inflammasome [131]. This was the first instance where NLRC4 inflammasome was found to be important for anti-fungal responses. Upon detailed examination, NLRC4 was predominantly found to be required in the non-hematopoietic compartment and in the local control of infection [131]. The collective

role of NLRP3 and NLRC4 inflammasomes in the *in vivo* infection model could in part, be due to inter-dependence of their expression on one another observed in oral mucosal tissues (Figure 1 in Tomalka, J. *et al*. 2011) [135].

NLRP3 and NLRC4 play a critical role in the induction of Th17 cytokines, anti-microbial peptides and leukocyte, in particular, neutrophil recruitment in the oral mucosal cavity against *C. albicans* [28,131]. Further, ASC and caspase-1, the molecules on which all the described inflammasome pathways converge upon, are not only critical for the protection of mice from disseminated candidiasis but are important for the development of Th1 and Th17 responses, evident from higher mortality, fungal burden and lower cytokine analysis in the KO mice shown by other studies [136]. Th1 and Th17 immunity are considered effective anti-fungal adaptive responses. Specifically, in the context of oral candidiasis, IL-17R and Th17 cells have been shown to be protective [28]. In line with that, patients with IL-17RA or IL-17F deficiency suffer from recurrent mucocutaneous candidiasis suggesting an important role of this subset in human anti-fungal responses as well [137]. IL-1R signaling in T cells along with IL-6 and IL-23 is critical for the Th17 cell differentiation [138]. Similar to IL-1β, Th17 cytokines also mediate the recruitment of neutrophils to the site of infection.

It is currently unclear how NLRC4 is activated during *C. albicans* infection. While it is not clear if any unknown *C. albicans* secreted proteins are similar to that rod proteins produced by intracellular bacterial pathogens, it is most likely

that the host bacterial species are detected by the NLRC4 inflammasome when the commensal environment is perturbed. In support of that argument, analysis of mucosal biofilms in a mouse model of OPC reveals a complex environment with *C. albicans* yeast, hyphae, host neutrophils and commensal bacteria such as *Enterococcus, Lactobacillus* and *Staphylococcus* species [139]. It is possible that macrophages and neutrophils recruited to the site of infection also respond to the bacterial PAMPs.

C. albicans is a dimorphic pathogen and can exist in various morphological forms such as yeast, early, later germ tubes and hyphae. The yeast form facilitates the dissemination of infection, while the hyphal forms invades tissues. Using engineered *C. albicans* strain which expresses *NRG1* (a negative regulator of filamentation) under the control of a tetracycline promoter, studies have examined the transition into hyphal phase at different stages during infection by the administration of doxycycline [140,141]. These studies have revealed that while yeast forms can be lethal under severely immune-compromised conditions, mice succumb to infection more readily with filamentous forms compared to yeast [140,141].

Our studies using formalin fixed preparations showed that, viability of *C. albicans* is not necessary for inflammasome activation and IL-1β production. Of the different stages of *C. albicans* tested, the yeast and germ tube stages were more immunostimulatory compared to the hyphal form in macrophages, with the

exception of GM-CSF differentiated dendritic cells which produced high levels of IL-1β in response to all forms. Other studies using live WT *C. albicans* or mutants of hyphal morphogenesis found that the ability to transition to hyphal form (germ tube or pseudophyphal stages), but not necessarily the hyphal filaments themselves, to trigger inflammasome activation [134,142]. It is possible that dendritic cells are more efficient at responding to hyphal forms of *C. albicans* and inducing higher IL-1β compared to macrophages and this might impact the innate immune response during the course of infection *in vivo*. One of the reasons attributed to the differential stimulatory capacity of these morphological forms is the accessibility and sensing of the underlying cell wall components beneath the mannan layer and difference in length of mannan fibril length between these forms [142].

Many fungal species have been identified to induce IL-1β production. While we used a clinical strain of *C. albicans*, isolated from a patient with denture stomatitis, or the lab strain SC5314, other studies have used the ATCC strain UC820, clinical isolate FC20, *C. krusei*, *C. tropicalis* or the common budding yeast *S. cerevisiae* [133,134]. Another common fungal pathogen, *A. fumigatus* has also been shown to trigger NLRP3 inflammasome dependent IL-1β production [143] indicating the relevance of these findings to fungal infections in general. Some of these studies, including that of *A. fumigatus* or *C. albicans,* showing that fungal secreted aspartic proteases induce NLRP3 mediated IL-1β production was performed in human cells [143,144]. Further, length polymorphism in NLRP3 has

been associated with recurrent vulvovaginal candidiasis in patients [145] indicating the application of these findings in human fungal infections as well.

One of the most important functions of IL-1β and IL-17 immunity in anti-fungal defenses is the recruitment of neutrophils. Neutrophils being one of the primary responders to the site of infection detect and directly kill the fungal pathogen through intracellular and extracellular mechanisms. Neutrophils secrete fibrous extracellular traps (NETs) formed by neutrophils can entrap and engulf yeast and fungal forms of *C. albicans* and mediate their killing by generating anti-microbial effectors including lytic enzymes and granules [146,147]. Expression of inflammasome components such as NLRP3, ASC, caspase-1 in neutrophils have been reported and serine proteases also appear to contribute to IL-1β processing in addition to caspase-1 [148,149]. NETs have also been linked to inflammasome activation [150]. Interestingly, NETs promote the NLRP3 inflammasome activation in macrophages and the resulting IL-1β can enhance the NET formation by neutrophils indicating a feed-forward loop of anti-fungal effector mechanism activated by the NLRP3 inflammasome at the site of infection [150,151].

Surprisingly, *Nlrp10*$^{-/-}$ mice have also been shown to display increased susceptibility, higher fungal burdens in systemic model of candidiasis in an intravenous model of infection [117]. *Nlrp10*$^{-/-}$ BMDM produced comparable levels of IL-1β to WT, yet, the contribution of NLRP10 appeared to be from the hematopoietic compartment [117]. On one hand, this stresses the highly specific

role of NLRP3 inflammasome in innate immune cells. On the other, these studies highlight the contribution of different NLRs such as NLRP3, NLRC4 and NLRP10 towards the inflammatory response to *C. albicans* in whole organism infection models [84,117,131]. An intriguing point to note is that NLRP10 does not have an LRR domain and may not directly detect a *C. albicans* associated ligand. In this case, it would be interesting to examine whether NLRs physically interact with each other or one NLR can activate the other NLRs through cell autonomous/non-autonomous mechanisms. Of note, NLRP3 and NLRC4 co-localize and are both required for IL-1β production in response to *S. typhimurium* [152]. It will be also be important to determine how NLRP3, NLRC4 and NLRP10 inflammasomes contribute to the production of other IL-1 family cytokines such as IL-1α, IL-18 or IL-33 in response to *C. albicans*. IL-1α$^{-/-}$ and IL-18$^{-/-}$ mice are more susceptible to intravenous infection of *C. albicans* which can lead to disseminated candidiasis [126,153]. IL-18 has also been demonstrated to induce IFNγ and TNF production in human blood cells in response to *C. albicans* [121]. Similarly, IL-1α is important to drive the production of cytokines such as IL-6, KC and IFNγ in in response to heat-killed *C. albicans* in macrophages and splenocytes respectively [126]. IL-33 also enhances the recruitment, microbicidal, phagocytic ability of neutrophils, promotes the clearance of *C. albicans* and pre-administration of IL-33 protects mice from fungal overgrowth and mortality [154]. However, the activation of IL-33 may not necessarily be dependent on inflammasome-mediated proteolysis and the full-length protein may be bioactive [155].

Together, we have demonstrated that NLRP3, but not NLRC4 inflammasome mediates IL-1β production to *C. albicans* in innate immune cells. Supporting our *in vitro* data, *in vivo* results from Dr. Hise group clearly shows the crucial role of NLRP3 inflammasome and NLRC4 particularly in the mucosal, stromal compartment in anti-fungal responses in an oral infection model[66,135]. These NLR inflammasomes induce anti-microbial peptides, neutrophil recruitment and development of Th17 immunity [135]. IL-1β production by macrophages was higher in response to germ tube stages of *C. albicans* compared to hyphal forms, consistent with other studies while the dendritic cells responded well to all forms [134,142]. The inflammasome activation and IL-1β production in response to *C. albicans* was dependent on the direct recognition and phagocytosis of the fungus and not indirectly through uric acid crystals released by dying cells in the culture. This led us to examine the role of phagocytic CLRs in fungal recognition and inflammasome activation and these results are presented in chapter III.

Materials and Methods

Formalin fixed preps of *C. albicans*: These were prepared in the Hise lab as previously described [66]. Briefly, *Candida albicans* strain GDH2346, a clinical strain originally isolated from a denture stomatitis patient [156] was used for *in vitro* studies. Stocks were maintained on Sabouraud Dextrose (SD) agar at 4°C. For different morphological forms, *C. albicans* was grown in RPMI + 10% fetal bovine serum (FBS) for 2, 4, or 6 hr (early, mid, or late germ tube formations) or for 24 hr for fully hyphal forms. Fungal cells were washed in sterile PBS, fixed in 1% formaldehyde for 10 mins, washed with sterile PBS for three times before re-suspending in fresh sterile PBS. Fixed preps were stored in -80C until use.

Mice: Mice were maintained and bred at UMASS Medical School in accordance with the Institutional Animal Care and Use Committee (IACUC). *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$, *Asc*$^{-/-}$ and *Casp1*$^{-/-}$ mice were from Millennium Pharmaceuticals. C57BL/6 and 129X1/SvJ mice were from Jackson laboratories.

Cell culture: Bone marrow progenitor cells were flushed out using PBS, pelleted, depleted of RBCs using the RBC lysis buffer (Sigma), resuspended in media depending on the required cell type as described below. For bone marrow derived macrophages (BMDM), progenitor cells were cultured in DMEM (Cell gro) with 10% heat inactivated fetal bovine serum (FBS-Atlas Biologicals), Pen-strep (Gibco) and 20% L929 supernatant (in house preparation) as the source of MCSF. Media was changed on day 3, 6 and 8 and cells were used for experiments between day 8-10. For bone marrow derived dendritic cells (BMDC),

progenitor cells were counted and plated at 8 x 10^6 cells/ 25cm^2 dish in RPMI with 10% heat inactivated serum, Pen-strep, 20ng/ml recombinant GM-CSF (Peprotech) and 50µM β-mercaptoethanol (Sigma) and cultured for 9-10 days as previously described [157]. For thioglycollate elicited peritoneal exudate cells (PECs), mice were injected intraperitoneally with 3ml of thioglycollate (Remel), were sacrificed on day 4, the peritoneal cavity was flushed with PBS containing 2% FBS to collect all cells. Cells were pelleted, RBCs depleted using RBC lysis buffer (Sigma), counted and used for experiments in supplemented DMEM culture media similar to BMDM. Density-gradient centrifugation using Ficoll Hypaque (GE Healthcare) was used to isolate fresh PBMCs from human blood. PBMCs and THP1 cells were maintained in RPMI with 10% FBS, Penstrep and additional supplements of sodium pyruvate (Gibco) and 50µM β -mercaptoethanol. THP1 cells were differentiated with 0.5µM Phorbol Myristate Acetate (PMA from Sigma) for 3h, washed with PBS, before using them for experiments.

Experiments: For ELISAs, cells were plated in 96 well plates at 1 x 10^6 cells/ml. BMDM, BMDC, PECs, THP1 cells or PBMCs were primed with LPS 200ng/ml for 3h and then stimulated with different formalin fixed preps of *C. albicans* (10^9/ml) at a vol:vol dilution of 1:5 in antibiotic free culture media for 6h. For controls, cells were stimulated with canonical inflammasome activators such as 1µg/ml pdAdT (Sigma) or 250µg/ml silica (MIN-U-SIL-15 from US Silica) for 6h, 10 µM nigericin (Invivogen) or 5mM ATP (Sigma) for 1h after priming. Where mentioned, primed

cells were pretreated with indicated concentrations of cytochalasin D (Sigma), uricase (Elitek-Sanofi Aventis), or 10μM z-YVAD-fmk (Calbiochem) for 0.5h, before stimulation with *C. albicans*. Cytokine levels were measured in triplicate and the presented data are representative of two or three independent experiments.

For western blot analysis, cells were plated at 2 x 10^6 cells/ well in 12 well plates in serum free, antibiotic free medium, primed and stimulated as described above. Supernatant proteins were precipitated with methanol and chloroform as previously described [127]. Briefly, supernatants were mixed with equal volume of methanol, 0.25 volumes chloroform, vortexed and centrifuged at 10,000 rpm for 10 min. The upper methanol layer was discarded, and the protein interphase was further re-suspended in same volume of methanol used in the previous step and centrifuged at 10,000 rpm for 5 min. The supernatant was discarded, pellet dried at 55°C for about 15-20 min and then re-suspended in 1X laemli buffer with 100mM DTT and boiled for about 10 min. Cells were lysed with 1% NP-40 lysis buffer and Bradford assay was performed to normalize the amount of protein. Samples were mixed with 4X SDS loading buffer, boiled for about 10 min and were run on 12% SDS-PAGE and transferred to nitrocellulose membrane. Western blotting was performed with the antibodies to caspase-1 p10 (sc-514, Santa Cruz Biotechnology), IL-1β (AF-401-NA, R&D Systems, 3ZD clone from National Cancer Institute Biological Resources Branch).

ELISA: BD Biosciences kit was used to measure the amount of IL-1β present in cell culture supernatants according to manufacturers' instructions.

Chapter III:

Inflammasome responses to β-glucans and *Candida albicans*

Chapter III: Copyright information

Most of the data and subject content of this chapter is extracted from the following manuscript/publication:

Ganesan S, Rathinam VAK, Bossaller L, Army K, Kaiser WJ, Mocarski ES, Dillon CP, Green DR, Mayadas TN, Levitz SM, Hise AG, Silverman N, Fitzgerald KA. Caspase-8 modulates Dectin-1 and CR3 driven IL-1β production in responses to β-glucans and *Candida albicans* (*Submitted to Journal of Immunology. In revision.*)

Vijay A. K. Rathinam performed the western blots shown in figure 12C.

Chapter III: Inflammasome responses to β-glucans and *Candida albicans*

Abstract

Inflammasomes are central mediators of host defense to a wide range of microbial pathogens. The NLRP3 inflammasome plays a key role in triggering caspase-1 dependent IL-1β maturation and resistance to fungal dissemination in *Candida albicans* infection. One major component of fungal cell walls that is known to trigger IL-1β secretion in both murine as well as human immune cells are the β-glucans. In this study, we sought to determine the contribution of β-glucans to *C. albicans*-induced inflammasome responses in mouse dendritic cells. We show that the NLRP3-ASC-caspase-1 inflammasome is absolutely critical for IL-1β production in response to β-glucans. Interestingly, we also found that both Complement Receptor 3 (CR3) and dectin-1 play an essential role in coordinating β-glucan-induced IL-1β processing as well as a moderate cell death response. In addition to the essential role of caspase-1, we identify an important role for the pro-apoptotic protease caspase-8 in promoting β-glucan-induced cell death and NLRP3 inflammasome-dependent IL-1β maturation. Complement Receptor 3 and caspase-8 was also required for cell death response and NLRP3 dependent IL-1β production in response to heat killed *C. albicans*, but not the live fungus. Together, these results define the importance of dectin-1, CR3 and caspase-8, in addition to the canonical NLRP3 inflammasome, in mediating β-glucan and *C. albicans* induced innate responses. Collectively, these findings establish a novel link between β-glucan recognition receptors and the

inflammatory proteases caspase-8 and caspase-1 in coordinating cytokine secretion and cell death in response to immunostimulatory fungal components.

Introduction

The pro-inflammatory cytokine IL-1β is an integral component of anti-fungal immune defenses, as discussed in the previous chapters. Mouse models of candidiasis and human *ex-vivo* studies have uncovered a critical role for IL-1β in triggering T-cell mediated production of IL-17 which is protective against fungal infection [121,123,125,126,130,138,158,159]. We and others have shown important roles for NLRP3, NLRC4 and NLRP10 in different aspects of anti-fungal immune responses [117,130,131,160]. In this chapter, we explore the molecular requirements and mechanisms by which dendritic cells activate the inflammasome in response to *Candida albicans*.

Infection by *Candida albicans* and other fungal pathogens is readily detected by an array of pattern recognition receptors as discussed in chapter I. Results in chapter II using formalin fixed *C. albicans*, suggested that the pathogen does not need to be viable to activate inflammasome and IL-1β. Detection of the fungal cell wall is the primary mechanism by which innate system senses infection [161,162]. Hence, to begin understanding the mechanisms underlying inflammasome activation, we focused our efforts on defining the contribution of fungal cell wall components. 90% of the yeast cell wall is comprised of polysaccharide structures and includes mannans, mannoproteins, β-glucans and chitin [163]. The outer matrix portion of the cell wall comprises of highly glucosylated proteins of which 90% of their molecular weight is mannans [26]. These proteins are glycosylphosphatidylinositol (GPI) anchored to the inner

β(1, 3) glucans through the β(1, 6) glucans. The skeletal framework and the inner layer of the fungal cell wall is made of β(1, 3) glucans covalently linked to β(1, 6) glucans and chitin [26].

β-glucans: Immunomodulators and immunostimulatory component of *C. albicans*

Interestingly, even though β-glucans are not the outer most part of the cell wall, they are considered highly immunostimulatory. A landmark study by Gantner, B. *et al*., showed that budding scar left during mother-daughter cell separation exposes this inner cell wall and allows its recognition by the mammalian pattern recognition receptors, while the hyphal forms shield their inner wall [164] [161]. In addition, detection of β(1,3) glucan and antibodies to β(1,3) glucan in the plasma are considered biomarkers of candidiasis, and many diagnostic tests that exploit this finding are under clinical development [165-167].

β-glucans also occur naturally in plants, bacteria, mushrooms, fungi but the human body does not produce enzymes to digest this polysaccharide. However, it is effectively eliminated from circulation and intestine by sampling and transfer of these particles by specialized M (microfold) cells of Peyer's patches to macrophages and DC, which underlie the intestinal epithelium [168]. These innate cells induce cytokines to activate T, NK cells and neutrophils amounting to mucosal and systemic immune responses [168]. Glucans can be categorized depending on length, branching, α or β isomers and their immunostimulatory capacity depends on a variety of factors such as, purity,

particulate nature, cell type involved, molecular weight, cellular receptor engaged, etc. β-glucans have been considered effective as immune supplements and possible vaccine adjuvants, however the precise molecular mechanisms have not been clear. Studies on inflammasome responses to β-glucans are key to advancing our understanding of host fungal sensing pathways and their mode of action as immunomodulators.

In this study, we investigated the molecular and mechanistic details of inflammasome responses to *C. albicans* by employing β-glucans and heat-killed *C. albicans* in conjunction with live *C. albicans*. While previous studies have yielded contrasting observations regarding the role of NLRP3 in β-glucan triggered inflammasome responses [169-171], we found the canonical NLRP3-ASC-caspase-1 inflammasome to be essential in mediating IL-1β production by both β-glucans and *C. albicans* (live or heat-killed) in mouse dendritic cells. The receptors linking β-glucan to inflammasome activation were found to be dectin-1 and Complement Receptor 3 (CR3). We also uncovered an essential role for CR3 in mediating IL-1β production to heat-killed (HK) and to a lesser extent to live *C. albicans*. Moreover, Dectin-1 and CR3 appear to also function non-redundantly in a less-well characterized cell death response to β-glucans and HK *C. albicans*.

Potential role of caspase-8 in inflammasome functions

A growing body of literature points to the existence of diverse and complex molecular platforms for inflammasomes including other caspases such as caspase-11 and -8 [128,169,172-174]. Gringhuis, S. I. *et al.* (2012) showed that IL-1β triggered by β-glucan and some heat-killed strains of *C. albicans* was caspase-8 dependent, but independent of caspase-1 in human cells [175]. Further, recent studies show that caspase-8 gets co-localized with ASC specks and is involved in inflammasome function during Salmonella infection or upon treatment of cells with pro-apoptotic, chemotherapeutic drugs or Fas ligand [172,176,177]. *Casp8*$^{-/-}$ mice are embryonic lethal due to RIP3-dependent necrosis [178]. This lethality is rescued by the genetic deletion of RIP3 [179,180]. Using the viable *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ double knockout and the *Rip3*$^{-/-}$ single knockouts as a control, we present evidence for caspase-8 involvement in β-glucan-triggered IL-1β production as well as cell death in mouse dendritic cells. Comparing the molecular requirements of inflammasome response between β-glucan and heat-killed HK *C. albicans* with that of live *C. albicans*, we identify a differential requirement for CR3, dectin-1 and caspase-8 in IL-1β and cell death responses. We attribute this differential requirement to higher exposure of β-glucans by HK *C. albicans* compared to that of live *C. albicans*.

Our findings suggest a β-glucan-specific dectin-1/CR3/caspase-8 pathway that synergizes with the canonical NLRP3-ASC-caspase-1 pathway for optimal inflammasome responses. This study gives rise to new insights into the

differences in host immune sensing of fungal molecular patterns such as β-glucans in contrast to live *C. albicans* infection.

Results

As described in chapter II, *C. albicans* induced IL-1β is mediated by the NLRP3-ASC-caspase-1 inflammasome [130,160]. To begin investigating the mechanism of inflammasome activation and the immunostimulatory component(s) of *C. albicans* responsible for this activity, we used heat-killed *C. albicans*. This also provided a way to block the virulence mechanisms of the fungus including filamentation and to capture the innate responses against the yeast and germ-tube forms. Interestingly, heat-killed *C. albicans* triggers levels of IL-1β production similar to that seen with live fungi, and this response is also completely dependent on the NLRP3-ASC-caspase-1 inflammasome in Pam2CSK4 primed mouse dendritic cells (Figure 1A and 1B). Even though *C. albicans* is efficient at inducing the synthesis of pro-IL-1β and inflammasome components, Pam2CSK4 priming provided a way to normalize the pro-IL-1β levels, especially when comparing cells of different genotypes, so that the phenotype could be attributed to signal 2 (inflammasome activation and pro-IL-1β processing). Of note, our previous studies utilized the *Casp1*$^{-/-}$ mice that carried the *Casp11* passenger mutation associated with the 129 mouse strain [128,129]. To carefully examine the role of caspase-1, the experiments in this chapter used *Casp1*$^{-/-}$ mice expressing caspase-11 from a bacterial artificial chromosome [128].

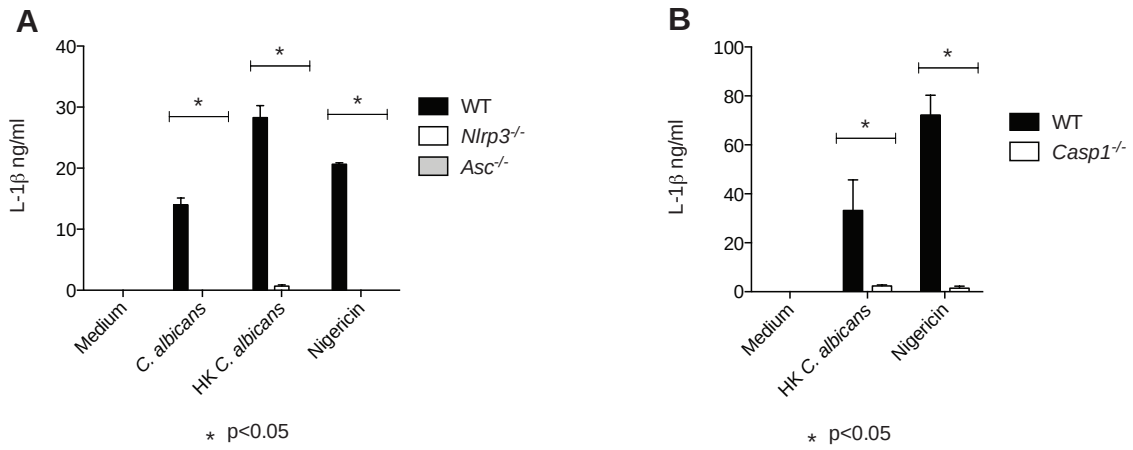


Figure 1. NLRP3-ASC-caspase-1 inflammasome is essential for *Candida albicans* induced IL-1β. Pam2CSK4 primed BMDC from *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ (A) *Casp1*$^{-/-}$ (B) and C57BL/6 mice were stimulated with live or heat-killed *C. albicans* at 10:1 for 6h, nigericin for 1h. Amount of IL-1β released in the supernatants was measured by ELISA (A, B).

β-glucan has been considered to be the most immune-stimulatory Pathogen Associated Molecular Pattern (PAMP) that is part of fungal cell walls and is likely exposed in heat-killed fungi [181]. Hence we sought to determine the contribution of the glucose polymers, β-glucans to inflammasome activation by *C. albicans*. Two representative β (1,3) glucans employed in this study are: (1) curdlan, a high molecular weight, water insoluble β(1,3) glucan derived from the bacterium *Alcaligenes faecalis;* and (2) dispersible Whole fungal Glucan Particles derived from the cell walls of the baker's yeast *Saccharomyces cerevisiae* (WGP agonist). The composition of the *S. cerevisiae* cell wall is highly similar to that of *C. albicans* [32,182]. β-glucan induced IL-1β production in mouse innate immune cells, such as thioglycollate-elicited peritoneal macrophages (Figure 2A), bone marrow derived macrophages (BMDM; Figure 2B) and dendritic cells (BMDC; Figure 2C), in the absence or presence of Pam2CSK4 (TLR2) priming. Dendritic cells produce markedly higher levels of IL-1β in response to β-glucans, compared to macrophages (Figure 2C). Pam2CSK4 priming greatly boosted this response. All remaining experiments in this study were performed in Pam2CSK4 primed dendritic cells, unless otherwise indicated. Further, results from chapter I indicated that dendritic cells are more efficient at sensing different morphological stages of *C. albicans* and hence would be the ideal system to understand the mechanisms by which inflammasome is activated in response to *C. albicans*.

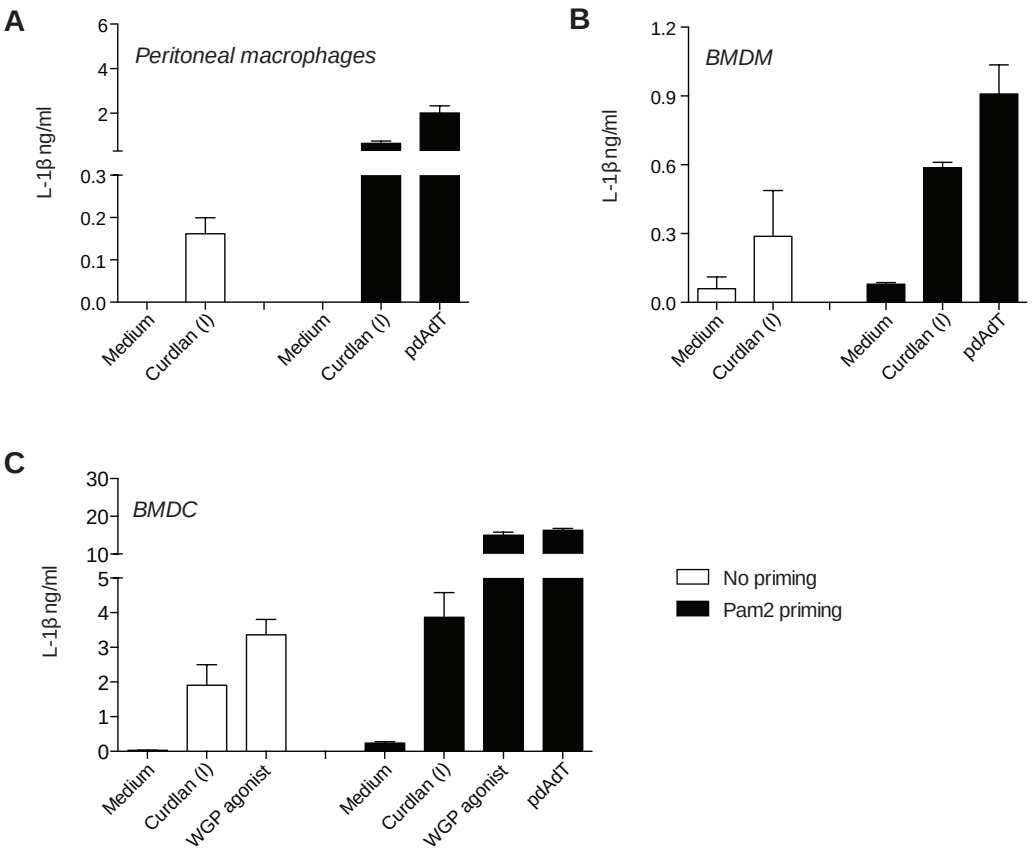


Figure 2. β -glucans induce IL-1β production in mouse immune cells. Peritoneal macrophages (A), BMDM (B) or BMDC (C) from C57BL/6 mice were unprimed or primed with Pam2CSK4 with 3h and stimulated with curdlan (I), dispersible fungal Whole Glucan Particles (WGP agonist) for 24h or pdAdT for 6h. Amount of IL-1β released in the supernatants was measured by ELISA (A-C).

We then set out to determine if β-glucan induced IL-1β production is also mediated by the NLRP3-ASC-caspase-1 inflammasome. To test this, Pam2CSK4-primed wild-type (WT), *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *Casp1*$^{-/-}$ BMDC were stimulated with curdlan and WGP agonist. IL-1β secretion was completely abrogated in *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *Casp1*$^{-/-}$ dendritic cells (Figure 3A and B) similar to HK *C. albicans*.

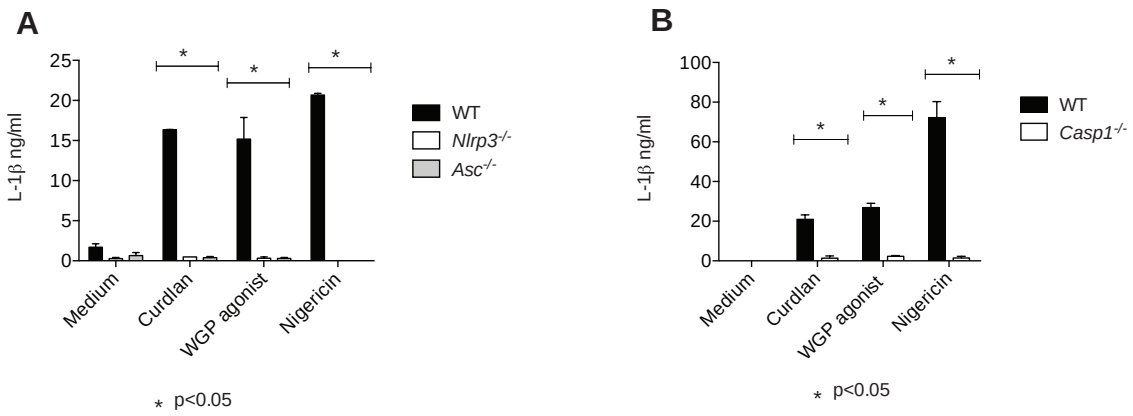


Figure 3. NLRP3-ASC-caspase-1 inflammasome is essential for β-glucan induced IL-1β. Pam2CSK4 primed BMDC from *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ (A) *Casp1*$^{-/-}$ (B) and C57BL/6 mice were stimulated with β-glucans for 24h or nigericin for 1h. Amount of IL-1β released in the supernatants was measured by ELISA (A, B).

To further evaluate the role of caspase-11 in *C. albicans* or β -glucan-induced IL-1β production, *Casp11*$^{-/-}$ cells were tested. Caspase-11 has been found to be essential for inflammasome activation in response to LPS sensing and gram negative bacteria [128,174]. As seen in figure 4, caspase-11 was not required for IL-1β production in response to β-glucans, live or heat-killed (HK) forms of the fungus (Figure 4A). These results show a critical and specific role for the caspase-1 in *C. albicans* as well as β-glucan induced IL-1β.

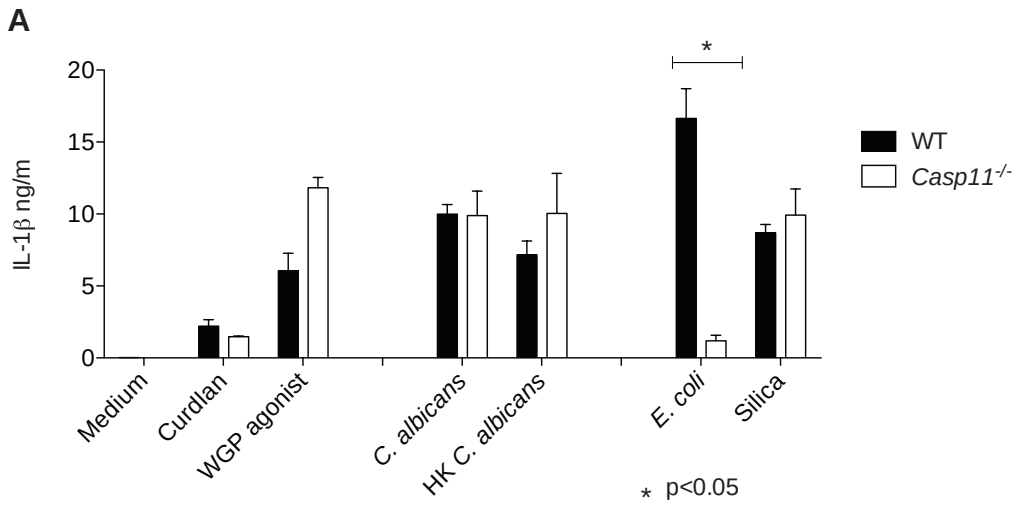


Figure 4. Caspase-11 deficiency has no effect on β-glucan or *C albicans* induced IL-1β. Pam2CSK4 primed BMDC from C57BL/6 or *Casp11*$^{-/-}$ mice (A) were stimulated with curdlan, WGP agonist, silica, live or heat killed *C. albicans* at 10:1 for 6h or EHEC for 24h. Amount of IL-1β released in the supernatants was measured by ELISA (A).

In order to ensure that the responses elicited by the curdlan and WGP preparations were specifically due to β-glucans, cells were pre-treated with a well-established antagonist, soluble β-glucan (WGP antagonist), to competitively bind and block signaling through host β-glucan receptors. WGP antagonist-treated cells were then stimulated with curdlan from two independent sources (Invivogen or Wako Chemicals) and IL-1β production was assayed. Curdlan is capable of triggering the synthesis (signal 1) and maturation (signal 2) of pro-IL-1β in dendritic cells, as observed in the experiments with unprimed cells shown in Figure 2. In the unprimed condition, WGP antagonist treatment led to an attenuated curdlan-induced IL-1β response (Figure 5A). Since the WGP antagonist could be blocking either of the two signals, the effect of WGP antagonist specifically on signal 2 was addressed by priming the cells with a non-β-glucan ligand, Pam2CSK4, to induce signal 1. WGP antagonist also attenuated IL-1β production in the primed BMDCs specifically in response to curdlan, but not pdAdT, (an activator of the AIM2 inflammasome) (Figure 5A), indicating that WGP antagonist inhibits β-glucan induced inflammasome activation and IL-1β release (signal 2). Together, these results highlight that receptor mediated sensing of β-glucan is important for inflammasome activation.

Considerable research is devoted to water-soluble chemical derivatives of β-glucans to improve the ease of their use in clinical applications. One such water-soluble derivative of curdlan, carboxy-methylated-curdlan (or CM-curdlan) was tested for its inflammasome triggering activity in BMDC. CM-curdlan induced IL-1β production was very weak compared to the same concentration of

unmodified curdlan in unprimed as well as Pam2CSK4-primed cells (Figure 5B), indicating water soluble CM-curdlan is a weaker activator of inflammasomes. Poor immune-stimulatory activity of CM-curdlan has also been observed *in vivo*, where pre-injection of curdlan, but not CM-curdlan protects mice from lethal *E. coli* infection [183]. The weak ability of CM-curdlan to trigger IL-1β production indicates that the particulate properties of β-glucans are important for triggering inflammasome activation. In line with that idea, chitosan, a deacetylated derivative of chitin, induces higher IL-1β in macrophages in particulate form compared to its soluble counterpart [184].

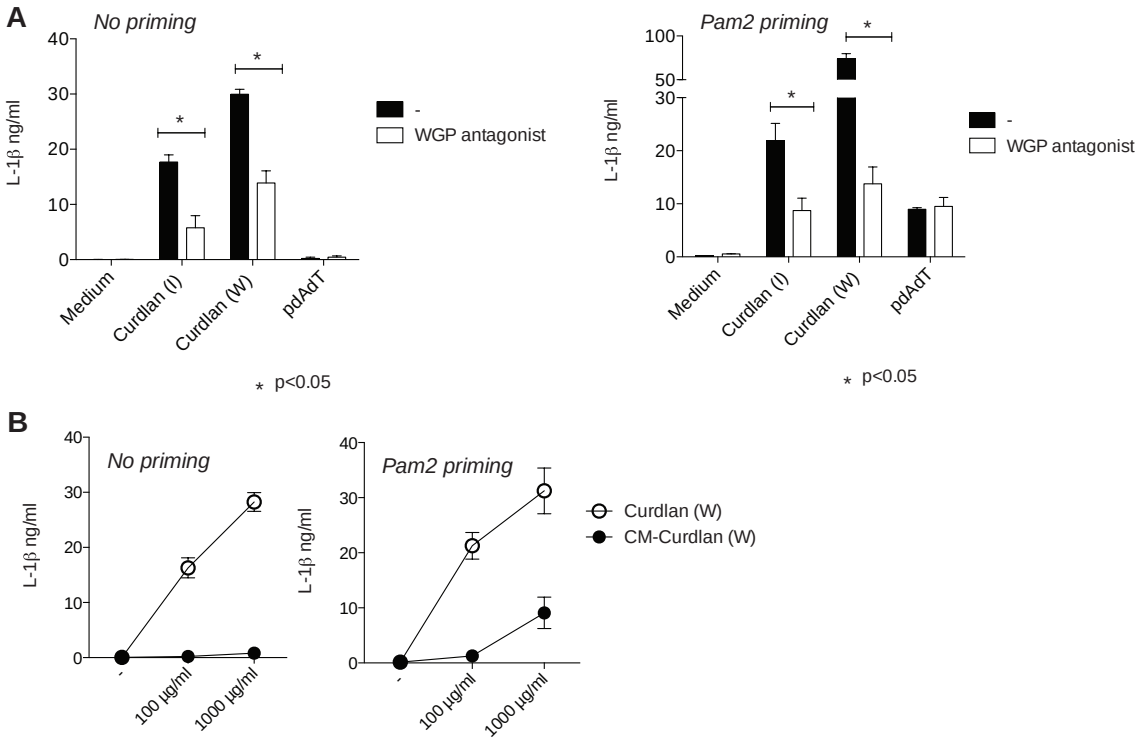


Figure 5. Sensing of particulate β -glucan triggers IL-1β production. Unprimed or Pam2CSK4 primed BMDC from C57BL/6 mice (A, B) were stimulated with curdlan (I or W) for 24h, pdAdT for 6h in the presence or absence of the soluble WGP antagonist (A). Cells were stimulated with curdlan or carboxymethylated curdlan from Wako Chemicals (W) at increasing concentrations (B). Amount of IL-1β released in the supernatants was measured by ELISA (A, B). Statistical analysis was performed on results in panel A as described in Materials and Methods section.

Since the particulate form of curdlan was more immunostimulatory and dendritic cells are highly phagocytic, we determined whether inflammasome responses to β-glucans require their uptake. Cells were pretreated with cytochalasin D, an inhibitor of actin re-arrangement and phagocytosis. Cytochalasin D severely reduced β-glucan triggered IL-1β production (Figure 6A), showing that ingestion of curdlan is required for the triggering of IL-1β release, similar to other particulate inflammasome activators. Interestingly, β-glucans have been known to cause apoptosis of cancer cells [185]. However the mechanism of β-glucan mediated cell death has not yet been characterized. Using a luciferase-based assay to measure intracellular ATP as an indicator of cell viability, we observed that β-glucans triggered a moderate decrease in the viability of dendritic cells (Figure 6B). Phagocytosis is essential for this process similar to that of the particulate stimulus silica (Figure 6B). Of note, when we measured lactate dehydrogenase (LDH) in the supernatants, no detectable increase was observed in β-glucan stimulated cells, unlike nigericin treated cells (Figure 6C), suggesting that either the LDH assay was not as sensitive as the ATP based assay or that LDH may not be released in the cell death process observed here. LDH release indicates loss of membrane integrity and cells that undergo apoptotic death may maintain their intact membranes.

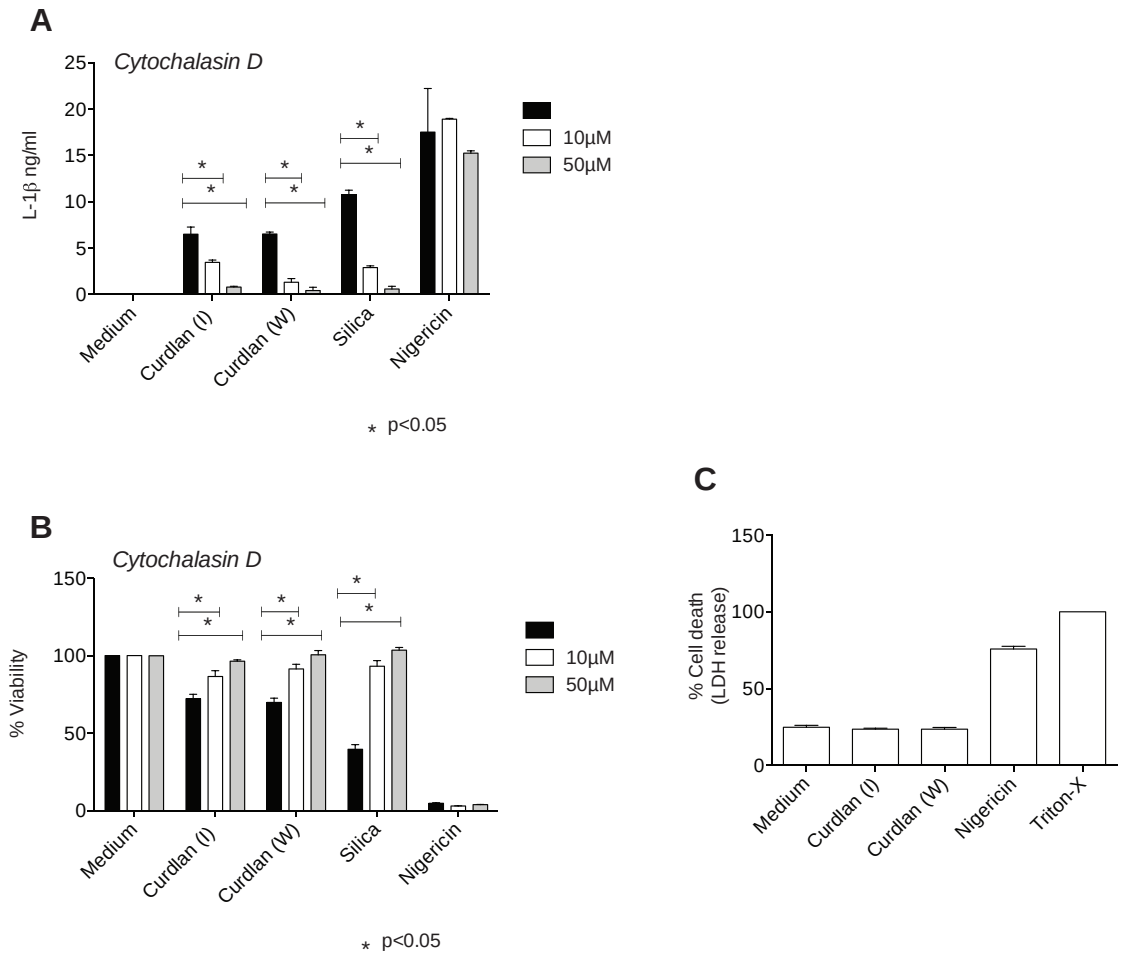


Figure 6. Phagocytosis of β-glucans is required for IL-1β production and cell death. Pam2CSK4 primed BMDC from C57BL/6 mice were pretreated with 50μM cytochalasin D for 0.5h and stimulated with curdlan (I or W) silica for 6h or nigericin for 1h. Amount of IL-1β released in the supernatants was measured by ELISA (A). Cell viability was assessed by cell titer-glo assay that measures intracellular ATP (B) and measuring LDH released in the supernatants (C). Statistical analysis was performed on results in panels A and B as described in Materials and Methods section.

The C-type lectin receptor family of proteins is considered the primary recognition and phagocytic receptors for fungal cell wall components, and dectin-1, in particular, plays an important role in anti-fungal immunity [31,38]. To identify innate immune receptors that couple β-glucan recognition to inflammasome activation, we first tested the role of dectin-1. Curdlan (I) induced IL-1β production was markedly decreased in Pam2CSK4-primed *Clec7a*$^{-/-}$ (dectin-1-deficient) BMDC (Figure 7A). Western blotting of precipitated supernatants from curdlan (I) stimulated cells reflected this decrease in the amount of processed IL-1β in *Clec7a*$^{-/-}$ BMDC (Figure 7B). Curdlan induced caspase-1 processing was also markedly reduced in *Clec7a*$^{-/-}$ BMDC, indicating that dectin-1 signaling is important for β-glucan induced caspase-1 activation (Figure 7B). CARD9 is an adaptor molecule that mediates NF-kB activation by many ITAM associated receptors including dectin-1 and is an indispensable component of anti-fungal innate immune responses in myeloid cells [186]. CARD9 mediates Th17 cell differentiation in response to *C. albicans* and dectin-1 agonists (adjuvants) serving as an important link between innate and adaptive immune responses [187]. Consistent with a role for dectin-1, IL-1β production by Pam2CSK4-primed *Card9*$^{-/-}$ BMDC was also decreased in response to curdlan (I) (Figure 7C). *Card9*$^{-/-}$ BMDC exhibited reduced IL-1β processing in response to curdlan (Figure 7D), indicating that the dectin-1-CARD9 signaling axis is critical for curdlan (I) induced IL-1β maturation.

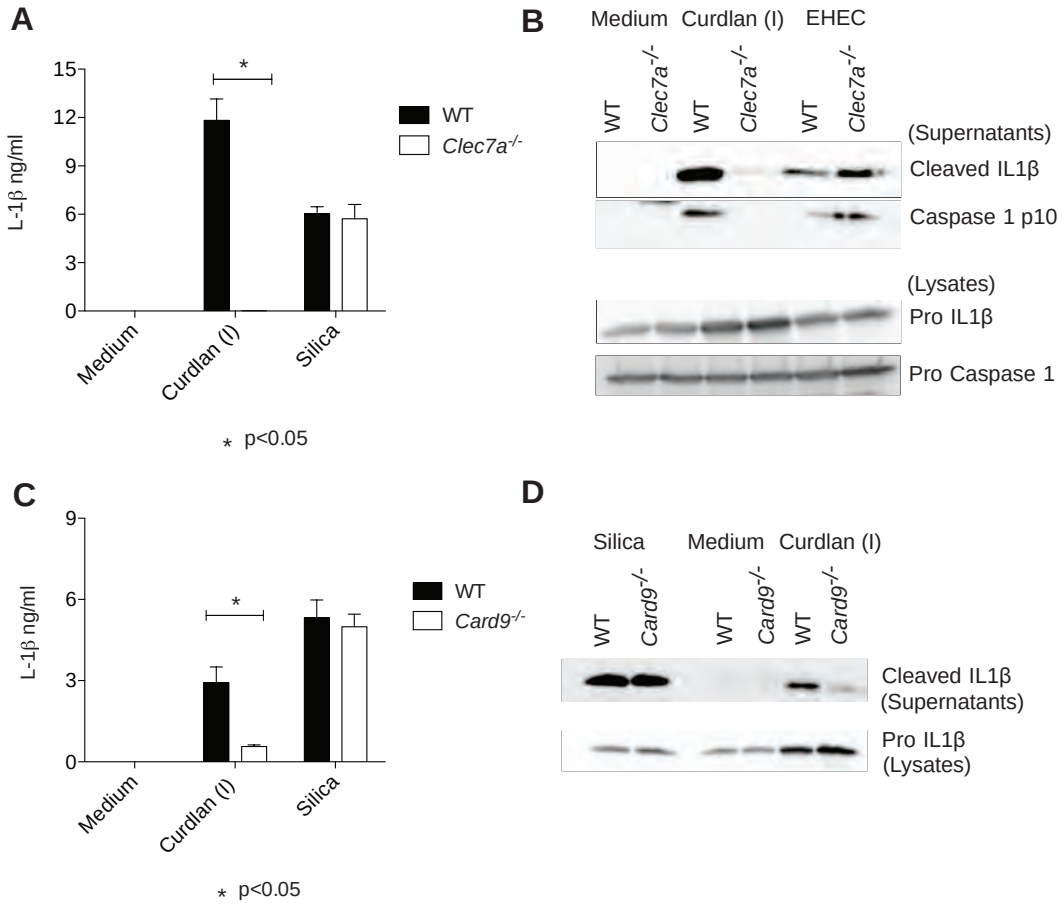


Figure 7. Dectin-1 and CARD9 signaling are important for β -glucan triggered IL-1β processing and release. Pam2CSK4 primed BMDC from *Clec7a*$^{-/-}$ (A, B) or *Card9*$^{-/-}$ (C, D) and C57BL/6 mice were stimulated with curdlan (I), EHEC or silica for 6h. Amount of IL-1β released in the supernatants was measured by ELISA (A, C). Cells were stimulated as indicated, lysates and precipitated supernatants were probed for processed IL-1β or caspase-1 p10 (B, D). Statistical analysis was performed on results in panels A and C as described in Materials and Methods section.

Another receptor that has been implicated in β-glucan recognition is CR3, an integrin composed of CD11b/CD18. In addition to binding the complement component iC3b, CR3 has a distinct lectin site for binding β-glucan [77,78]. Similar to other leukocyte integrins, upon receiving intracellular signals, CR3 undergoes conformational changes required for it to optimally bind its ligand. Recently, it was shown that dectin-1 activates inside-out signaling of CR3 that in turn promotes CR3 binding of *C. albicans* and subsequent neutrophil anti-fungal effector functions [81]. The role of CR3 in inflammasome activation generally and particularly in response to β-glucans or *C. albicans* has not been investigated to date. To test the role of CR3 in this pathway, Pam2CSK4-primed *Itgam*$^{-/-}$ (CR3-deficient) BMDC were stimulated with curdlan (I), and IL-1β release was measured. CR3-deficient BMDC (*Itgam*$^{-/-}$ BMDC) displayed severely impaired IL-1β production in response to curdlan (Figure 8A). Curdlan induced IL-1β processing was also drastically reduced in the *Itgam*$^{-/-}$ BMDC, while that induced by silica was unaffected (Figure 8B). Remarkably, curdlan triggered caspase-1 processing was also impaired in CR3 deficient cells, suggesting that CR3 is important for caspase-1 activation in response to β-glucans. The role of CR3 observed in caspase-1 activation and IL-1β production appears to be complement-independent, since all of these experiments were performed in serum-free media.

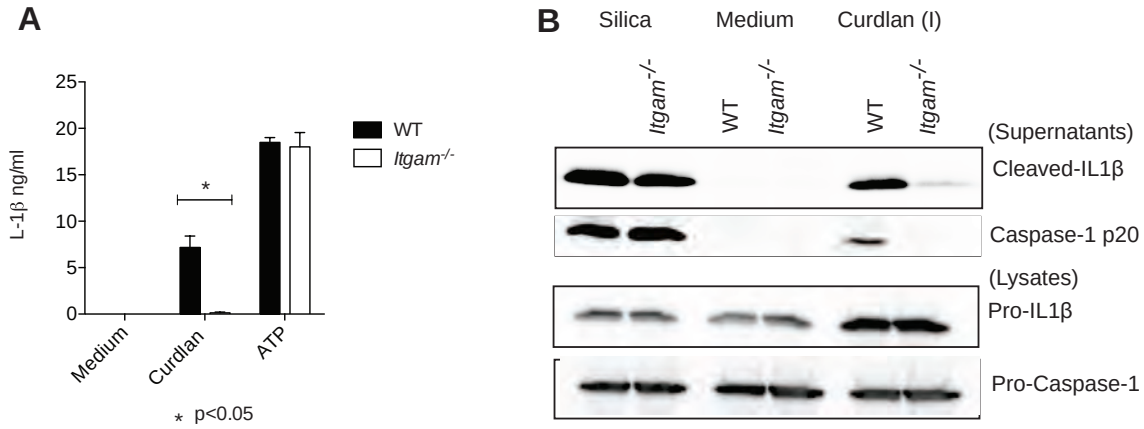


Figure 8. Complement Receptor 3 is critical for β -glucan induced inflammasome activation and IL-1β production. Pam2CSK4 primed BMDC from C57BL/6 or *Itgam*$^{-/-}$ mice (A, B) were stimulated with curdlan (I) for 6h or ATP for 1h. Amount of IL-1β released in the supernatants was measured by ELISA (A). Cells were stimulated as indicated, lysates and precipitated supernatants were probed for IL-1β or caspase-1 p20 (B). Statistical analysis was performed on results in panel A as described in Materials and Methods section.

Dectin-1 and CR3 deficiency also protected cells from β-glucan induced cell death (Figure 9A and B), indicating that these receptors not only trigger inflammasome activation and IL-1β processing, but also cell death. However, CARD9 deficiency did not affect β-glucan induced cell death (Figure 9C), indicating the specificity of this process. Further, this result suggests that while CARD9 mediates dectin-1 induced IL-1β processing, it is not required for dectin-1 activated cell death response. This hints that the dectin-1 signaling pathways diverge at the receptor level. Together, these results indicate an essential role for dectin-1 and CR3 pathways in innate immune responses to β-glucans.

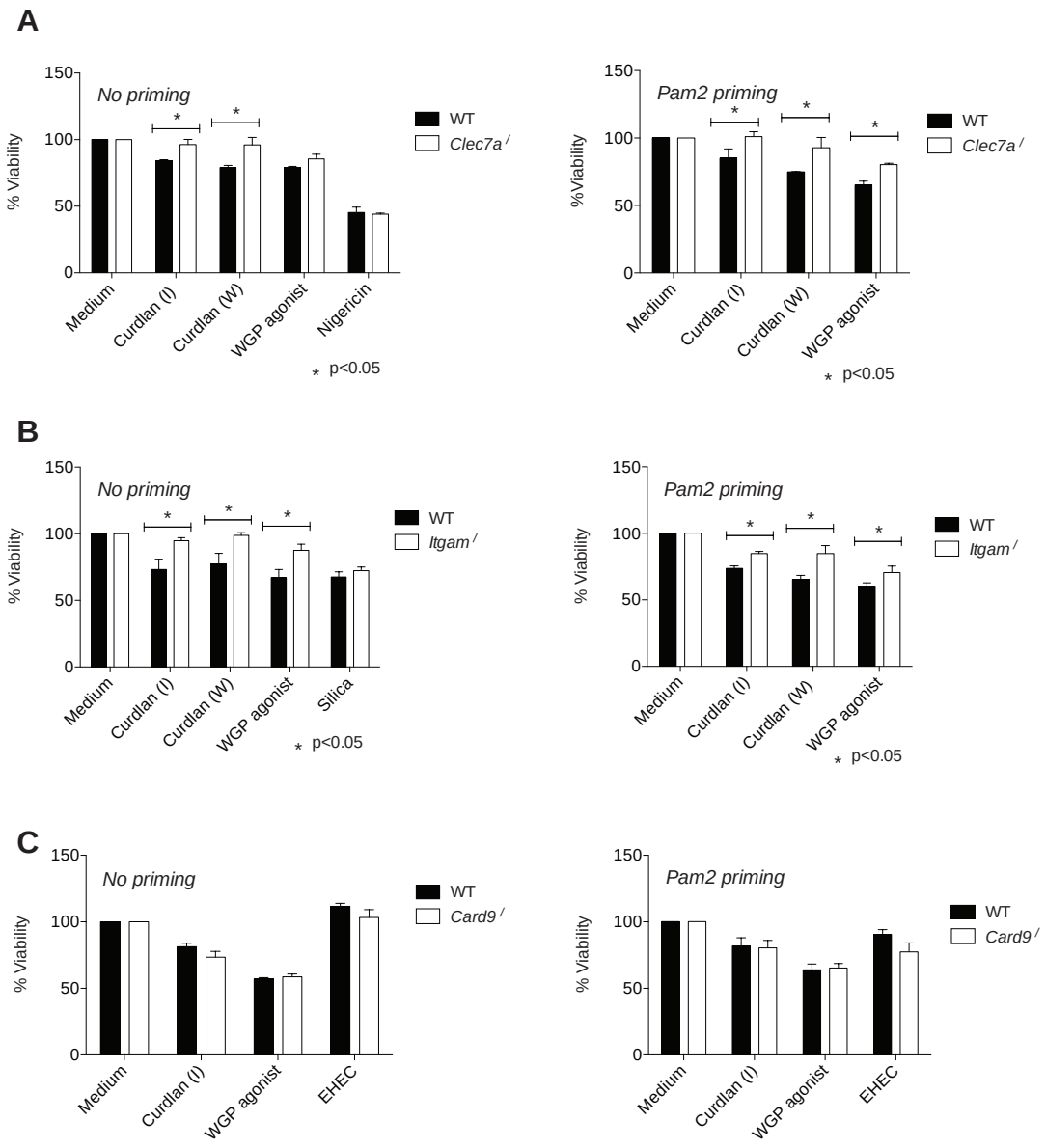


Figure 9. Dectin-1 and Complement Receptor 3, but not CARD9, mediate β-glucan induced cell death. Unprimed or Pam2CSK4 primed BMDC from *Clec7a*$^{-/-}$ (A), *Itgam*$^{-/-}$ (B), *Card9*$^{-/-}$ (C) and C57BL/6 mice were stimulated with curdlan (I or W), WGP agonist, silica for 6h or nigericin for 1h. Cell viability was assessed by cell titer-glo assay (A-C). Statistical analysis was performed on results in all panels as described in Materials and Methods section.

We have shown that dectin-1 is required not only for β-glucan induced IL-1β maturation, but also for dendritic cell death (Figures 7A and 9A). A recent study reported that dectin-1 mediated caspase-8 activation is essential for β-glucan induced IL-1β responses in human dendritic cells [169]. We have also linked caspase-8 to Fas ligand-induced IL-1β responses in mouse macrophages [172]. Thus, we investigated if caspase-8 is involved in β-glucan triggered IL-1β production as well as cell death in mouse BMDCs. To define the role of caspase-8 in this pathway, we utilized *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice which are viable [179,180], in contrast to the embryonically lethal *Casp8*$^{-/-}$ [178]. First, we stimulated BMDC from C57BL/6, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ and littermate *Rip3*$^{-/-}$ mice with curdlan, WGP agonist, pdAdT or nigericin and measured both the release and processing of IL-1β. Additionally, we treated the cells with Fas ligand-containing vesicles, as a positive control for caspase-8 dependent IL-1β production. We observed that caspase-8 deficiency reduced curdlan and WGP agonist mediated IL-1β production in Pam2CSK4 primed BMDC (Figure 10A). Curdlan from other sources such as Sigma or Wako chemicals also showed a similar phenotype in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ BMDC (Figure 10A). In contrast, the activation of the AIM2 inflammasome by pdAdT and NLRP3 inflammasome by nigericin was intact in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ BMDC. This effect was observed across various time points (Figure 10B). Notably, even though BMDM do not respond to β-glucan as robustly as BMDC, we also observed a similar reduction of IL-1β release in BMDM (Figure 10C).

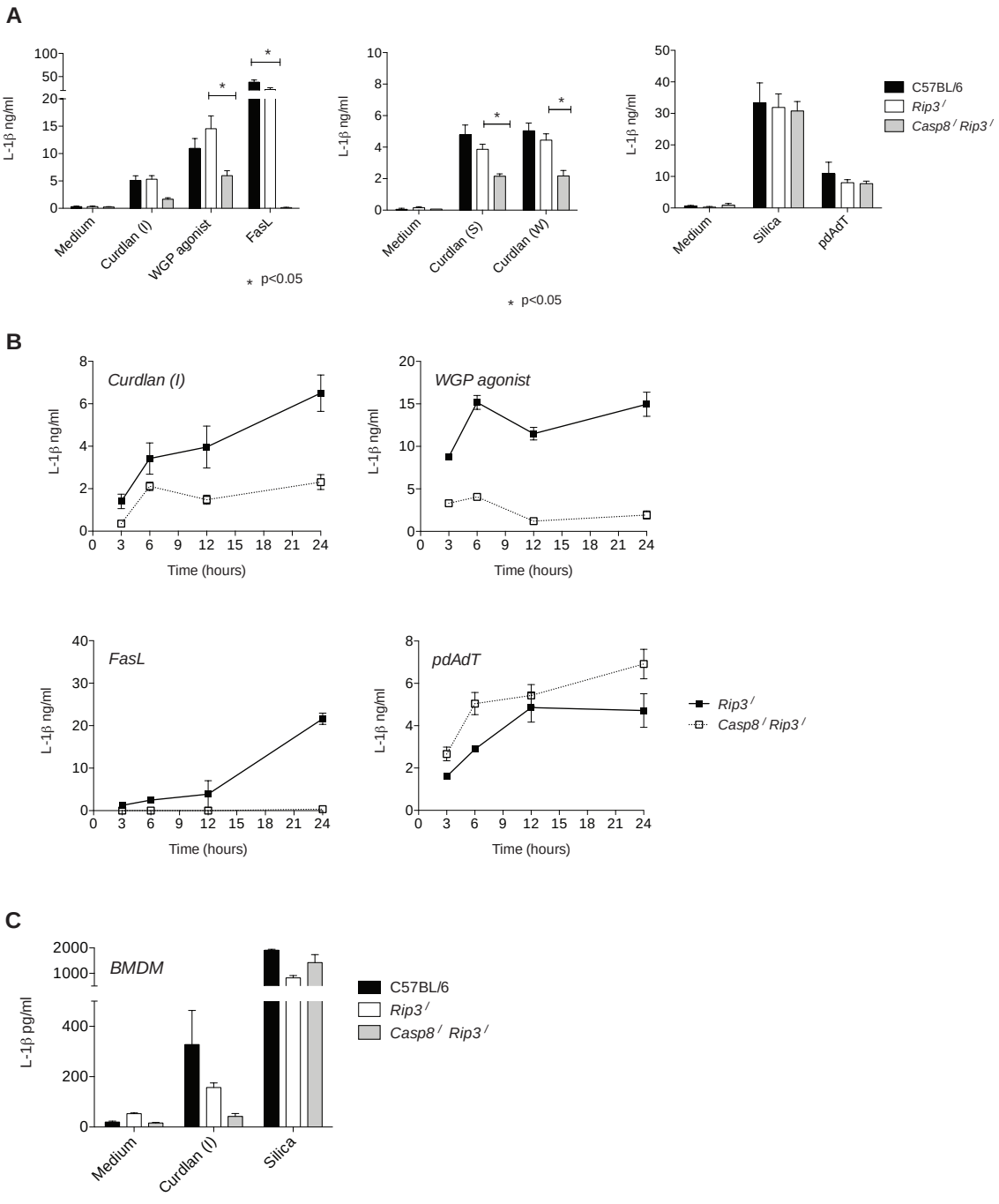


Figure 10. Caspase-8 is required for β-glucan induced IL-1β production. Pam2CSK4 primed BMDC (A, B) or BMDM (C) from C57BL/6, littermate *Rip3*$^{-/-}$, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice were stimulated with β-glucans for 6h or as indicated (BMDC) or 24h (BMDM), FasL, silica, pdAdT for 6h or nigericin for 1h. Amount of IL-1β released in the supernatants was measured by ELISA (A-C). Statistical analysis was performed on results in panel A as described in Materials and Methods section.

Many previous studies have suggested a role for caspase-8 in NF-kB activation in T cells, B cells and MEFs [188-190], yet this remains controversial [191,192]. Pro-IL-1β is a transcriptional target of NF-kB and hence, we first examined the impact of caspase-8 deficiency on pro-IL-1β synthesis in response to β-glucans. In the absence of Pam2CSK4 priming, pro-IL-1β mRNA levels were similar in WT, *Rip3*$^{-/-}$, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ BMDC stimulated with curdlan (Figure 11A), suggesting that caspase-8 deficiency does not affect curdlan-induced signal 1. Pro-IL-1β protein levels in the *Casp8*$^{-/-}$*Rip3*$^{-/-}$ DC were also comparable to that in WT or *Rip3*$^{-/-}$ cells (Figure 11B), when cells were primed with the TLR2 agonist Pam2CSK4, prior to curdlan (I) stimulation similar to most of the experiments in this study. These results indicate that caspase-8 does not play a role in β-glucan or lipopeptide triggered signal 1 (NF-kB mediated pro-IL-1β induction) and suggest a defect in signal 2 (inflammasome mediated pro-IL-1β processing).

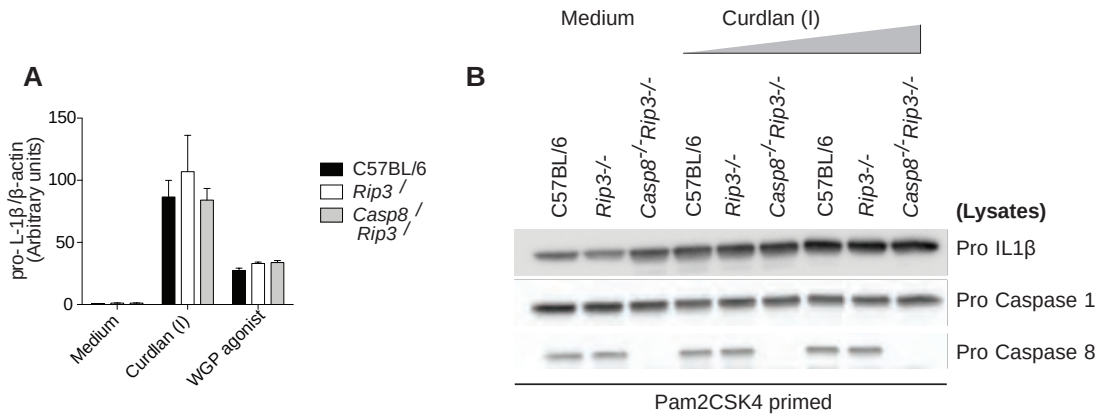


Figure 11. Role of caspase-8 in TLR2 and β-glucan induced pro-IL-1β synthesis. Unprimed BMDC from C57BL/6, *Rip3*$^{-/-}$, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice were stimulated with β -glucan ligands for 4h and pro-IL-1β mRNA levels was measured by qRT-PCR (A). Pam2CSK4 primed cells were stimulated with the indicated ligands for 6h and cell lysates were probed for IL-1β, caspase-1 and caspase-8 (B).

We then examined if *Casp8*$^{-/-}$*Rip3*$^{-/-}$ cells are deficient in IL-1β processing. As seen in figure 12A, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ BMDC exhibited reduced levels of processed IL-1β in the supernatants of stimulated cells compared to the control *Rip3*$^{-/-}$ BMDC. IL-1β processing detected in the supernatants of stimulated macrophages was also decreased in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ compared to WT and the littermate *Rip3*$^{-/-}$ cells (Figure 12B). Since caspase-1 was also found to be critical for β -glucan induced IL-1β production (Figure 3B), and caspase-8 is also required for this process (Figure 10A), we wanted to determine if caspase-8 mediates the activation and processing of caspase-1. Surprisingly, caspase-1 processing was intact in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ BMDCs, suggesting that caspase-8 regulates IL-1β processing independent of caspase-1 (Figure 12C).

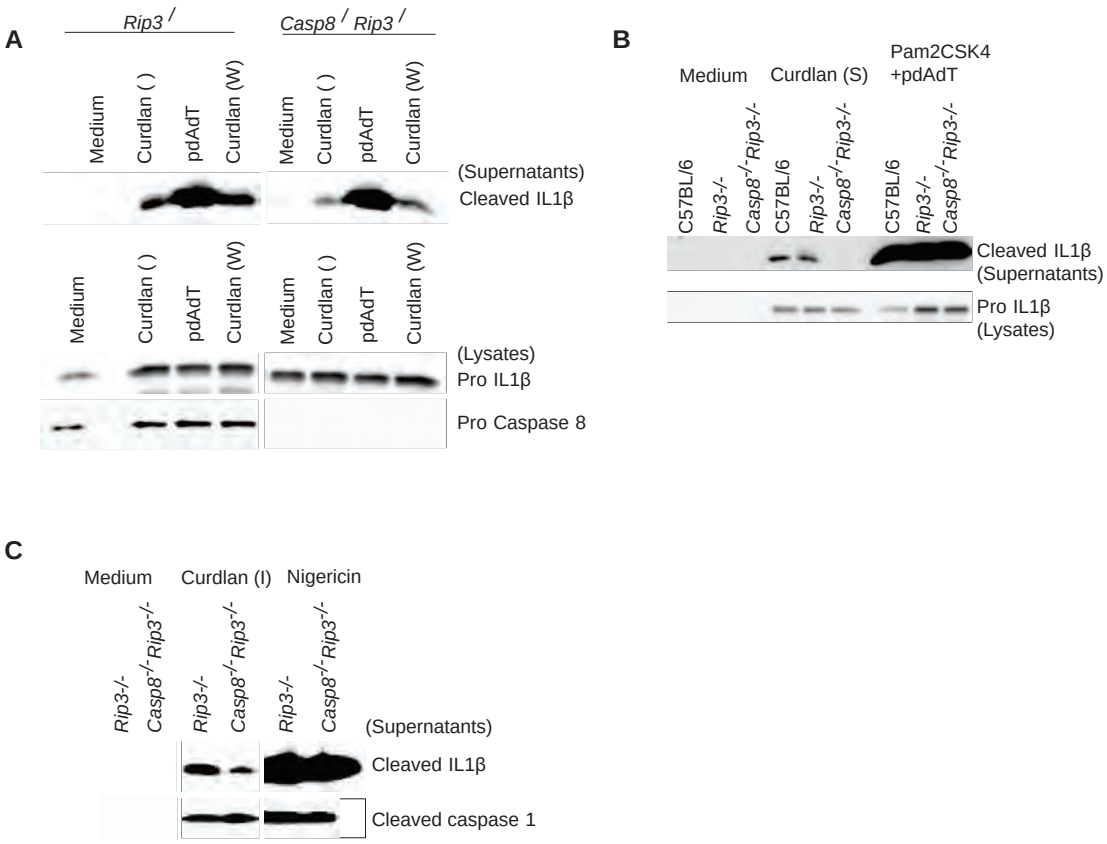


Figure 12. Caspase-8 deficiency reduces β -glucan induced IL-1β processing with no defect in caspase-1 activation. Pam2CSK4 primed BMDC (A, C) or unprimed BMDM (B) from C57BL/6, *Rip3*$^{-/-}$, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice were stimulated with curdlan (I), curdlan (W) for 6h (BMDC) or curdlan (S) for 24h (BMDM), pdAdT for 6h or nigericin for 1h. Cell lysates and precipitated supernatants were probed for processed IL-1β, caspase-1 or caspase-8.

Similarly, when we examined the effect of caspase-8 or caspase-1 deficiency on the cell death triggered by β-glucans, caspase-8, but not caspase-1 deficiency protected cells from decrease in viability (Figure 13A and B). These data indicate that β-glucan-induced IL-1β maturation requires both caspase-8 and caspase-1 and the two caspases co-ordinate this process independent of each other. However, the cell death response is mediated by caspase-8 and not caspase-1.

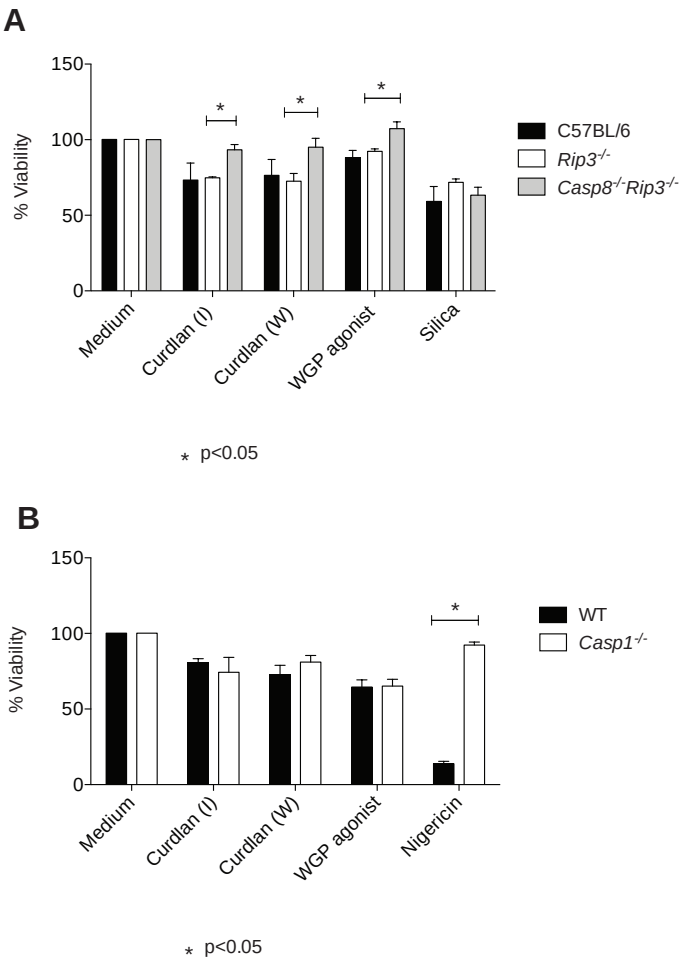


Figure 13. Caspase-8 and not caspase-1, mediates β-glucan induced cell death. Pam2CSK4 primed BMDC from C57BL/6, littermate *Rip3*$^{-/-}$, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ (A) or *Casp1*$^{-/-}$(B) mice were stimulated with β-glucans, silica for 6h or nigericin for 1h. Cell viability was measured by the cell-titer glo assay (A, B). Statistical analysis was performed on results in panels A and B as described in Materials and Methods section.

Having identified the innate immune pathways involved in the execution of IL-1β and cell death responses to β-glucan, we sought to explore the relevance of these signaling pathways during infection with *C. albicans*. C57BL/6, *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ and littermate *Rip3*$^{-/-}$ BMDC were infected with *C. albicans* at a multiplicity of infection 1:1 or 10:1. To facilitate higher β-glucan presentation by *C. albicans*, we also used heat-killed (HK) fungus. As expected, IL-1β production by *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ cells was significantly decreased in response to HK *C. albicans.* However, live log phase *C. albicans*, did not require caspase-8 for IL-1β production (Figure 14A and B). Consistent with the IL-1β phenotype, HK *C. albicans* triggered cell death was protected in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ cells, whereas the response to live *C. albicans* was unaffected (Figure 14C and D). We also measured LDH release in the supernatants of WT BMDC stimulated with HK *C. albicans* and live *C. albicans* (Figure 14C). While live *C. albicans* and nigericin treatments led to a robust release of LDH, HK *C. albicans* resulted in a very moderate release (Figure 14C). Similar to β-glucans, minimal to moderate release of LDH and a caspase-8 requirement in response to HK *C. albicans* may indicate a different kind of cell death compared to that of live *C. albicans*.

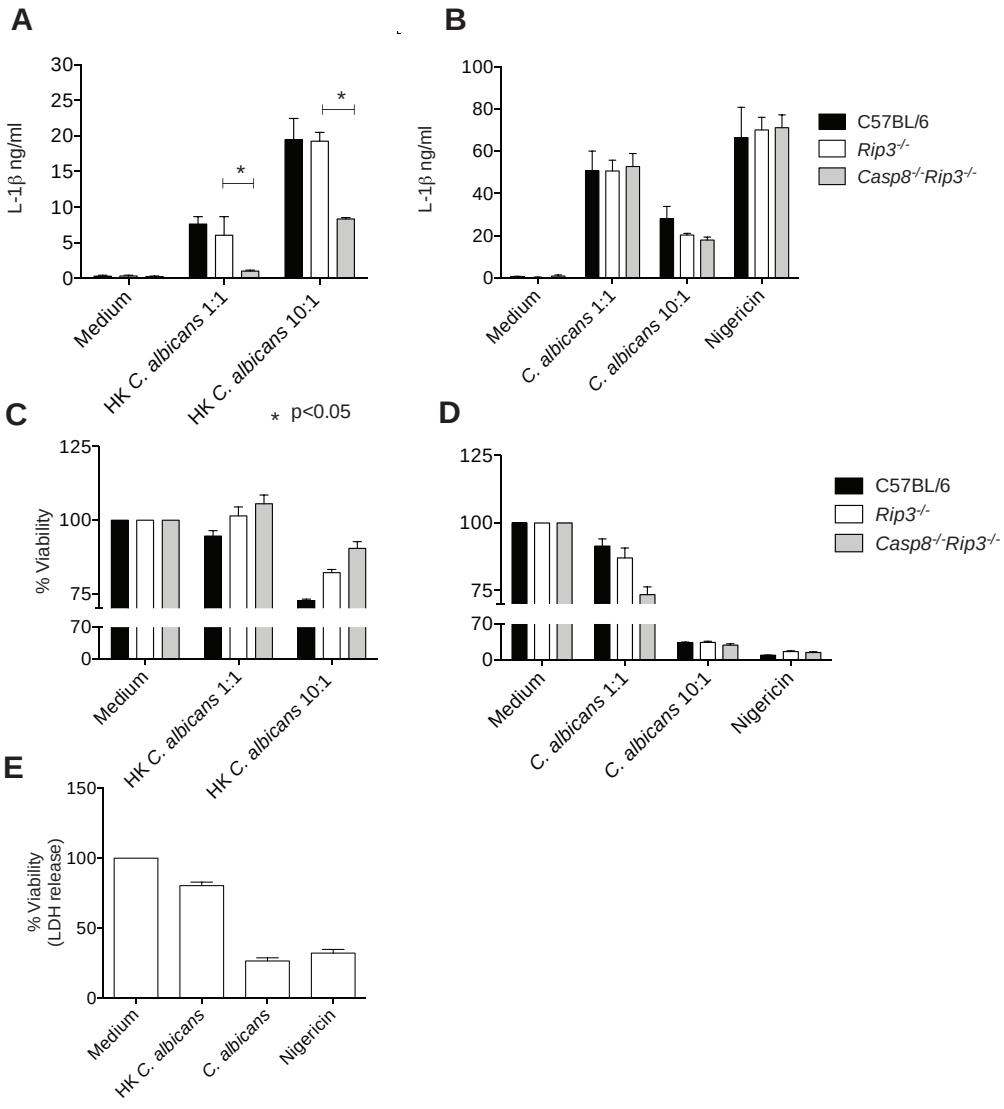


Figure 14. Differential requirement of caspase-8 for HK versus live *C. albicans* induced IL-1β and cell death. Pam2CSK4 primed BMDCs from C57BL/6, littermate *Rip3*$^{-/-}$, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice (A-D) were stimulated with live or HK *C. albicans* at 1:1, 10:1 or nigericin. Amount of IL-1β released in the supernatants was measured by ELISA (A, B). Cell Viability was measured by the cell titer-glo assay (C, D). Statistical analysis was performed on results in panel A as described in Materials and Methods section.

Considering the similarity between β-glucan and HK *C. albicans*, we employed the WGP antagonist to test whether HK *C. albicans* induced responses are more β-glucan dependent than that of its live counterpart. Cells treated with HK *C. albicans,* but not live *C. albicans* exhibited reduced IL-1β and protection from cell death in the presence of the antagonist (Figure 15A-C), validating that the exposed β-glucans are the main immunostimulatory components of HK *C. albicans*.

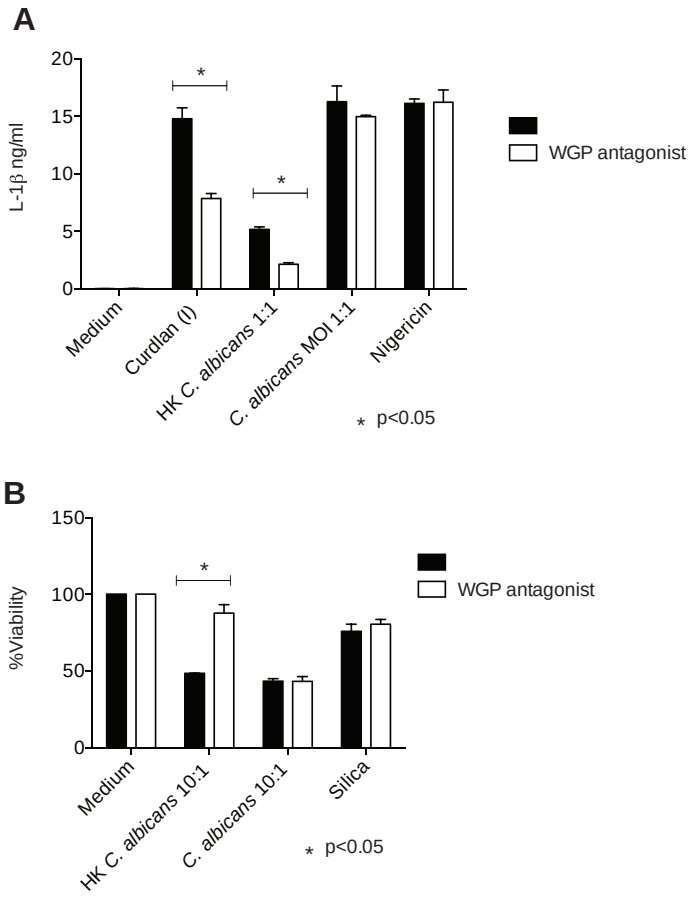


Figure 15. Differential requirement of β-glucan sensing for HK versus live *C. albicans* induced IL-1β and cell death. Pam2CSK4 primed BMDC from C57BL/6 mice (A-C) were stimulated with live or HK *C. albicans* at 1:1, 10:1, pdAdT or silica for 6h. Amount of IL-1β released in the supernatants was measured by ELISA (A, B). Cell Viability was measured by the cell titer-glo assay (C). Statistical analysis was performed on results in panels A and B as described in Materials and Methods section.

Although β-glucans and HK *C. albicans* show an additional requirement for caspase-8, the canonical NLRP3 inflammasome was essential for all for these stimuli (Figure 1). We monitored caspase-8 activity in cells exposed to HK *C. albicans.* Although this response was weak compared to FasL-vesicles, caspase-8 activity was independent of ASC (Figure 16A). The assay was specific for caspase-8, since FasL-vesicles induced activity was undetectable in the *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ cells (Figure 16A). Moreover, NLRP3 or ASC deficiency did not protect cells from HK *C. albicans* induced cell death (Figure 16B), indicating that caspase-8 is engaged independently of the NLRP3 inflammasome to trigger dendritic cell death.

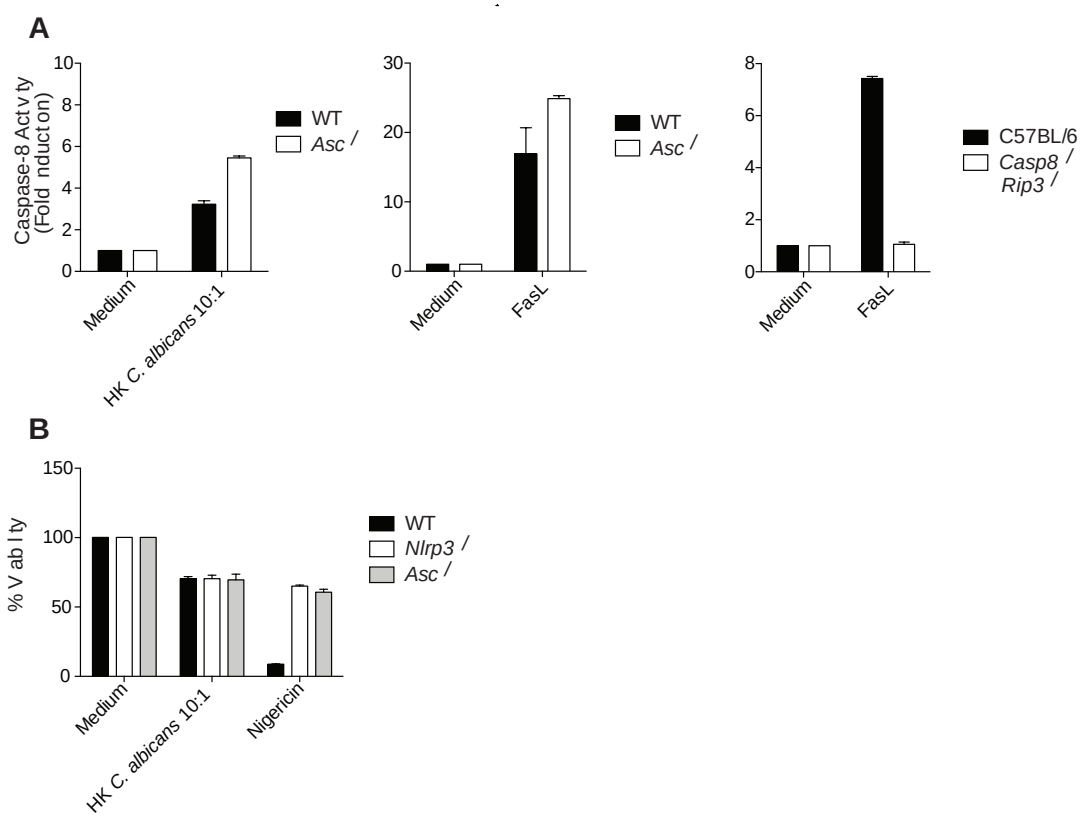


Figure 16. NLPR3 inflammasome does not play a role in HK *C. albicans* triggered caspase-8 activity or cell death. BMDC from C57BL/6, *Casp8*$^{-/-}$*Rip3*$^{-/-}$, *Nlrp3*$^{-/-}$ or *Asc*$^{-/-}$ mice were stimulated with HK *C. albicans*, FasL for 6h or nigericin for 1h. Caspase-8 activity in the lysates (A) and cell viability (B) were measured.

To determine if the distinct phenotypes observed with live and HK *C. albicans* are due to the difference in the upstream receptor engaged, we tested if dectin-1 or CR3 was required for *C. albicans* induced IL-1β in our system. No significant reduction in IL-1β production was noticed in *Clec7a*$^{-/-}$ BMDC treated with HK or live *C. albicans* (Figure 17A). On the other hand, HK *C. albicans* showed a nearly complete requirement for CR3, and the live fungus also exhibited reduced IL-1β production (Figure 17B), suggesting that CR3 may in fact be the dominant receptor involved in *C. albicans* induced IL-1β. Cell death triggered by HK, but not live *C. albicans*, was protected to a certain extent in *Clec7a*$^{-/-}$ as well as *Itgam*$^{-/-}$ BMDC (Figure 17C and D) indicating that both CR3 and dectin-1 are involved in the cell death response to heat-killed fungi.

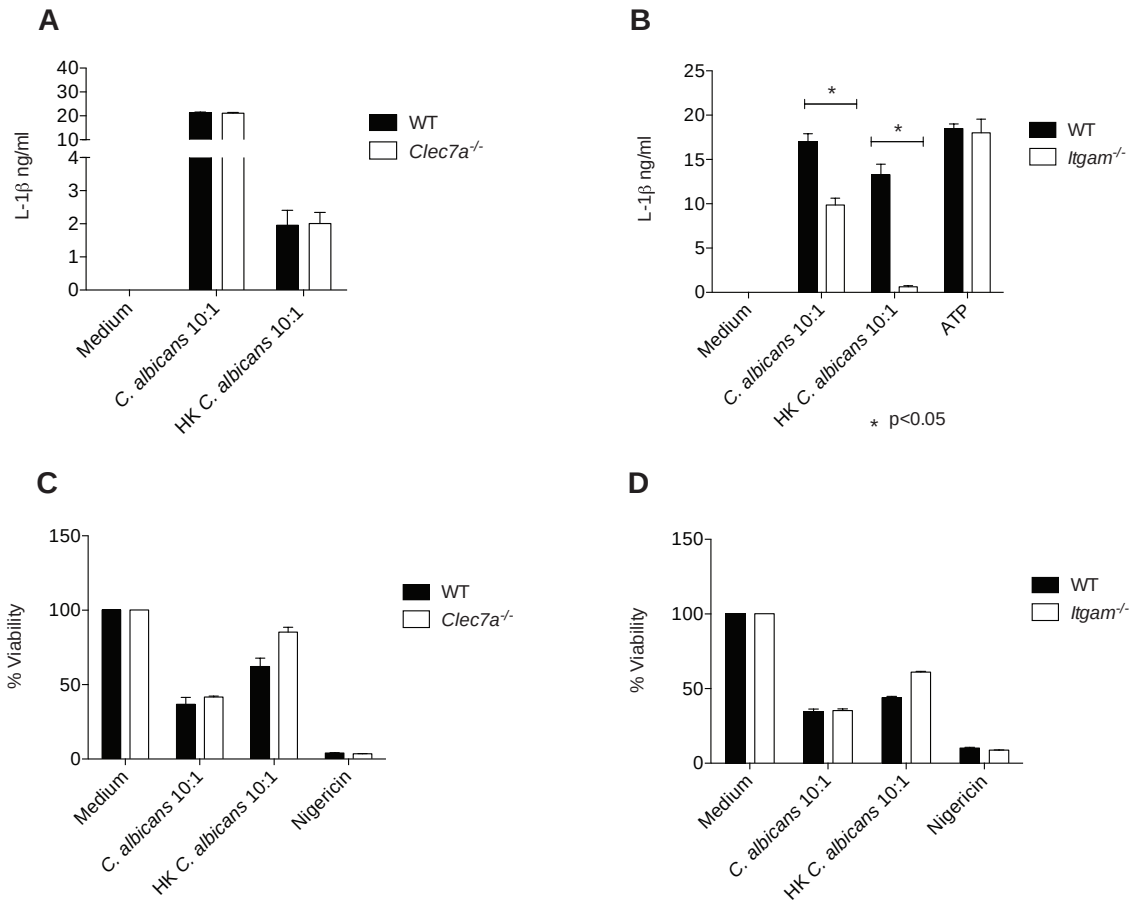


Figure 17. HK *C. albicans* requires CR3 for IL-1β, CR3 as well as dectin-1 for cell death, while live *C. albicans* partially requires CR3 for IL-1β production. Pam2CSK4 primed BMDC from C57BL/6, *Clec7a*$^{-/-}$ (A, C), *Itgam*$^{-/-}$ (B, D) mice were stimulated with HK *C. albicans* or live *C. albicans* MOI 10:1 for 6h, ATP or nigericin for 1h. Amount of IL-1β released in the supernatants was measured by ELISA (A B) and cell viability by cell-titer glo assay (C, D). Statistical analysis was performed on results in panels A and B as described in Materials and Methods section.

Thus, none of the entities tested such as NLRP3 inflammasome, caspase-8, dectin-1 and CR3 were required for live *C. albicans* induced cell death. However, NLRP3 inflammasome and CR3 to an extent was required for *C. albicans* induced IL-1β. We then examined if the IL-1β and cell death response to live *C. albicans* is due to cells phagocytosing the fungus. Dendritic cells were pretreated with an inhibitor of actin polymerization, cytochalasin D and then infected with *C. albicans.* As observed in figure 18A and B, IL-1β and cell death to *C. albicans* was drastically reduced in cytochalasin D treated cells similar to the case of particulate stimulus, silica. This indicates that even though cell death does not require many molecules tested in this study, phagocytosis as a process was indeed required, indicating the validity of this response. This also uncouples the host IL-1β and cell death pathways, since even though phagocytosis promotes both responses, their molecular requirements do not completely overlap.

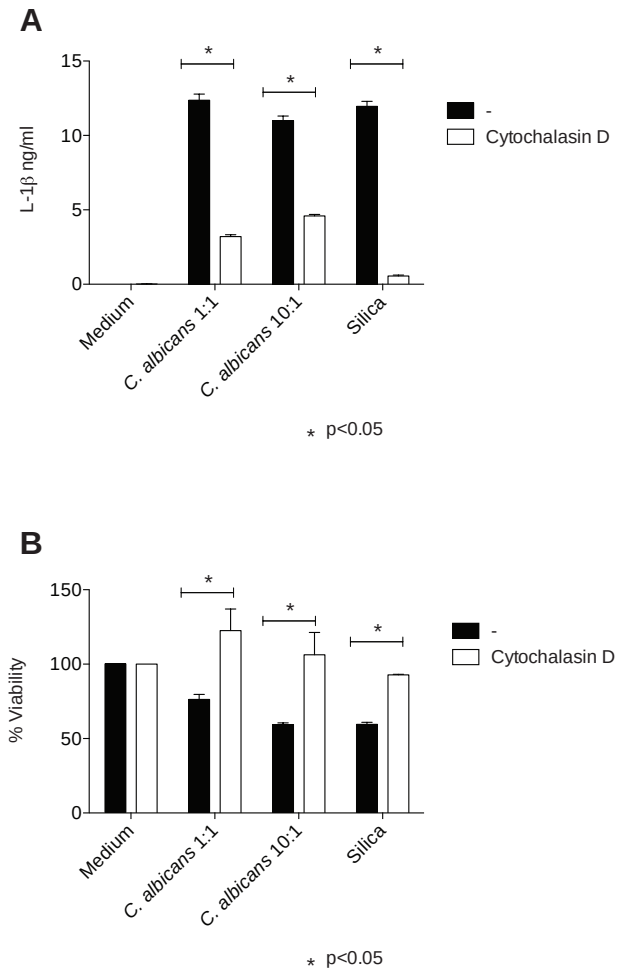
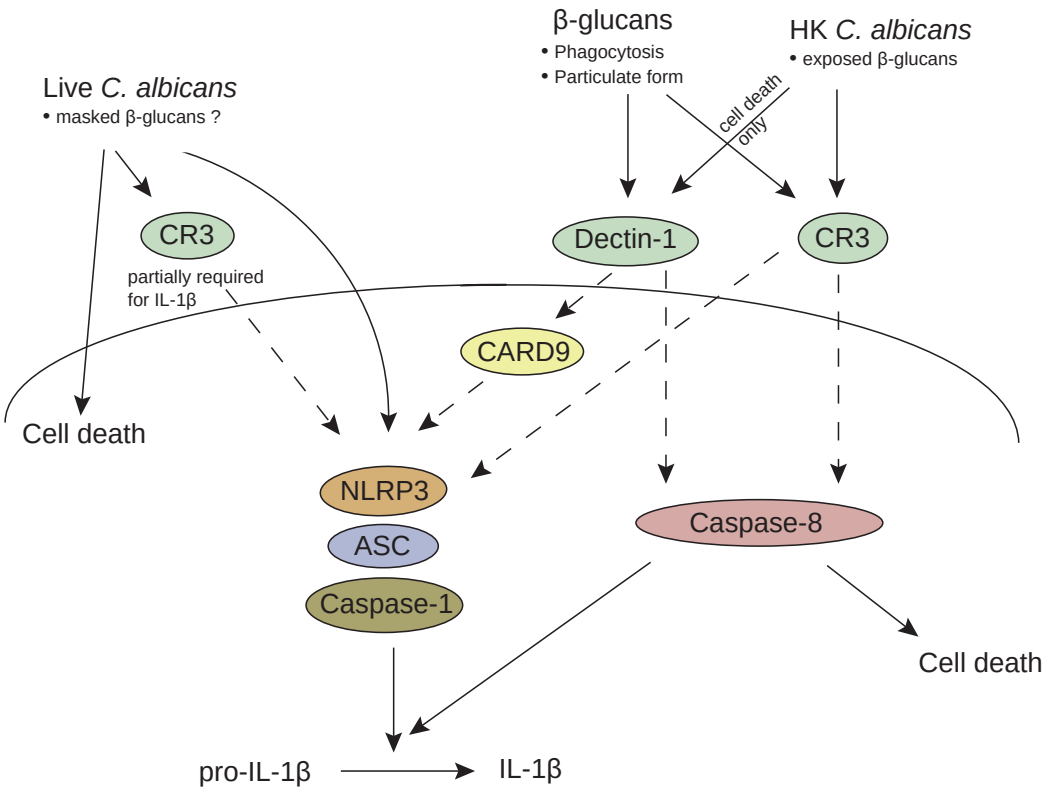


Figure 18. Phagocytosis of *C. albicans* is required to induce IL-1β and cell death responses. Pam2CSK4 primed BMDC from C57BL/6 mice were stimulated with live *C. albicans* MOI 1:1 or 10:1 or silica for 6h Amount of IL-1β released in the supernatants was measured by ELISA (A) and cell viability by cell-titer glo assay (B). Statistical analysis was performed on results in panels A and B as described in Materials and Methods section.



Schematic model 5. Modulation of inflammasome responses to β-glucans and *Candida albicans.* β-glucans heat-killed (HK) as well as live *C albicans* trigger IL-1β maturation and cell death in dendritic cells. All these stimuli absolutely require NLRP3- ASC-caspase-1 inflammasome for pro-IL-1β processing and release. Both dectin-1 and CR3 are essential for β -glucan triggered inflammasome activation, IL-1β production and cell death. CARD9 mediates β-glucan induced IL-1β production, but not cell death. CR3 is essential for HK *C. albicans* induced IL-1β production and cell death, but is only partially required for live *C. albicans* induced IL-1β Dectin-1 is required for HK *C. albicans* induced cell death, but not for HK or live *C. albicans* triggered IL-1β. Caspase-8 promotes IL-1β maturation and mediates inflammasome independent cell death specifically in response to β -glucans and HK *C. albicans.* Thus, caspases-1 and -8, dectin-1, CR3 and the NLRP3 inflammasome co-ordinate to mount innate immune responses against the fungal PAMP, β -glucan and the pathogen *C. albicans.* Bullet points indicate the attributes of the stimuli that contribute to the responses studied. Dotted lines indicate inferred functional associations from our findings.

Discussion

The data presented here demonstrate that the canonical NLRP3 inflammasome (NLRP3, ASC and caspase-1), dectin-1, CARD9, CR3 and caspase-8 all contribute to β -glucan-induced inflammasome responses (Schematic model 5). These molecular requirements are specific to β -glucans, since dectin-1, CARD9, CR3 and caspase-8 were dispensable for IL-1β induced by some of the canonical inflammasome triggers such as silica, nigericin, pdAdT or ATP. Our data, for the first time, implicates CR3 in addition to dectin-1 as a critical sensor coupling β -glucan recognition to inflammasome activation. Strikingly, dectin-1 and CR3 were both essential, and appeared to function in a non-redundant manner in the IL-1β and cell death response to β-glucans. Dectin-1 has previously been shown to activate the integrin CR3 through inside-out signaling, to recognize *C. albicans* PAMPs [81], indicating a potential functional connection between these 2 receptors. Thus, dectin-1 and CR3 could be acting sequentially for sensing and signaling in response to β -glucans. Another possibility is that dectin-1 and CR3 could form a complex to recognize β-glucans. This idea is supported by a recent observation that dectin-2 and dectin-3 form heterodimers to recognize *C. albicans* hyphae indicating co-ordination among fungal PRRs [193].

We used an ATP-based luminescence assay to measure the metabolic activity of dendritic cells, as surrogate for cell viability. It is possible that LDH assay is not as sensitive at detecting viability as the ATP assay or that LDH may

not be released in some of these scenarios (depending on the type of cell death). However, intracellular ATP can fluctuate in response to other physiological and mitochondrial perturbations and hence, an alternate interpretation of this assay, in the case of β-glucans in particular, is that reduction in metabolic activity, rather then cell death. Interestingly, decrease in ATP inversely correlates with IL-1β release indicating that these processes go hand in hand.

C. albicans induced IL-1β and cell death profiles showed some similarities and some differences from that of β-glucans (Schematic model 5). Live or HK *C. albicans* triggered IL-1β production didn't require dectin-1. Instead, CR3 appeared to the critical receptor for *C. albicans* induced IL-1β production, with heat-killed and live fungus showing a complete or partial requirement respectively. Both dectin-1 and CR3 were important for heat-killed *C. albicans* induced cell death. Our results are corroborated by previously reported findings that CR3 drives caspase-8 activation and neutrophil apoptosis in response to phagocytosis of serum-opsonized yeast [194]. We observed that the recognition of β-glucan is important for HK *C. albicans* triggered IL-1β and cell death, in contrast to live *C. albicans*. This is consistent with many published studies that have indicated that β-glucan is masked within the cell wall of live fungus [195], which might impede dectin-1/CR-3 recognition of live *C. albicans*. The differential requirement of these receptors for heat-killed and live *C. albicans* may represent their independent and redundant function or alternatively the involvement of receptors for other PAMPs, such as mannans, in triggering inflammasome

activation. Similar to mouse cells, human PBMC stimulated with heat-killed *C. albicans* uses β-glucan dependent mechanisms to induce higher amount of TNF, IL-6, IFNγ whereas the live form induces lesser cytokine levels which are TLR4 and MR dependent but not β-glucan dependent [181].

Several instances during the process of infection can facilitate the exposure or shedding of fungal β-glucans in the host. Mucosal biofilms reveal the presence and exposure of β-glucans in an environment of *C. albicans* yeast and hyphae [139]. Further, *in vivo* studies suggest that β-glucan that is masked by live *C. albicans*, especially in the yeast form, gets exposed later during the course of infection in organs such as the kidneys [195]. Varied degree of β-glucan unmasking that occurs with hyphal formation in conjunction with yeast and hyphal dissemination is expected to elicit a dectin-1, CR3 and caspase-8 dependent cytokine response. Anti-fungal drugs such as azoles, polyenes, echinocandins to treat infections with *Candida, Aspergillus and Cryptococcus spp.* target the ergosterol of fungal membranes or cell wall β(1,3) glucan synthase [196]. These drugs can cause cause pore formation, ion leakage and exposure of *C. albicans* PAMPs such as β-glucans [197]. Further studies are required to determine if anti-fungal drugs acting on the live fungus and exposing their β-glucans will elicit dectin-1, CR3 and caspase-8 dependent inflammasome responses in the host. While our studies used the glucan particles derived from the yeast form *Saccharomyces cerevisiae* (WGP), β-glucans extracted from hyphae of *C. albicans* has been shown to induce higher amount of IL-1β in human PBMC, in a

dectin-1 dependent manner compared to that from the yeast [198]. It will be very interesting to see how these signaling pathways are fine-tuned in response to yeast versus hyphal β-glucans [198].

Our findings present striking similarities and differences with that of Gringhuis *et al.* 2012, where IL-1β maturation in human dendritic cells, triggered by curdlan and some strains of HK and live *C. albicans* was dependent on dectin-1, ASC and caspase-8, but independent of NLPR3, caspase-1 and phagocytosis [175]. Our studies in mouse dendritic cells, using genetic models, similarly show key roles for dectin-1 and caspase-8, but also a critical requirement for NLPR3, ASC and caspase-1 for optimal IL-1β maturation following β-glucan stimulation. Other studies have also linked the NLRP3 inflammasome to β-glucan induced IL-1β production in mouse and human macrophages and dendritic cells [170,171]. In contrast to β-glucans, dectin-1 and caspase-8 were not required for live *C. albicans* induced IL-1β production in mouse dendritic cells.

For dimorphic fungi such as *C. albicans*, germinating hyphae are capable of physically invading cells and tissues, pierce the macrophage membrane and lyse the cells. Lower pathogen: target cell ratio (2:1) has been shown to activate caspase-1 dependent pyroptosis with no effect on *C. albicans* survival [199,200]. However, a trend towards caspase-1 independent macrophage death was observed when higher MOI or longer co-culture times were studied [199]. We observed dendritic cell death only at an MOI of 10, not at 1 and this death didn't

require the NLRP3 inflammasome or caspase-8. In contrast, particulate β-glucans and HK *C. albicans* trigger a host-programmed caspase-8 dependent cell death pathway mediated by dectin-1 and CR3. The differential cell death responses to live *C. albicans* versus β-glucans and HK *C. albicans* may reflect not only the infectious, invasive capacity of the fungus, but also of an active mechanism by which the pathogen bypasses the shutting down of the responding phagocytic host cell.

The mechanism by which caspase-8 is engaged by β-glucan-sensing pathways is currently unclear. One unique aspect of the β-glucan induced IL-1β production is that caspase-8 is not required for the up regulation of pro-IL-1β, in contrast to other systems such as Fas ligand, *Y. pestis*, *S. typhimurium* or TLR4 priming and hence points to a specific and peculiar role in enhancing the inflammasome effector functions (refer to chapter IV) [176,177]. Caspase-8 deficiency did not have a profound impact on curdlan-induced caspase-1 processing, suggesting that NLRP3 inflammasome assembly and caspase-1 activation are intact in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ cells. This is consistent with a recent study which linked caspase-8 to Salmonella induced pro-IL-1β synthesis and processing in a manner that is independent of caspase-1 [176]. Further, caspase-8 mediated cell death in response to HK *C. albicans* is not dependent on NLRP3 or ASC, even though all of these molecules are important for IL-1β production. These results position caspase-8 on a parallel β-glucan activated pathway that is independent of inflammasome assembly but converges with the NLRP3 pathway

to promote IL-1β maturation (Schematic model 5). In addition to NLRP3, the role of caspase-8 in augmenting NLRC4 mediated IL-1β production [176], will also be relevant for anti-fungal responses, since NLRC4 inflammasome was also found to be essential for host protective responses in mouse model of OPC [135].

Future studies will dissect the mode of action of caspase-8 to determine if caspase-8 promotes the activation and processing of IL-1β through direct or indirect mechanisms. Interestingly, recombinant caspase-8 has been shown to cleave pro-IL-1β at the same site as caspase-1, in an *in vitro* system [173], suggesting that caspase-8 may directly cleave pro-IL-1β. Caspase-1 and caspase-8 have CARD and DED domains respectively, which are related protein-protein interaction domains involved in a number of distinct homotypic interactions. Thus, one intriguing possibility would be direct interaction between these two caspases. It would also be interesting if caspase-8 regulates caspase-1 through activating cellular inhibitors of apoptosis proteins, cIAPs, and K63 ubiquitination [201]. In our own studies, we have shown that the *Drosophila* caspase-8-like factor DREDD triggers IAP-dependent K63-ubiqutination [202] and perhaps some aspects of these pathways are conserved. Overall, the role of caspase-8 in bridging β -glucan sensing with cell death and NLRP3 inflammasome dependent IL-1β processing is reminiscent of the role of caspase-11 in gram-negative bacterial infection, where caspase-11 integrates LPS sensing with cell death and NLRP3 dependent IL-1β maturation [174].

Extrinsic cell death pathways show significant overlap with innate immune signaling mechanisms and share molecular components including RIP1 and cIAPs. Ripoptosomes are dynamic cytosolic multi-protein complexes, which determine cell fate along the survival/apoptosis/necrosis axes [203,204]. Recent studies have elucidated their role in regulation of IL-1β processing in addition to their well-characterized roles in cell death. Two recent reports indicate that RIP3 triggers NLRP3-ASC dependent caspase-1 activation and IL-1β processing when caspase-8 is deficient or when cIAPs are depleted [205,206]. Surprisingly, IL-1β production observed in these scenarios required just a priming signal by TLRs. This suggests a tight quality control of the ripoptosome complex where removal of caspase-8 or cIAP proteins skews the ripoptosome to feed into the NLRP3-ASC-caspase-1 pathway for IL-1β processing. We noticed that *Rip3*$^{-/-}$ DC exhibit IL-1β and cell death responses comparable to WT, in response to all stimuli examined (Figures 10A, 13A and 14).

Our efforts to characterize the activation of caspase-8 in response to HK *C. albicans* revealed that induction of caspase-8 activity, albeit detectable, was very weak as compared to that elicited by FasL-vesicles (Figure 16). However, consistent with our data, caspase-8 activation has been observed when dectin-1 is activated by HK *C. albicans* or when CR3 phagocytoses opsonized yeast in other cell types [169,194]. Catalytic functions of caspase-8 are regulated by a non-catalytic homologue and partner, cFLIP, which is also involved in NLRP3 and AIM2 inflammasome responses [207]. Recent evidence that hemizygotic cFLIP

deficient cells exhibit higher caspase-8 mediated IL-1β production specifically in response to HK *C. albicans* supports the important role of caspase-8 activity in the β-glucan signaling pathway [207].

We observed that particulate but not soluble glucans stimulate IL-1β production in dendritic cells. Further, soluble glucans antagonize IL-1β and cell death responses to particulate, immunostimulatory glucans. Our results are consistent with previous studies indicating that higher the molecular weight and the particulate nature of the glucan, better is the level of cytokine induction in dendritic cells [168]. The reason for this higher activity was demonstrated by an elegant study by Goodridge, H. S. *et al.* 2011 [208]. They showed that recognition of particulate β-glucans leads to clustering, activation of dectin-1 receptor and exclusion of inhibitory tyrosine phosphatases CD45 and CD148 from this junction described as “phagocytic synapse”. This was proposed to be a mechanism by which signaling could be activated by β-glucan in the context of the microbe and not by the floating PAMP [208]. Similar to glucans, particulate and not the soluble form of chitosan, triggers inflammasome activation and IL-1β production [209].

Phagocytosis of particulate glucans is mediated by dectin-1 and CR3 to different extent depending on the opsonization status of the glucans [210]. In fact, other cell wall components N- and O- linked mannan, cell wall proteins, or hyphae specific elements do not appear to contribute significantly towards ingestion of yeasts in macrophages [200]. Bone marrow derived dendritic cells were

found to be more responsive to glucans compared to bone marrow derived macrophages or thioglycollate elicited macrophages representing an interesting difference between the responsiveness of various cell types. The higher responsiveness of dendritic cells compared to macrophages has been attributed to dectin-1 expression and dectin-1-CARD9 signaling that can be boosted by GM-CSF or IFNγ priming [211]. For myeloid cells other than BMDC, it has been shown that GM-CSF priming is necessary to induce a stronger cytokine response to glucans of varying sizes (range of ~3μM for Glu-mp to 0.2mm for curdlan) and recruit peritoneal neutrophils in mice [212]. A phenomenon of "frustrated phagocytosis" results when macrophages attempt to engulf huge particles of curdlan, heightening their innate immune responses [212]. Curiously, insoluble glucans phagocytosed by macrophages by dectin-1 recognition can be processed/degraded by oxidative mechanisms into smaller fragments that are released and can activate other cells such as neutrophils or NK cells in a CR3 dependent manner [168,213] thus amplifying this loop.

Currently, no anti-fungal vaccines are available for humans, but β-glucan conjugates have shown promise as vaccines in rodent experimental models [214]. Laminarin, a β-glucan derived from the alga, *Laminaria digitata* conjugated with a diphtheria toxoid conferred protection to mice against systemic *C. albicans* and *A. fumigatus* infection that was attributed to β-glucan specific antibodies [215]. Moreover, β-glucans also serve as delivery systems for antigens and in eliciting potent antibody, Th1 and Th17 responses against protein antigens [187,216] [210].

Hence, it is imperative to understand the signaling pathways underlying their therapeutic and adjuvant roles.

β(1,3) glucans are considered to possess higher antitumor and immunoprotective potential compared to β(1,4) or β(1,6) glucans [217,218,168]. It would be exciting to determine if the anti-cancer and/or adjuvant properties of β-glucans results from engagement of caspase-8 and/or caspase-1 pathways. β-glucans appear to stimulate many cell types such as macrophages, DC, neutrophils, NK, T and B cells [168] which increases their relevance as immunomodulators. Binding of neutrophil CR3 to a macrophage-processed 25kD fragment of β-glucan was found to effectively prime them to target opsonized tumor cells for killing [219]. Similar mechanism was attributed to the CR3 dependent cytotoxic ability of neutrophils towards iC3b opsonized tumors when β(1,3) glucans were orally administered in mice [78,220]. CR3 has also been found to contribute to antigen specific Th17 cytokine induction elicited by the delivery of antigen loaded glucan particles [210] adding CR3 to an array of molecules NLRC4, ASC, Caspase-1 and NLRP10 that mediate the development of Th17 immunity.

Intriguingly, even though mycobacteria do not contain β-glucans, many studies have implicated dectin-1 in *M. tuberculosis* induced pro-inflammatory cytokine production, Th1 and Th17 responses in mouse and human cells, raising the possibility that dectin-1 may also other unidentified ligands. Although a recent report showed that dectin-1 KO do not show worse pulmonary pathology,

survival compared to WT in an *M.tb* aerosol infection model (reviewed in Marakalala, M. J. *et al.* 2011)[221] it is worth exploring if activating dectin-1 signaling by pre-administration of β-glucans will enhance host-immunoprotective responses against *M.tb* and potentially other bacterial infections.

The classical belief of non-specificity for innate immunity has been challenged by a recent concept of "training the innate immune cells" to mount better protective responses for subsequent infectious encounters [222]. Pretreatment of PBMC or monocytes with β-glucans enhances the cytokine responses to subsequent TLR stimuli, possibly by inducing epigenetic change to increase the expression and efficiency of PRRs [223] [222]. Overall, the clinical applications of β-glucans are myriad and future studies should continue to uncover the underlying signaling pathways so that they can be manipulated to achieve the desired treatment outcome.

Altogether, the findings presented here re-emphasize the specific role of NLRP3-ASC-caspase-1 inflammasome in IL-1β maturation, not only for live *C. albicans*, but also the heat-killed form of the fungus and β-glucans. We uncover roles for CR3, dectin-1 as well as caspase-8 in coordinating cell death and inflammasome responses to β-glucans and the contribution of these pathways to innate immune responses against the pathogen *C. albicans*. These studies significantly enhance our mechanistic understanding of β-glucan elicited

inflammatory responses, their immunomodulating properties and anti-fungal innate immune responses.

Materials and Methods

Glucans: Curdlan was purchased from Sigma, Invivogen or Wako Chemicals. Carboxy-methylated curdlan was also from Wako chemicals. Curdlan was re-suspended in sterile PBS at 10mg/ml and used at 100µg/ml to stimulate BMDC or 1mg/ml for BMDM, unless otherwise indicated. Fungal whole glucan particle agonist or antagonist (WGP dispersible or WGP soluble) was purchased from Invivogen. WGP (dispersible/soluble) was used at 100µg/ml, unless otherwise indicated.

Mice: Mice were maintained and bred at UMASS Medical School in accordance with the Institutional Animal Care and Use Committee (IACUC). *Casp8*$^{+/-}$*Rip3*$^{-/-}$ mice were kindly provided by Dr. Douglas Green (St. Jude Children's Research Hospital) and in some cases *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ and *Casp8*$^{+/+}$ *Rip3*$^{-/-}$ femurs were from Dr. Bill Kaiser and Dr. Edward S. Mocarski (Emory University School of Medicine), *Card9*$^{-/-}$ femurs were from Dr. Ramnik Xavier (Massachusetts General Hospital) [224] and *Itgam*$^{-/-}$ femurs from Dr. Tanya N. Mayadas (Brigham and Women's Hospital) [225]. *Casp1*$^{-/-}$ and *casp11*$^{-/-}$ mice were kindly provided by Dr. Vishva Dixit (Genentech) [128], *Clec7a*$^{-/-}$ (Dectin-1$^{-/-}$) mice were from Gordon Brown (University of Aberdeen) [38]. *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice were from Millennium Pharmaceuticals. *Casp8*$^{+/-}$*Rip3*$^{-/-}$ mice were on a mixed C57/BL6-129 background and were intercrossed to generate *Casp8*$^{-/-}$*Rip3*$^{-/-}$ and *Casp8*$^{+/+}$*Rip3*$^{-/-}$ mice, which were used as littermate controls in all of our experiments. All other mice were on a C57/BL6 background.

***Candida albicans* and EHEC culture conditions:** *C. albicans* UC820 (ATCC MYA-3573) strain, obtained from Dr. Mihai Netea (Nijmegen Institute for Infection, Inflammation and Immunity), was used in all experiments [142]. *C. albicans* was maintained as glycerol stocks and cultured in the yeast-phase in Sabouraud Dextrose (SBD) broth at 30°C, 250 rpm. Overnight cultures were re-inoculated 1:20 in SBD broth for 3-4h. Live log phase cultures were washed twice and re-suspended in PBS, counted and used directly or heat-killed (95°C for 30') and used for BMDC stimulation. EHEC (Enterohemorrhagic *Escherichia coli*, strain O157:H7, EDL 933) was maintained as glycerol stocks and cultured overnight in LB media, at 37°C, 250 rpm and used in stationary phase for experiments as previously described [174].

BMDC and BMDM culture: As described in chapter II, for bone marrow derived macrophages, progenitor cells were cultured in DMEM with 10% heat inactivated serum, Pen-strep and 20% L929 supernatant as the source of MCSF. Media was changed on day 3, 6 and 8 and cells were used for experiments between day 8-10. For dendritic cells, bone marrow progenitor cells were counted and plated at 8 x 10^6 cells/ 25cm^2 dish in RPMI with 10% heat inactivated serum, Pen-strep, 20ng/ml recombinant GM-CSF and 50µM β-mercaptoethanol and cultured for 9-10 days as previously described [157].

For ELISAs, BMDC or BMDM were plated in 96 well plates at 1 x 10^6 cells/ml. Cells were unprimed or primed with Pam2CSK4 at 100ng/ml for 3h and stimulated with curdlan, WGP agonist (100µg/ml) or EHEC MOI 25 for 6h or 24h.

Media was replaced with fresh gentamicin containing media for EHEC infected cells after 1h of infection. For controls, cells were stimulated with canonical inflammasome activators such as pdAdT or silica for 6h, nigericin or ATP for 1h after priming (as described in chapter II). For experiments with the WGP antagonist, unprimed or primed cells were pretreated with 100µg/ml antagonist for 1h, before stimulation with β-glucan ligands. For *C. albicans* infection, cells were infected with heat-killed or live log phase cultures at multiplicity of infection 1:1, 10:1 for 6h post priming.

For western blot analysis, cells were plated at 2 x 10^6 cells/ well in 12 well plates in serum free, antibiotic free medium, primed and stimulated as described above. Supernatant proteins were precipitated with methanol and chloroform. Cells were lysed with 1% NP-40 lysis buffer and Bradford assay was performed to normalize the amount of protein. Samples were run on 12% SDS-PAGE and transferred to nitrocellulose membrane. Western blotting was performed with the antibodies to caspase-1 p10 (sc-514, Santa Cruz Biotechnology), caspase-1 p20 (Casper1 clone; Adipogen), IL-1β (AF-401-NA, R&D Systems), caspase-8 (1G12, Enzo Life Sciences), RIP3 (2283, Pro-Sci) and α-tubulin (2125, Cell signaling).

ELISA: BD Biosciences or eBioscience kits were used to measure the amount of IL-1β and TNF present in cell culture supernatants according to manufacturers' instructions. RANTES in the supernatants was measured by a kit from R&D

systems (DY478). For IFNβ, a sandwich ELISA that has been previously described was used [226].

Quantitative RT-PCR: RNA was extracted from cells using RNeasy kit (QIAGEN). iScript Select cDNA synthesis kit (Bio-Rad) was used to synthesize cDNA from 1µg total RNA from each sample. Quantitative RT-PCR for pro-IL-1β and β-actin was performed using iQ SYBR green supermix (Bio-Rad). Primers used for pro-IL-1β were 5'-TCCCCAGCCCTTTTGTTGA-3' (F), 5'-TTAGAACCAAATGTGGCCGTG-3' (R) and β-actin were 5'-TTGAACATGGCATTGTTACCAA-3' (F), 5'-TGGCATAGAGGTCTTTACGGA-3' (R). Levels of pro-IL-1β mRNA were normalized to that of β-actin.

Caspase-8 Activity and Cell death Assay: Caspase-8 activity in cell lysates was assayed using Caspase-Glo 8 Assay kit (Promega, Cat # G8200). Cell viability was assessed using Cell-Titer Glo kit (Promega, Cat # 7570) according to manufacturers' instructions.

Statistics: Data were analyzed by the two-way analysis of variance followed by Bonferroni test using PRISM software. P value less than 0.05 was considered significant. For cytokine measurements, experiments were performed in triplicates and the graphs depicted are representative of atleast two or three independent experiments. The triplicates of the representative experiment shown were analyzed using two-way ANOVA. The significant comparisons are marked

by an asterisk (p value < 0.05) and the unmarked groups in the same graph can be assumed to not be significant. In the case of experiments with *Casp8*$^{+/+}$ *Rip3*$^{+/+}$, *Casp8*$^{+/+}$ *Rip3*$^{-/-}$ and *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ dendritic cells, the groups *Casp8*$^{+/+}$ *Rip3*$^{-/-}$ and *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ were compared to assess significance.

Chapter IV:

Characterization of the role of caspase-8 in TLR signaling

Chapter IV: Copyright Information

Some of the data and subject content of this chapter are part of the following manuscript/publications:

Ganesan S, Rathinam VAK, Bossaller L, Army K, Kaiser WJ, Mocarski ES, Dillon CP, Green DR, Mayadas TN, Levitz SM, Hise AG, Silverman N, Fitzgerald KA. Caspase-8 modulates Dectin-1 and CR3 driven IL-1β production in responses to β-glucans and *Candida albicans* (*Submitted to Journal of Immunology. In revision.*)

Ganesan S, Aggarwal K, Paquette N, Silverman N. NF-κB/Rel proteins and the humoral immune responses of *Drosophila melanogaster*. Curr Top Microbiol Immunol. 2011 349:25-60.

Chapter IV: Characterization of the role of caspase-8 in TLR signaling

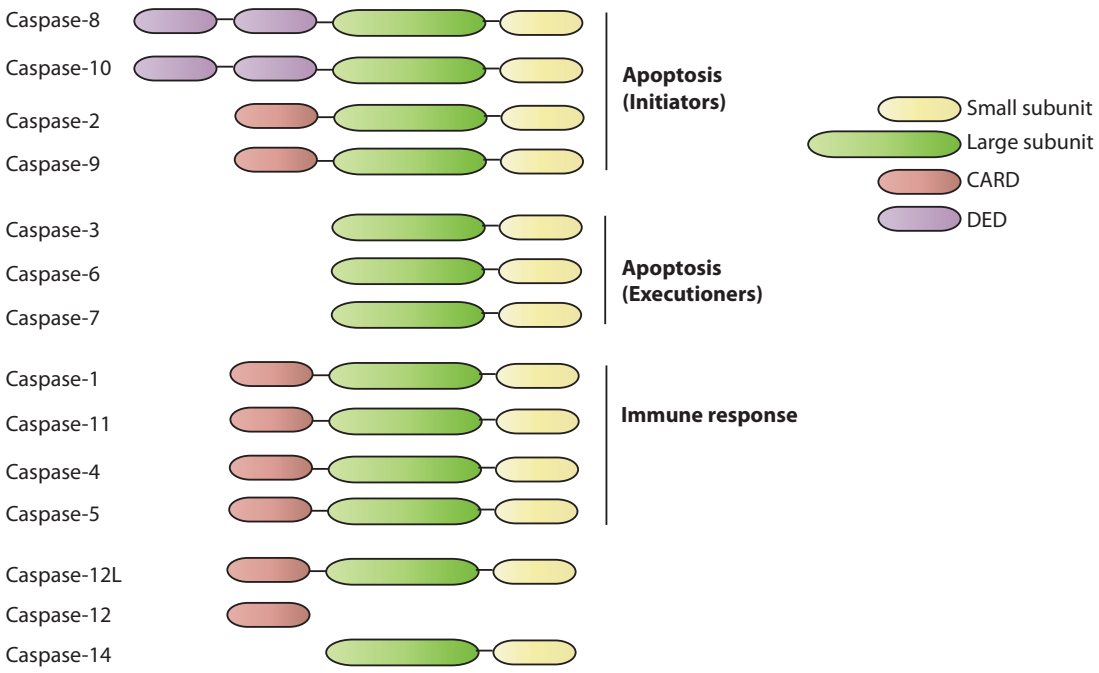
Abstract

Caspases are highly conserved cysteine proteases that play myriad roles in cellular processes such as inflammation, cell death and proliferation. Previous studies from our group have demonstrated that the *D. melanogaster* homologue of mammalian caspase-8, namely DREDD plays a critical role in the *Drosophila* IMD pathway, which activates the NF-κB homolog, Relish. At the receptor level, DREDD cleaves IMD thereby exposing a neo-N-terminal IAP binding motif in IMD. This in turn facilitates the binding of the E3 ligase dIAP2 to IMD and conjugation of K63 ubiquitin chains on IMD [227]. DREDD also cleaves the NF-κB homolog Relish, which allows the N-terminal transactivating domain to translocate into the nucleus. We hypothesized that caspase-8, through its proteolytic activity may also be important for mammalian NF-κB pathways, owing to the high evolutionary conservation of IMD signaling with that of the mammalian TLR and RLR pathways. We set out to determine the role of caspase-8 in NF-κB pathways using *casp8*$^{-/-}$ *Rip3*$^{-/-}$ macrophages and dendritic cells, *casp8*$^{-/-}$ embryonic fibroblasts and conditional caspase-8 KO (deficient in myeloid lineage) macrophages. We demonstrate that caspase-8 is specifically required for TLR4 induced TNF and pro-IL-1β synthesis, while it negatively regulates TRIF mediated IFNβ and RANTES depending on the cell type. This requirement was also observed in IL-1β response to gram-negative bacterial pathogen, EHEC, which activates TLR4. Surprisingly, immortalized MEFs *casp8*$^{-/-}$ MEFs were found to have lost the expression of RIP3, presumably to sustain against RIP kinases

mediated necrosis. Reconstitution of *casp8*$^{-/-}$ MEFs with caspase-8 or RIP3 indicated that while the requirement in TLR4 signaling might specifically be due to caspase-8, the negative regulatory role in TLR3 mediated RANTES production may be due to caspase-8 as well as RIP3. LysMcre *casp8*$^{-/-}$ macrophages exhibits a “hyper-inflammatory” phenotype with escalated IL-1β production and cell death in response to TLR ligands and hence, masks the direct role of caspase-8 in these pathways. Together, our results show an intricate interplay between caspase-8 and RIP3 and how these molecules modulate TLR signaling pathways to differentially regulate pro-inflammatory cytokines. Studies in this chapter emphasize the importance of studying these proteins in an all-inclusive manner for precise therapeutic requirements.

Introduction

Caspases are a family of highly conserved cysteine proteases involved in a variety of cell-death and inflammatory pathways (Schematic model 6). Caspases -1, -11, -4, -5 are involved in immune signaling pathways as seen in chapters II and III. Caspases -9, -8, -10, -3, and -7 have well characterized roles in inducing a “silent” form of cell death called apoptosis. Interestingly, caspase-8 is involved in both immune signaling as well as apoptosis. Based on their role in apoptosis, caspases can be classified as initiators (caspase-8, -10, -9) or executioners (caspase-3, -7) as reviewed in McIlwain, D. R. *et al*. 2013 [228]. Most caspases contain an N-terminal pro-domain, which can be a CARD (CAspase Recruitment Domain) or a DED (Death Effector Domain) that mediates their homotypic interactions with other proteins. Their C-terminal domain comprises of a small and large subunit (p10 and p20 respectively) that harbors its catalytic site (Schematic model 6). The enzymatic activity of caspases is targeted towards a specific aspartate site (P1) in their substrates and controls many cellular processes including cell death.



Schematic model 6. The caspase family of proteins regulates cell death and inflammation. This figure depicts the family of caspases present in either humans or mice only or in both (caspase-11 is a mouse member, while caspase-4, -5, -10 are human members). They contain a small and large subunit, which includes their catalytic site and an N-terminal CARD or DED domain. Caspases function in immune responses and in mediating extrinsic as well as intrinsic apoptosis. Caspase-12 is expressed in humans usually as a short 'CARD domain' only form due to a premature stop codon [229]; however, a SNP containing full length form has been identified in a subset of humans [230]. The functions of caspases in other cellular processes beyond their defined roles (as shown above) are being uncovered. Model figure inspired from the review, McIlwain, D. R. *et al*. 2013 [228].

Certain intrinsic and extrinsic stimuli can trigger caspase-mediated cell death. Intrinsic cell death process originates from a change in the mitochondrial membrane potential, release of cytochrome c into cytosol and activation of caspase-9 in the apoptosome complex in the cytosol. In contrast, extrinsic cell death is triggered by soluble inflammatory mediators such as TNF, TRAIL or Fas ligand, which signal through the cell surface receptors TNFR, TRAIL receptor or Fas (CD95). In particular, engagement of these receptors can lead to the activation of caspase-8 in a receptor associated signaling platform called DISC, Death Induced Signaling Complex. “Activation” of caspase-8 usually involves homo-dimerization and auto-proteolytic cleavage to separate the N-terminal prodomain (DED) from its C-terminal domain. Active caspase-8 comprising of the large and small subunits along with its catalytic site targets its cellular substrates including caspase-3 and -7 to trigger apoptosis. Apoptosis through extrinsic pathway can also lead to the cleavage of the pro-apoptotic protein, BID into truncated BID (tBID) which can activate intrinsic apoptosis through caspase-9 (reviewed in McIlwain, D. R. *et al*. 2013)[228].

Some caspases also regulate many inflammatory pathways, such as inflammasomes in the case of caspase-1 or caspase-11 (Schematic model 6). Interestingly, caspase-8 was found to regulate inflammasome functions as well (Chapter III). In addition, caspase-8 has long been implicated in NF-κB activation and cell proliferation in various cell types. The first indication of a non-apoptotic role of caspase-8 was observed with a human family of genetically inherited

mutation in caspase-8 (Arg248-Trp)[231]. As expected, peripheral blood mononuclear cells (PBMCs) from these individuals were defective in Fas induced apoptosis similar to patients with autoimmune lymphoproliferative syndrome (ALPS). But surprisingly, these cells were found to be impaired in the activation of T, B and NK cells in response to TCR and TLR signals [231,232]. Following that, many studies have aimed to characterize this immune-related function of caspase-8 using genetic mice models.

Caspase-8 knockout is embryonic lethal at day 11.5 during gestation [178]. Defects observed in the knockout mice include impaired vasculature and muscle development and higher number of erythrocytes [178]. In fact, caspase-8 KO appears to be the most detrimental of all single caspase deficiencies (reviewed in Li, J. and Yuan, J. 2008)[233]. Hence, conditional deficient cells have been used to study the functions of caspase-8 in different cell types. Unexpectedly, T cells lacking caspase-8 are deficient in antigen induced proliferation, cytokine production and anti-viral responses [234]. However, with aging, T cell specific deletion results in hyper activation, proliferation and infiltration in organs resulting in reduced mice survival [235]. Similar immunodeficient phenotype was observed in B cells. B-cell specific deletion of caspase-8 using CD19 promoter was shown to decrease antibody production in response to viral infection and reduce cell-viability in response to TLR 2, 3 and -4 signaling [188]. Interestingly, caspase-8 was recruited to IKK complex and required to promote NF-κB activation and translocation in response to LPS [188]. Similar to defects in cell surface TLR4 and

TLR3 signaling, transfected dsRNA induced expression of NF-κB target genes TNF and IL-6 were markedly decreased in caspase-8 KO mouse embryonic fibroblasts (MEFs) and in HEK293 cells following knockdown of caspase-8. These results indicated a potential defect downstream of intracellular RNA sensors such as RIG-I [236]. Thus, these studies indicated that caspase-8 plays an important role in TLR-2, -3, -4 and RLR signaling; and activation of T, B and NK cells in various cell types [232].

On the other hand, other studies showed that caspase-8 deletion in B cells does not impair their proliferation or NF-κB activation in response to LPS and poly(I:C) [or pIC] [237]. Similarly, T cells lacking caspase-8 also were shown to not be impaired in NF-κB activation in response to CD3-CD28 stimulation or TLR ligands [191,237]. Caspase-8 deficiency did however lead to decrease in cell numbers or impaired proliferation of T cells in response to these stimuli [191,237]. This alternate form of cell death in T cells required RIP1 and was blocked by the inhibitor of its kinase activity, necrostatin-1 [191]. These studies brought to light that cells die even in the absence of caspase-8. This other programmed cell death pathway mediated by RIP kinases, and blocked by caspase-8, was termed “necrosis”. This indicated that the role of caspase-8 in NF-κB activation and cell death needed to be explored further.

Based on the evidence that caspase-8 prevents necrotic cell death, two groups re-visited the generation of caspase-8 knockout mice. They found that the

embryonic lethality observed in caspase-8 single knockout can be rescued by the deletion of the kinase RIP3 [179,180] indicating that RIP3 dependent necrosis is detrimental to embryonic development. However, around 4 months of age, due to the lack of two cell death pathways, these mice start developing symptoms of lymphoadenopathy, similar to Fas receptor deficient mice with higher T cell populations in the spleen and lymph node [238]. In addition, they develop a peculiar CD4, CD8 double negative T cell population ($CD4^{-}CD8^{-}CD3^{+}B220^{+}$) [179]. Caspase-8 actively blocks RIP3 from activating necrosis. This non-apoptotic, yet protective, function of caspase-8 was attributed to its ability to form a heterodimeric complex with cFLIP, a non-catalytic homologue of caspase-8 [180]. Together, these studies revealed a new functional aspect of caspase-8.

Strikingly, *Drosophila* caspase-8 like molecule DREDD also plays an essential role in one of the two *Drosophila* NF-κB signaling pathways, the IMD pathway. Understanding the role of DREDD in the IMD pathway gave us important mechanistic insights into the potential role of caspase-8 in mammalian NF-κB signaling. A brief overview of *Drosophila* NF-κB signaling and the IMD pathway is presented here to underline these similarities.

Drosophila **innate immune signaling**

Drosophila melanogaster mounts potent humoral immune responses against microbes. Similar to the mammalian innate immune responses, recognition of conserved microbial cell wall structural components such as peptidoglycan (PGN) is the primary mechanism by which infection is detected.

The two major pattern recognition pathways in *D. melanogaster* are the Toll and the IMD pathways. Toll and IMD signaling pathways culminate in the activation of the NF-κB transcription factors Dorsal, DIF and Relish. IMD pathway is activated by DAP-type peptidoglycan which occurs in most Gram-negatives and various *Bacillus spp*. whereas Lys-type peptidoglycan found in Gram-positive microbes activate the Toll signaling pathway [239,240]. Activation of both Toll and IMD pathways by bacterial PGNs requires peptidoglycan recognition proteins (PGRPs). Of the 13 PGRP genes in *Drosophila*, PGRP-SA and PGRP-SD are two soluble PGN receptors that function in the Toll pathway [241,242], while PGRP-LC and PGRP-LE recognize DAP-type PGN and trigger the IMD pathway [243-246].

IMD signaling

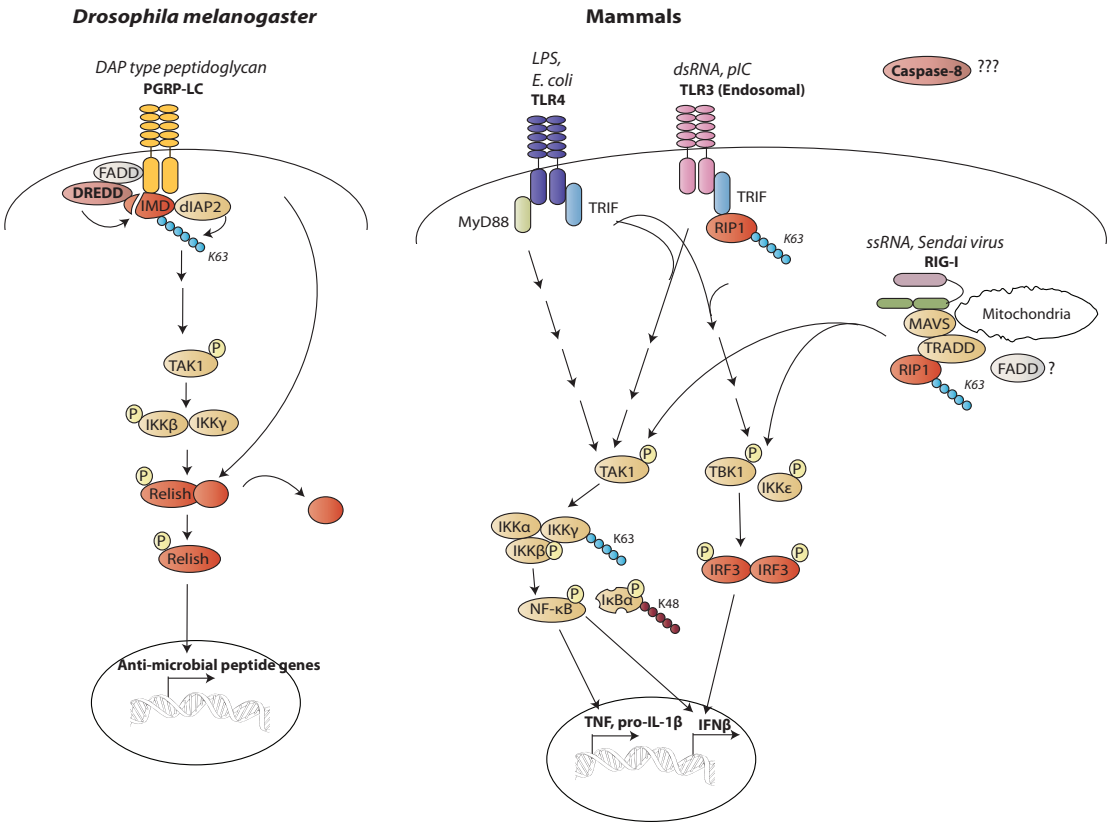
Detection of DAP-type PGN by PGRP-LC and PGRP-LE triggers the IMD pathway [239,243-248]. PGRP-LC is a transmembrane receptor with an extracellular PGRP domain while PGRP-LE lacks a transmembrane domain and functions as an intracellular receptor [246,249]. Small monomeric PGN fragments like TCT that enter cells or from intracellular bacteria are recognized by the cytosolic receptor PGRP-LE[249], reminiscent of the mammalian NOD pathways.

PGRP-LC and PGRP-LE detect DAP-type PGN through their C-terminal PGN-binding PGRP domain and transduce signal through their N-terminal domains [246,250]. A short stretch of about 20 amino acids is common to their N-terminal domains and is essential for signal propagation [246]. This conserved motif has some weak similarity to the RHIM motif found in RIP1 and TRIF, signaling

proteins in the mammalian TLR pathways [251]. PGRP-LC and PGRP-LE recruit IMD, a death domain-containing protein which is similar to the mammalian kinase RIP1 [246,250,252,253]. While IMD in *Drosophila* also possesses the death domain and the RHIM motif similar to RIP1, it does not contain the kinase domain. It is likely that IMD facilitates the recruitment of the *Drosophila* FADD homolog via a homotypic death domain (DD) interaction [254]. FADD is then known to interact with the caspase DREDD (homolog of mammalian caspase-8) via a homotypic interaction between the death effector domain (DED) in these proteins [255,256] (Schematic model 7).

DREDD is critical for the initiation of downstream signaling events in the IMD pathway [257,258]. In response to PGN stimulation, IMD is cleaved in a DREDD-dependent manner at a caspase cleavage site in the N-terminal region (LEKD^{30}) [259]. Immediately following this cleavage site is a consensus IAP-binding motif (IBM, ^{31}AAPV). Both the caspase site and the IBM are highly conserved in multiple species of *Drosophila* as well as the *Anopheles gambiae* mosquito [259]. DREDD-mediated cleavage of IMD exposes the IBM, allowing IMD to associate with the *Drosophila* inhibitor of apoptosis 2 protein (DIAP2). The BIR2 and BIR3 domains of DIAP2 are responsible for interacting with the IBM of cleaved IMD, similar to the IBM–BIR interactions observed in the regulation of caspase-mediated programmed cell death [260]. DIAP2, through its RING domain mediates the robust ubiquitination of cleaved IMD [227,261] (Schematic model 7). IMD conjugated with K63-polyubiquitin chains, is similar to that observed with the

mammalian RIP1 in TNFR signaling [262,263] . Polyubiquitinated IMD has been proposed to act as a scaffold for recruiting the downstream kinases TAK1 and IKK, via ubiquitin binding domains found in their partners TAB2 and IKKγ, respectively, similar to mammalian NF-κB signaling [264-267]. Once recruited and activated, TAK1 activates IKK, which in turn phosphorylates the NF-κB precursor protein Relish [258,268-270] (Schematic model 7). Relish is also cleaved, by the caspase DREDD, resulting in the uncoupling of the C-terminal IκB-like domain from the N-terminal NF-κB module, thereby allowing the N-terminal Rel Homology Domain (RHD) to translocate into the nucleus to initiate the transcription of antimicrobial peptides such as Diptericin [258] (Schematic model 7).



Schematic model 7. Molecular conservation between *Drosophila* IMD and mammalian innate immune pathways. *Drosophila* IMD pathway responds to DAP type PGN through the PRR, PGRP-LC. Upon ligand recognition, FADD, DREDD and IMD are recruited to the receptor. DREDD cleaves IMD to facilitate its K63 ubiquitination by dIAP2. These upstream signaling events are important for TAK1 activation and phosphorylation, subsequent IKKβ activation and phosphorylation. Relish is activated by IKKβ mediated phosphorylation and DREDD mediated cleavage. Active N-terminal cleaved Relish translocates to the nucleus to induce the transcription of anti-microbial peptides such as Diptericin. Signal transduction in mammalian TLR4, TLR3 and RIG-I pathways are highly similar to that of the IMD pathway. TLR4 responds to LPS and gram-negative bacteria such as *E. coli* while TLR3 senses dsRNA. They engage the adaptors MyD88 (TLR4) and TRIF (TLR4 and TLR3). Intracellular RIG-I senses ssRNA and viruses such as Sendai virus and binds to the mitochondria-associated adaptor protein MAVS and recruits TRADD, RIP1 and likely FADD. These pathways activate the kinases TAK1, IKK to phosphorylate the inhibitor of NF-κB, IκBα and target it for K48 ubiquitination and proteasomal degradation. This event frees NF-κB to translocate into the nucleus to induce transcription of genes such as TNF and pro-IL-1β. TRIF and RIG-I signaling can also activate TBK1 and IKKε. These kinases phosphorylate IRF3, which dimerizes and translocates into the nucleus to activate the transcription of IFNβ. While mammalian FADD and caspase-8 have also been implicated in these pathways in other cell types, their precise functions are not clear.

Mammalian TLR4, TLR3 and RIG-I signaling pathways

As described in chapter I, TLRs sense a wide variety of microbial molecules. Upon recognition of their corresponding ligand, TLRs homo- or hetero-dimerize to bring together their cytoplasmic signaling tails and trigger downstream signaling cascades. Two critical adaptor molecules that facilitate TLR signaling are Myeloid Differentiation Primary Response gene 88 (MyD88) and TIR domain containing adaptor inducing Interferon-β (TRIF) [271-273]. With the exception of TLR3, which utilizes TRIF only, all TLRs require MyD88 to potentiate signaling. TLR4 is unique in that, it triggers both MyD88-dependent as well as TRIF-dependent signaling [271]. Two additional adaptors MyD88 adapter like (Mal) and TRIF-related adaptor molecule (TRAM) bridge the interaction between TLR4 and MyD88 or TRIF, respectively [271,274]. Some of the key upstream components that are common to many of the TLR signaling pathways include the upstream kinases IRAK-1, -2, -4 or RIP1 and the E3 ligases TRAF-2, -3, -6. In a basal state, NF-κB is kept contained in the cytoplasm by its inhibitor, IκBα. TLR signaling leads to the recruitment of the kinase TAK1 and its adaptor TAB2 to the upstream signaling complex, possibly through K63 ubiquitinated upstream components. For example, RIP1 and IKKγ appear to get K63 ubiquitinated in response to TLR and TNFR signals [275,276]. Recruitment to the upstream signaling complex leads to the phosphorylation and activation of TAK1, which in turn activates the kinase IKKβ that is in a complex with the other kinase IKKα, and the regulatory IKKγ. Active IKKβ in turn phosphorylates IκBα and targets it for K48 ubiquitination and proteasomal degradation. This event frees

NF-κB to translocate into the nucleus to induce the transcription of its target genes such as TNF, pro-IL-1β (Schematic model 7). Additionally, IKKβ also phosphorylates NF-κB (p65) itself to increase its transcriptional activity, similar to that observed with Relish [270,277]. The kinases IKKε and TBK1 also phosphorylate the transcription factor IRF3 which dimerizes, translocates to the nucleus and activates the transcription of IFNβ [278] (Schematic model 7). FADD has been implicated in TLR3 and TLR4 signaling in B cells, however its precise role requires further investigation [279].

The intracellular receptor RIG-I which senses 5' triphosphate RNA and single stranded RNA viruses [280,281] also employs some of the above mentioned signaling intermediates to activate NF-κB and IRF3. RIG-I binds to the adaptor protein MAVS on the surface of mitochondria, through CARD-CARD interactions [282-285]. MAVS in turn recruits TRADD and RIP1 and possibly FADD to activate signaling [276,286,287](Schematic model 7). These events lead to the activation of TAK1, IKK complex and TBK1 as described above. The RIG-I family of receptors also includes the intracellular MDA5 that responds to longer dsRNA [288]. RIG-I and MDA5 both signal through MAVS and trigger robust induction of type I IFN and interferon stimulated genes (ISGs) and potent antiviral responses.

Potential role for mammalian caspase-8 in TLR and RLR signaling

As described above, a dynamic molecular event in the IMD pathway that is critical for the signal propagation is the cleavage of IMD by DREDD. *Drosophila* IMD shares many structural as well as functional features with the

mammalian RIP1 such as the presence of death and RHIM –like domains, K63 and K48 ubiquitination, phosphorylation and regulation by caspase-mediated cleavage. RIP1 is a signaling mediator downstream of death receptors such as TNFR, Fas and also PRRs such as TLR3, TLR4, RIG-I and likely certain DNA sensing receptors [289]. RIP1 is critical for NF-κB activation in TNF signaling [290] and its K63 ubiquitination at a conserved site K377 facilitates the recruitment of TAK1 and IKK complex through IKKγ [262,291]. RIP1 has a serine/threonine kinase domain at its N-terminus, however, its kinase activity has been found to not be required for NF-κB oriented functions [292,293]. This aligns with the lack of kinase activity for the *Drosophila* IMD. Further, the molecular state and regulation of RIP1 is a crucial factor that decides the fate of the cell [203,204]. TNF signaling, in addition to activating NF-κB, can activate apoptotic or necrotic cell death under appropriate conditions. For example, cleavage of RIP1 by caspase-8 at D324 is a crucial step in the switch of signaling from NF-κB activation to apoptosis [294]. On the other hand, kinase active RIP1 stabilizes the complex between RIP1 and RIP3 and mediates necrosis [295].

The conservation of molecules and mechanisms between *Drosophila* and mammalian NF-κB pathways extends beyond RIP1 and caspase-8. Other shared molecules include, but are not limited to, the adaptor TAB2, kinases TAK1 and IKKs and their functions. In addition, FADD an adaptor molecule in *Drosophila* IMD pathway as well as mammalian TNFR signaling has been implicated in TLR and RLR signaling pathways, in conjunction with RIP1 and caspase-8 [189,279,287].

We set out to determine if caspase-8 plays a role in cytokine production in the mammalian RIP1 pathways such as TLR3 and TLR4. It is important to understand the role of caspase-8 in innate immune signaling pathways specifically in myeloid cells such as macrophages and dendritic cells since these are main contributors of cytokines during infection. Since caspase-8 single knockout mice are embryonic lethal, caspase-8 RIP3 DKO, caspase-8 deficient immortalized MEFs or conditional caspase-8 single KO (LysMcre) were used.

Results

To determine if caspase-8 has a role in pro-inflammatory cytokine production in TLR and RIG-I signaling pathways, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice and littermate *Rip3*$^{-/-}$ mice were used. A few studies have shown that catalytically active caspase-8 and FADD are required for the development of hematopoietic myeloid cells in response to differentiating factors or cytokines such as M-CSF [296,297]. Of note, we didn't observe any difficulty in differentiating bone marrow cells of caspase-8 RIP3 DKO mice to mature macrophages or dendritic cells using L929 supernatant (M-CSF source for macrophages) or GM-CSF (dendritic cells) respectively. Bone marrow derived macrophages (BMDM) and dendritic cells (BMDC) were profiled for TNF production in response to a variety of ligands. TLR4 ligand, LPS, induced TNF production was dramatically reduced in *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ macrophages compared to *Rip3*$^{-/-}$ cells (Figure 1A). poly I:C (pIC), a TLR3 ligand induced TNF production also appeared to be reduced in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ cells, however, this decrease was discounted since the control *Rip3*$^{-/-}$ macrophages displayed the same phenotype as *Casp8*$^{-/-}$*Rip3*$^{-/-}$. This effect of caspase-8 on TLR4 pathway is specific, since ligands such as synthetic lipopeptides Pam2CSK4 and Pam3CSK4 (TLR2), imidazoquinoline compound R848 (TLR7/8) or the ssRNA Sendai virus (RIG-I) induced TNF production was unaffected in the KO (Figure 1B). Similar to BMDM, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ BMDC also produced decreased TNF in response to LPS, compared to the controls (Figure 1C). This indicated that caspase-8 is specifically required for optimal TNF

production in response to LPS in the TLR4 pathway, in macrophages and dendritic cells.

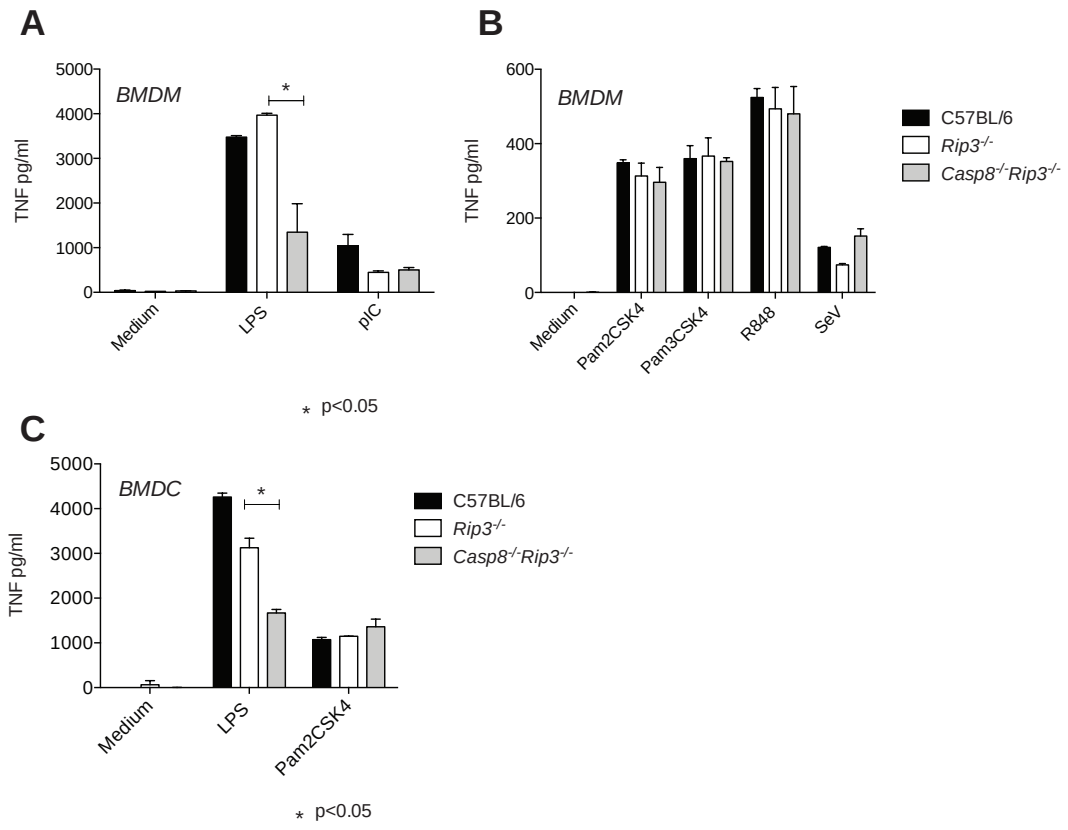


Figure 1. Caspase-8 is required for TLR4 induced TNF production in macrophages and dendritic cells. Bone marrow derived macrophages (BMDM-A, B) or dendritic cells (BMDC-C) from C57BL/6, littermate *Rip3*$^{-/-}$, *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ mice were stimulated with LPS, pIC, Pam2CSK4, Pam3CSK4, R848 or SeV for 24h. Amount of TNF released in the supernatants was measured by ELISA (A-C). Statistical analysis was performed on results in all panels as described in Materials and Methods section.

Next, the induction of pro-IL-1β, the precursor of the pro-inflammatory cytokine IL-1β critical in the context of infections (as discussed in chapters I, II and III) was examined. *Casp8*$^{-/-}$*Rip3*$^{-/-}$ and control *Rip3*$^{-/-}$ dendritic cells were stimulated with LPS at two concentrations 10 or 100ng/ml and Pam2CSK4 at 100ng/ml for 6h. Since in the case of IL-1β, there are two forms, the full-length pro-IL-1β and the cleaved IL-1β, western blotting was used to detect pro IL-1β synthesis in the lysates. As seen in Figure 2A, amount of pro IL-1β in the lysates was particularly lower for LPS treated *Casp8*$^{-/-}$*Rip3*$^{-/-}$ cells compared to that of Pam2CSK4, while tubulin levels mark equal loading across samples. NF-κB, being the dominant transcription factor for TNF as well as pro IL-1β production, these results indicated that caspase-8 is required for optimal NF-κB activation specifically in response to LPS (TLR4).

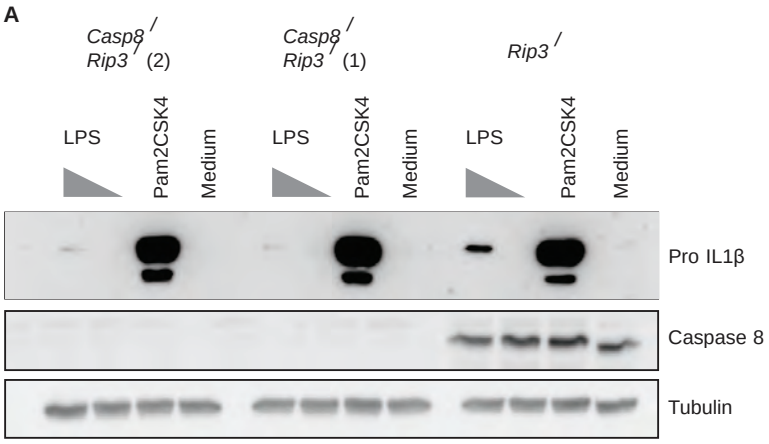


Figure 2. Caspase-8 is important for TLR4 induced pro-IL-1β synthesis. Bone marrow derived dendritic cells from littermate *Rip3*$^{-/-}$, *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ mice were stimulated with Pam2CSK4 or LPS at different concentrations for 6h. pro-IL-1β, caspase-8 and tubulin levels in the lysates were detected by western blotting (A).

In addition to NF-κB, TLR4 and TLR3 signal through the adaptor TRIF to activate IRF3, a transcription factor that induces the synthesis of type I Interferons (IFN). Type-I interferons (IFN) such as IFNβ, signals via the IFN-Receptor (IFNR) in the infected and bystander cells to induce the production of IFNα and several IFN stimulated genes (ISGs) that help in resisting viral infection and replication. To determine if caspase-8 plays a role in the production of IFNβ, C57BL/6, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ and control *Rip3*$^{-/-}$ macrophages and dendritic cells were treated with LPS and pIC at different concentrations for 6h. Early time point was used for the ease of detection of LPS triggered IFNβ. Surprisingly, caspase-8 deficient macrophages and dendritic cells displayed higher IFNβ production in response to both LPS as well pIC (Figure 3A and B). These results showed that caspase-8 negatively regulates TRIF mediated IFNβ production in these cells in complete contrast to the TNF and pro-IL-1β phenotype. Thus, caspase-8 differentially modulates NF-κB and IRF3 dependent cytokines.

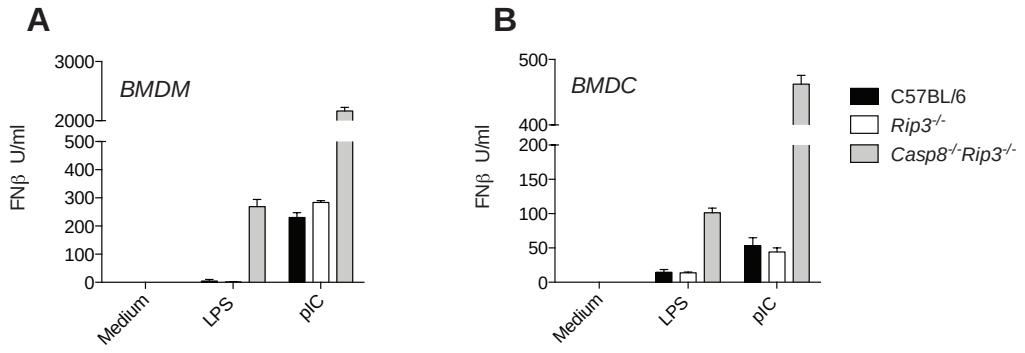


Figure 3. Caspase-8 negatively regulates TRIF induced IFNβ production in macrophages and dendritic cells. Bone marrow derived macrophages (BMDM-A) or dendritic cells (BMDC-B) from C57BL/6, littermate *Rip3*$^{-/-}$, *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ mice were stimulated with LPS or pIC for 6h. Amount of IFNβ released in the supernatants was measured by ELISA (A, B).

We then investigated the status of a pro-inflammatory cytokine whose transcription is regulated by both NF-κB as well as IRF3, such as RANTES [298,299]. C57BL/6, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice and control *Rip3*$^{-/-}$ macrophages and dendritic cells were stimulated with LPS or pIC for 24h. Intriguingly, we didn't observe any striking difference in RANTES levels across the genotypes (Figure 4A-B). Together, these results indicated that while caspase-8 is required for TNF, pro-IL-1β (NF-κB dependent) and suppresses IFNβ (IRF3 dependent) production, RANTES is unaffected, indicating that in the case of cytokines which require both transcription factors, there could be a combinatorial phenotype.

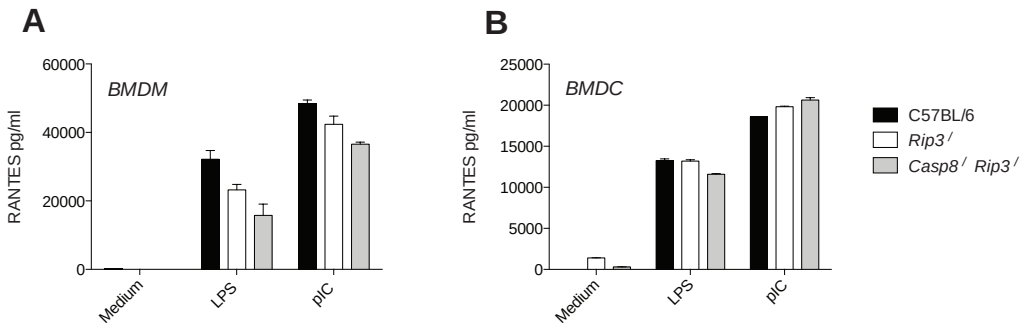


Figure 4. Caspase-8 deficiency does not affect TLR4 and TLR3 induced RANTES production in macrophages and dendritic cells. Bone marrow derived macrophages (BMDM-A) or dendritic cells (BMDC-B) from C57BL/6, littermate *Rip3*$^{-/-}$, *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ mice were stimulated with LPS or pIC for 24h. Amount of RANTES released in the supernatants was measured by ELISA (A, B).

In order to determine if the role of caspase-8 holds in non-immune cell-types, immortalized *Casp8*$^{+/+}$ and *Casp8*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were used. Since MEFs are not efficient producers of TNF or pro-IL-1, our hypothesis that caspase-8 deficiency impairs LPS triggered NF-κB activation, was directly investigated in MEFs. Cells were stimulated with LPS for different time points and phosphorylated IκBα was detected by western blotting. As described in the introduction of this chapter, when TLRs are activated, IκBα is phosphorylated, K48 ubiquitinated and targeted for proteasomal degradation to release NF-κB. It was observed that phosphorylation of IκBα was greatly reduced in *Casp8*$^{-/-}$ MEFs compared to *Casp8*$^{+/+}$ cells, while the levels of total proteins were unaffected (Figure 5A). Further, unpublished data from Dr. Egil Lien's lab shows impaired IκBα degradation in response to LPS in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ macrophages. In addition, caspase-8 has been implicated in TLR4 induced NF-κB activation in various lymphoid cells, as described in the introduction. In summary, these results strongly emphasized the requirement of caspase-8 for LPS induced NF-κB responses as a common phenomenon across different cell types.

A

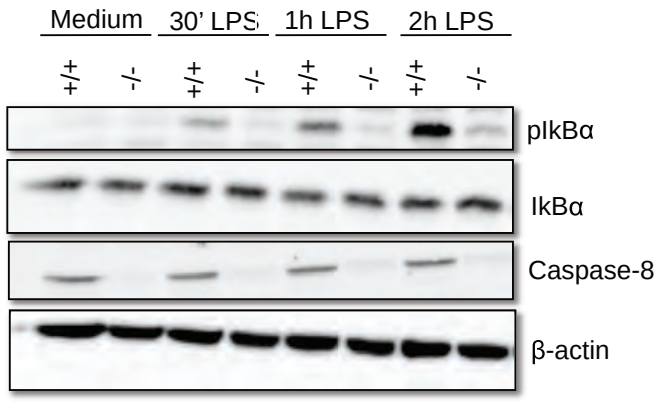


Figure 5. Caspase-8 is required for LPS induced NF-κB activation in MEFs. Immortalized *Casp8*$^{+/+}$, *Casp8*$^{-/-}$ MEFs were stimulated with LPS for different time points. pIκBα, total IκBα, caspase-8, RIP3, β-actin in the lysates were detected by western blotting (A).

To determine the relevance of these findings in the context of an infection, Enterohaemorrhagic *Escherichia coli* (EHEC), a gram-negative bacterium was used. LPS sensing by TLR4 and the production of cytokines such as IL-1β is critical for innate immune responses against EHEC. To evaluate this, C57BL/6, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ and control *Rip3*$^{-/-}$ macrophages and dendritic cells were used to evaluate the synthesis of the precursor pro-IL-1β and then its release mediated by inflammasome activation. As observed in figure 6A, the induction and synthesis of pro-IL-1β stimulated with EHEC for 4h was severely decreased in the *Casp8*$^{-/-}$*Rip3*$^{-/-}$ BMDC compared to controls. Consequently, cells were primed with a TLR2 agonist Pam2CSK4 since caspase-8 was not required for TLR2 triggered TNF or pro-IL-1β thereby providing a tool to normalize cellular pro-IL-1β levels between the three genotypes. When Pam2 primed BMDC (A) as well as BMDM (B) were infected with EHEC for 24h, IL-1β released in the supernatants as a result of inflammasome activation, was still found to be severely reduced in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ cells compared to the controls (Figure 6B and C). This suggests that caspase-8 may be required for “signal 2” of the inflammasome activation (pro-IL-1β processing and release) in addition to “signal 1” (pro-IL-1β synthesis), in the case of *E. coli*. This shows that the caspase-8 requirement observed in the LPS-TLR4 pathway can be extended to cytokine responses in bacterial infections.

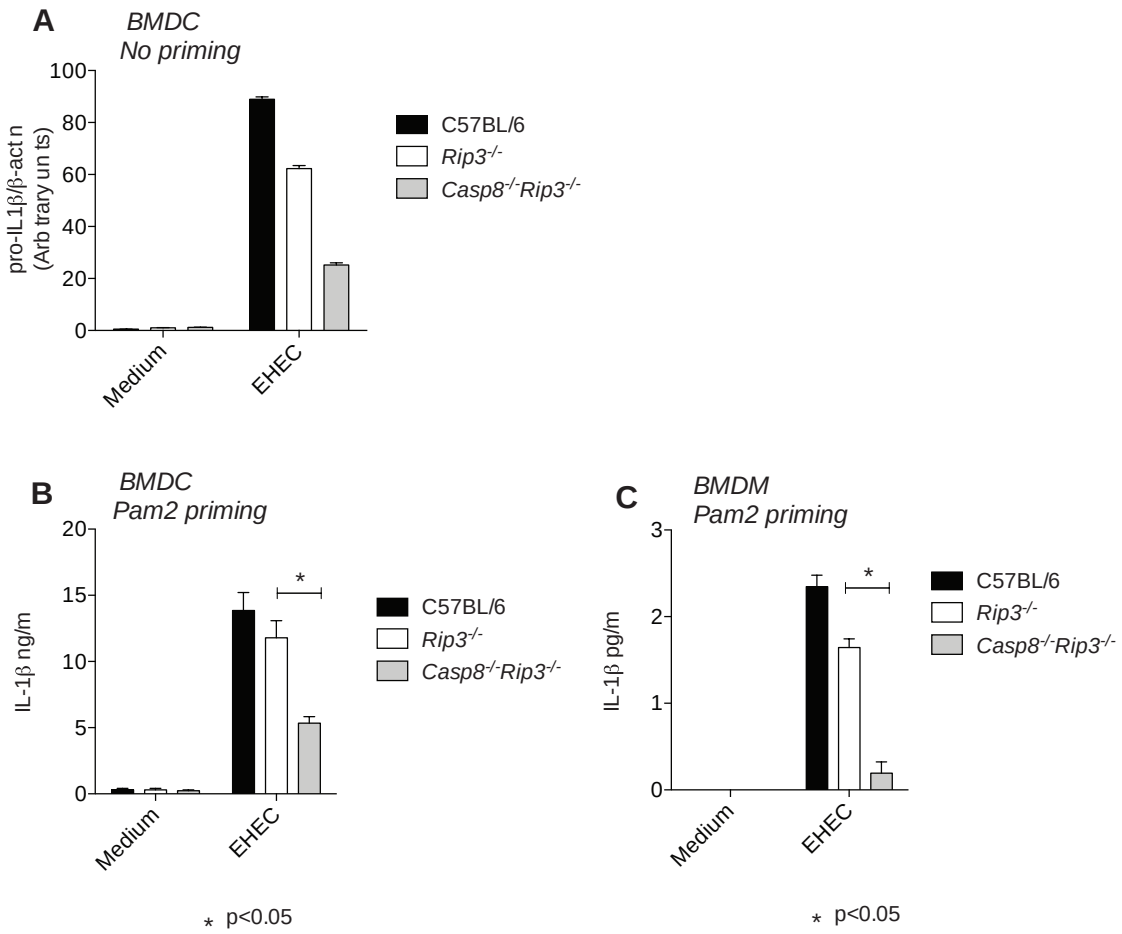


Figure 6. Gram-negative bacterium EHEC induced IL-1β production requires caspase-8 in macrophages and dendritic cells. Pam2 primed bone marrow derived dendritic cells (A, B) or macrophages (C) from C57BL/6, littermate *Rip3*$^{-/-}$, *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ mice were infected with EHEC for 24h, or nigericin for 1h. Amount of IL-1β released in the supernatants was measured by ELISA (A-C). Statistical analysis was performed on results in panels B and C as described in Materials and Methods section.

Since MEFs are known not to secrete much of TNF, IL-1β in response to LPS and pIC, RANTES production was measured. Strikingly, LPS induced RANTES production was drastically reduced in *Casp8*$^{-/-}$ MEFs (Figure 7A), while pIC induced RANTES was elevated in stark contrast (Figure 7B). This led to the hypothesis that the difference in LPS and pIC phenotypes could arise from the engagement of MyD88 vs TRIF, in TLR4 vs TLR3 pathways, for RANTES induction in MEFs. To test this, WT and *Trif*$^{-/-}$ MEFs were stimulated with LPS, pIC overnight. As seen in figure 7C, both LPS and pIC induced RANTES was almost completely dependent on TRIF, while TRIF was not required for SeV. This showed that TRIF is an essential common signaling component of both TLR4 as well as TLR3 signaling. Yet, LPS and pIC exhibit completely opposite phenotypes in the context of RANTES production. These results imply that caspase-8 may play opposite roles in MyD88 and TRIF signaling pathways which impacts RANTES production in a contrasting manner since TLR4 additionally engages MyD88.

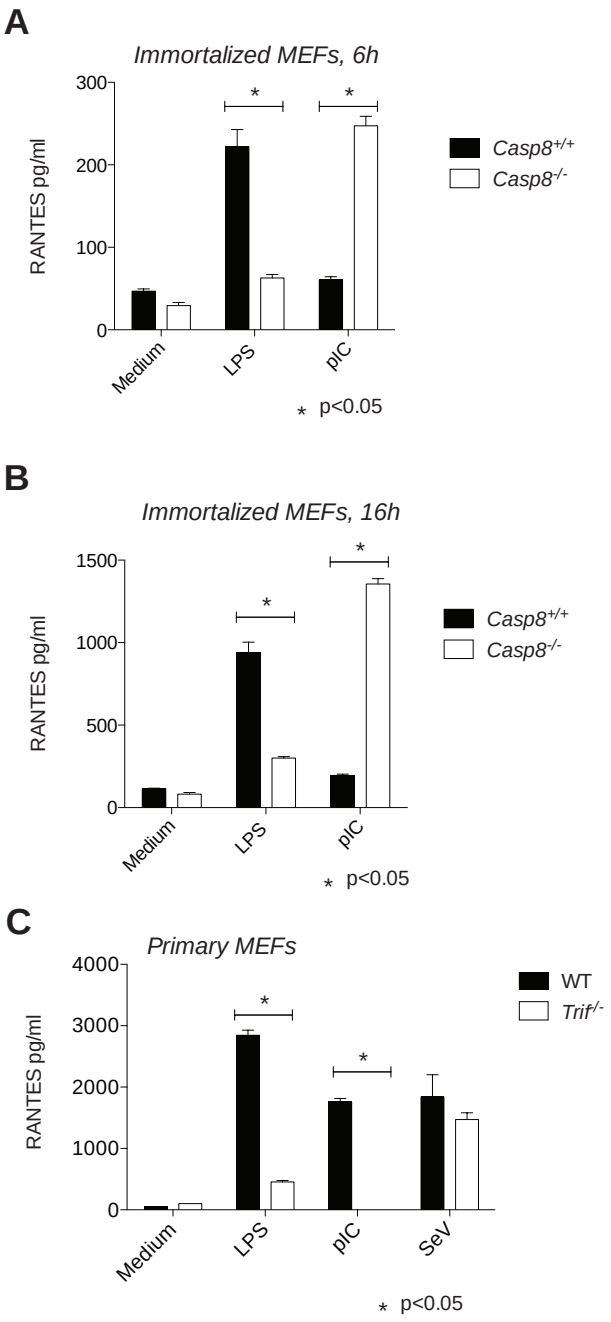


Figure 7. Caspase-8 regulates TRIF induced RANTES production in MEFs. Immortalized *Casp8*$^{+/+}$, *Casp8*$^{-/-}$ MEFs (A, B) or primary *Trif*$^{-/-}$ and C57BL/6 MEFs (C) were stimulated with LPS, pIC or SeV for 6h (A), 16h (B-C). Amount of RANTES released in the supernatants was measured by ELISA (A-C). Statistical analysis was performed on results in all panels as described in Materials and Methods section.

Strikingly, we observed that *Casp8*$^{-/-}$ MEFs completely lacked the expression of RIP3 protein, and resembled RIP3 KO cells (Figure 8A). Further investigation showed that *Casp8*$^{-/-}$ MEFs were completely deficient in the transcription of *Rip3* mRNA compared to *Casp8*$^{+/+}$ MEFs as witnessed by qRT-PCR (Figure 8B). The specificity of *Rip3* primers was verified using *Rip3*$^{-/-}$ BMDM (Figure 8C). However, the known RIP3 targeted allele that was used to make the *Rip3*$^{-/-}$ mice [300] was not detected in *Casp8*$^{-/-}$ MEFs (Figure 8D). Caspase-8 may regulate the expression of *Rip3*. However, there is a possibility that when cre was expressed to delete caspase-8 in immortalized MEFs, the expression of *Rip3* was suppressed to maintain their survival, since the embryonic lethality of *Casp8*$^{-/-}$ mice is rescued by RIP3 deficiency [238,301].

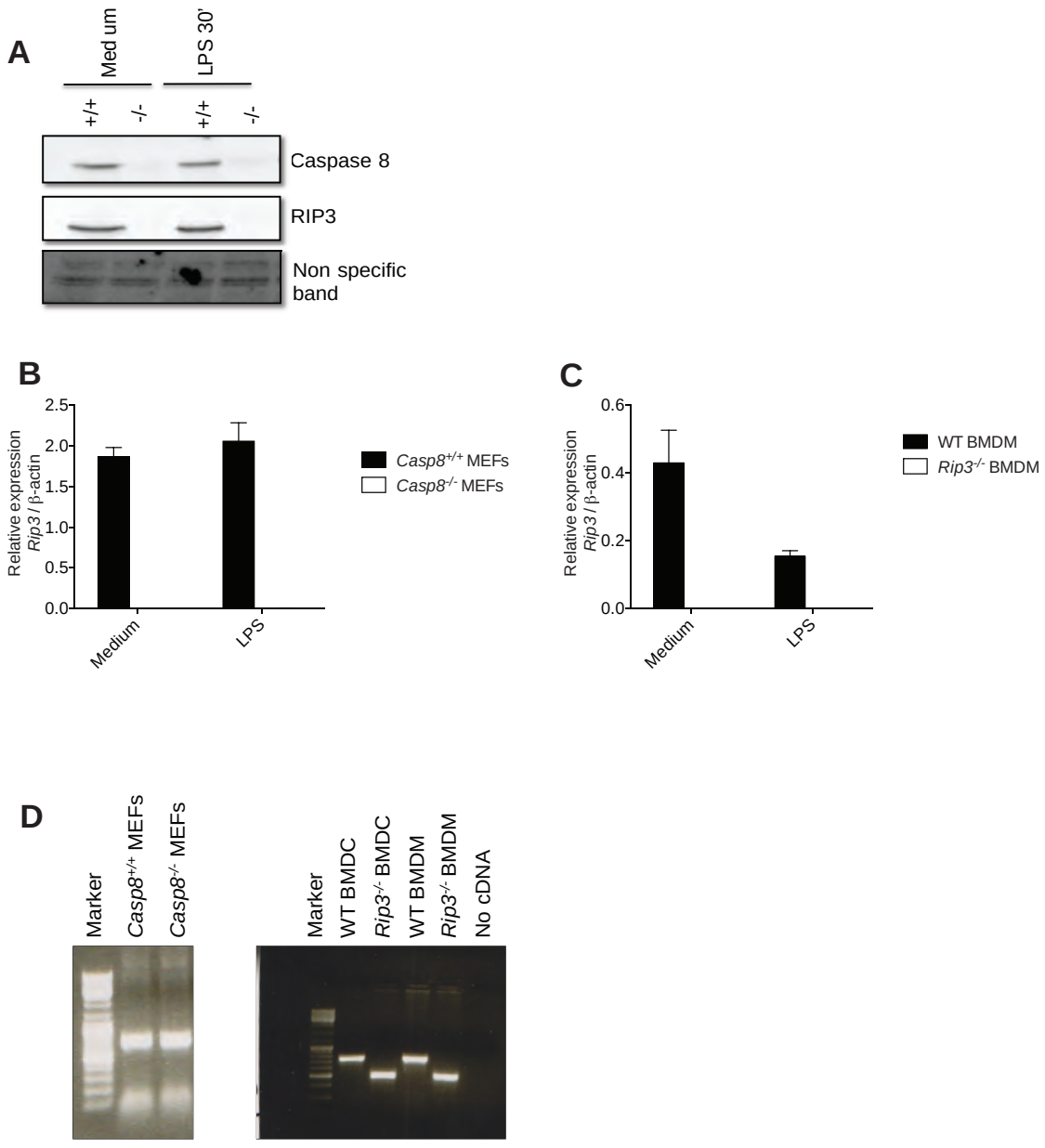


Figure 8. Lack of RIP3 expression in *Casp8*$^{-/-}$ MEFs. Immortalized *Casp8*$^{+/+}$, *Casp8*$^{-/-}$ MEFs were left untreated or stimulated with LPS for 30' (A) or 4h (B) and cell lysates were probed for caspase-8, RIP3 by western blotting (A). Non-specific band in the caspase-8 blot indicates equal loading. Levels of *Rip3* mRNA in *Casp8*$^{+/+}$, *Casp8*$^{-/-}$ MEFs (B) and *Rip3*$^{+/+}$, *Rip3*$^{-/-}$ BMDM (C) were quantified by qRT-PCR using the same set of primers. *Casp8*$^{+/+}$, *Casp8*$^{-/-}$ MEFs and *Rip3*$^{+/+}$, *Rip3*$^{-/-}$ BMDM/BMDC were genotyped for the presence of targeted *Rip3* allele generated by Newton, K. et al. [300](C).

To address if the cytokine phenotypes observed in MEFs were due to the absence of RIP3 or caspase-8, *Casp8*$^{-/-}$ MEFs were separately reconstituted with RIP3 or caspase-8 by retroviral transduction. Empty pQCXIH vector (EV), or encoding full-length mouse RIP3 or RIP3 whose 4 amino acid RHIM domain has been substituted with alanines (ΔRHIM) were used (Figure 9A). Similarly, *Casp8*$^{-/-}$ MEFs were reconstituted with empty pMSCV vector (EV), encoding full-length mouse caspase-8 (FL), its catalytic site mutated (C362A) or a self-processing mutant (D387A) (Figure 9B). Constitutive expression of reconstituted RIP3 or caspase-8 was detected using antibodies to Flag or HA tags (Figure 9A and B). Interestingly, reconsititution of caspase-8 didn't revert RIP3 expression, indicating that caspase-8 may not directly control RIP3 expression (Figure 9B). These reconstituted cells provided a tool to attribute the RANTES phenotype in MEFs to the loss of RIP3 or caspase-8 directly.

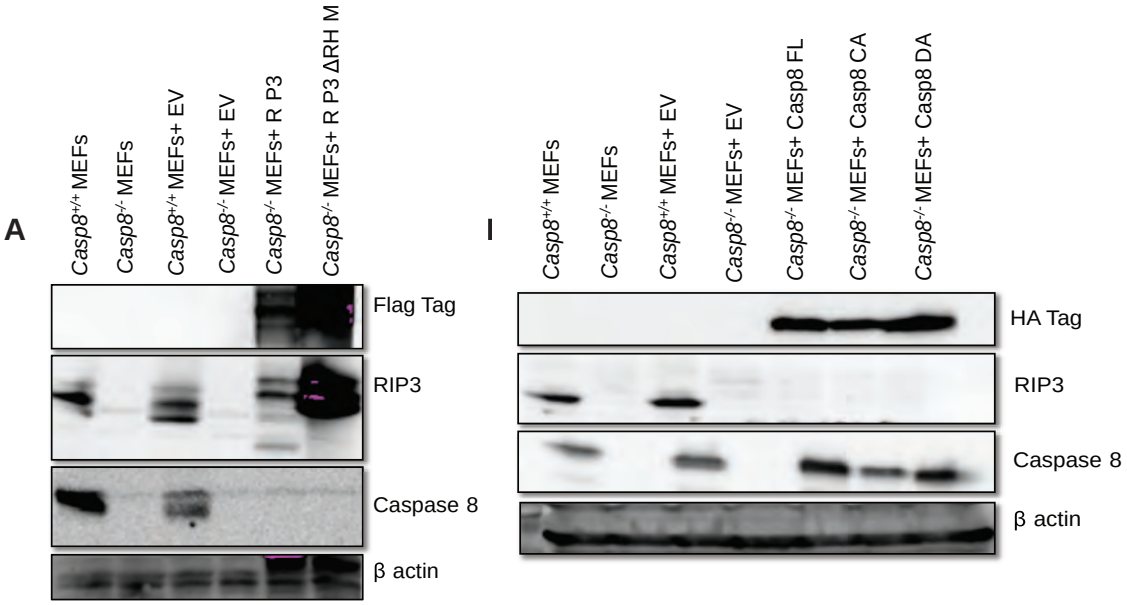


Figure 9. Reconstitution of caspase-8 or RIP3 in *Casp8*$^{-/-}$ MEFs. Immortalized *Casp8*$^{+/+}$, *Casp8*$^{-/-}$ MEFs were reconstituted with retroviral vectors pQCXIH encoding empty vector (EV), full length mouse RIP3, or RIP3 with mutated RHIM domain (A) or pMSCV with full length mouse caspase-8, catalytic mutant [CA] or self-processing mutant [DA] (B). Anti-Flag (A) or anti-HA (B) antibodies to detect the vector-expressed proteins, and endogenous caspase-8, RIP3, β-actin in the lysates were detected by western blotting (A, B).

First, the RIP3 reconstituted MEFs were tested for RANTES production in response to LPS and pIC. While the elevated RANTES production in pIC stimulated *Casp8*$^{-/-}$ MEFs was substantially suppressed by the presence of FL RIP3, RIP3 with a mutated RHIM could not replicate this effect (Figure 10A). In contrast to pIC, the reduced RANTES production in response to LPS witnessed in *Casp8*$^{-/-}$ MEFs was not rescued by FL or RHIM mutated RIP3 (Figure 10B). On the other hand, when caspase-8 reconstituted MEFs were tested for RANTES production in response to pIC, once again, the higher RANTES in *Casp8*$^{-/-}$ MEFs was decreased by the expression of FL as well as self-processing deficient caspase-8, but not catalytically the inactive molecule (Figure 10C). Spontaneous RANTES was observed basally in untreated empty vector or caspase-8 reconstituted cells (Figure 10C) which masked the reduced RANTES phenotype with LPS observed in non-reconstituted *Casp8*$^{-/-}$ MEFs (Figure 7A and B). Hence, these cells could not be successfully employed to study the effect on LPS induced RANTES. Together, these results suggested that both catalytically active caspase-8 (but not necessarily cleavable) and RHIM sufficient RIP3 are involved in blocking pIC induced RANTES production. Since RIP3 was not required for LPS induced RANTES production, it can be inferred that caspase-8 may solely be responsible for this phenotype in MEFs.

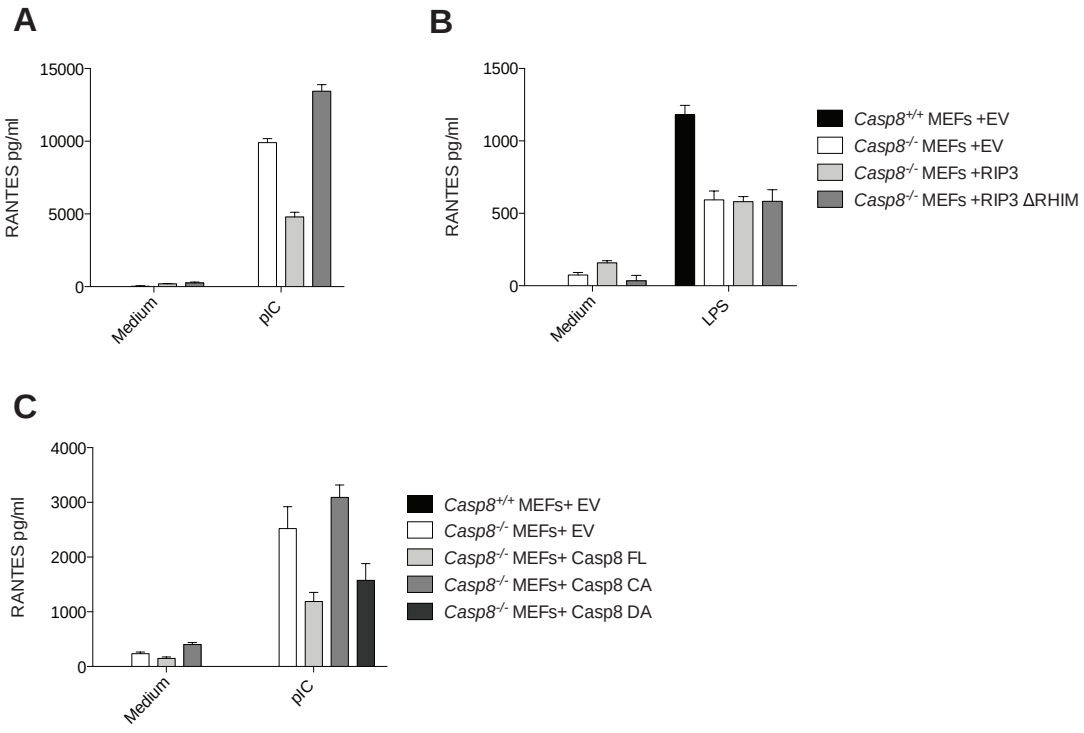


Figure 10. RANTES phenotype in reconstituted *Casp8*$^{-/}$ MEFs. Immortalized *Casp8*$^{+/+}$, *Casp8*$^{-/-}$ MEFs reconstituted with empty vector, full length or mutated versions of RIP3 (A-B) or caspase-8 (C) were stimulated with LPS or pIC for 16h. Amount of RANTES released in the supernatants was measured by ELISA (A-C).

Since the reconstitution experiments in MEFs indicated that caspase-8 and RIP3 co-regulate some of the cytokine phenotypes, we sought to verify whether the phenotypes that were observed in macrophages and dendritic cells were due to caspase-8 or RIP3. To this end, we utilized BMDM from mice that had been constructed with floxed *Casp8* allele expressing cre under the control of the myeloid cell lineage specific LysM promoter (generated by D. Shayakhmetov). Amount of caspase-8 protein was markedly decreased in the lysates of the LysMcre *casp8*$^{-/-}$ macrophages indicating that this was a good system to study the specific role of caspase-8 (Figure 11A). Since caspase-8 deficiency has been shown to lead to hyper-inflammation and RIP3 mediated necrosis [302,303], we directly tested the production of the cytokine IL-1β, which is normally regulated by 2 steps of synthesis and processing in wild-type cells. Indeed, treatment with LPS and pIC, which induce pro-IL-1β synthesis, but not processing or release in WT cells, triggered high levels of IL-1β production in *casp8*$^{-/-}$ macrophages (Figure 11B). Such a spontaneous IL-1β production was not observed in *casp8*$^{-/-}$*Rip3*$^{-/-}$ cells stimulated with just Pam2CSK4 or LPS (Figure 11C), indicating that IL-1β noticed in *casp8*$^{-/-}$ macrophages is due to RIP3. Since caspase-8 is also known to suppress RIP3 mediated necrosis, cell death was examined in these WT and *casp8*$^{-/-}$ macrophages. Once again, even though LPS or pIC normally do not trigger macrophage death (Figure 11D), *casp8*$^{-/-}$ cells which harbor increased RIP3 expression showed higher loss in viability especially in response to LPS and pIC. This could be due to the ability of the adaptor molecule TRIF to recruit caspase-8 and RIP3. Thus, on one hand,

experiments in *casp8*$^{-/-}$*Rip3*$^{-/-}$ macrophages and dendritic cells showed that caspase-8 is required for TLR4 induced IL-1β. On the other hand, caspase-8 deleted macrophages exhibit RIP3 mediated loss in inflammasome regulation, elevated IL-1β production and cell death in response to just TLR signals. Caspase-8, in addition to directly modulating TLR pathways, strongly suppresses RIP3 whose regulatory effects are unleashed only when caspase-8 is absent, but not in WT, *Rip3*$^{-/-}$ or in *casp8*$^{-/-}$*Rip3*$^{-/-}$ cells. In essence, these results show intricate interplay between RIP3 and caspase-8.

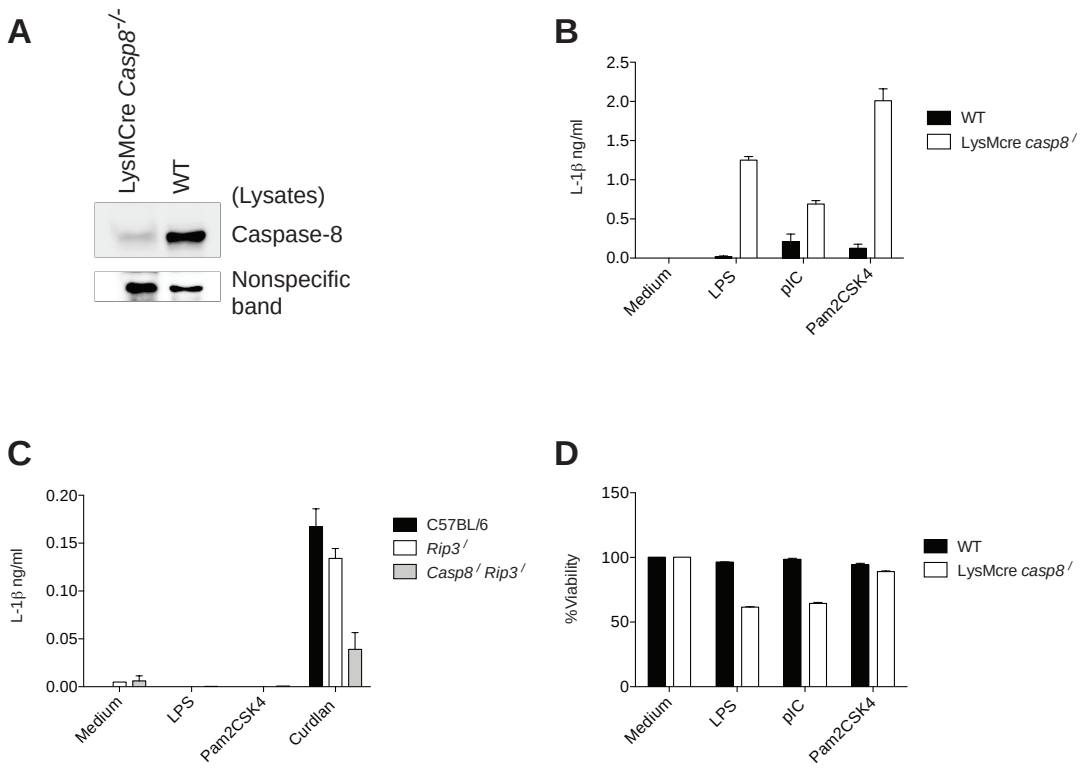
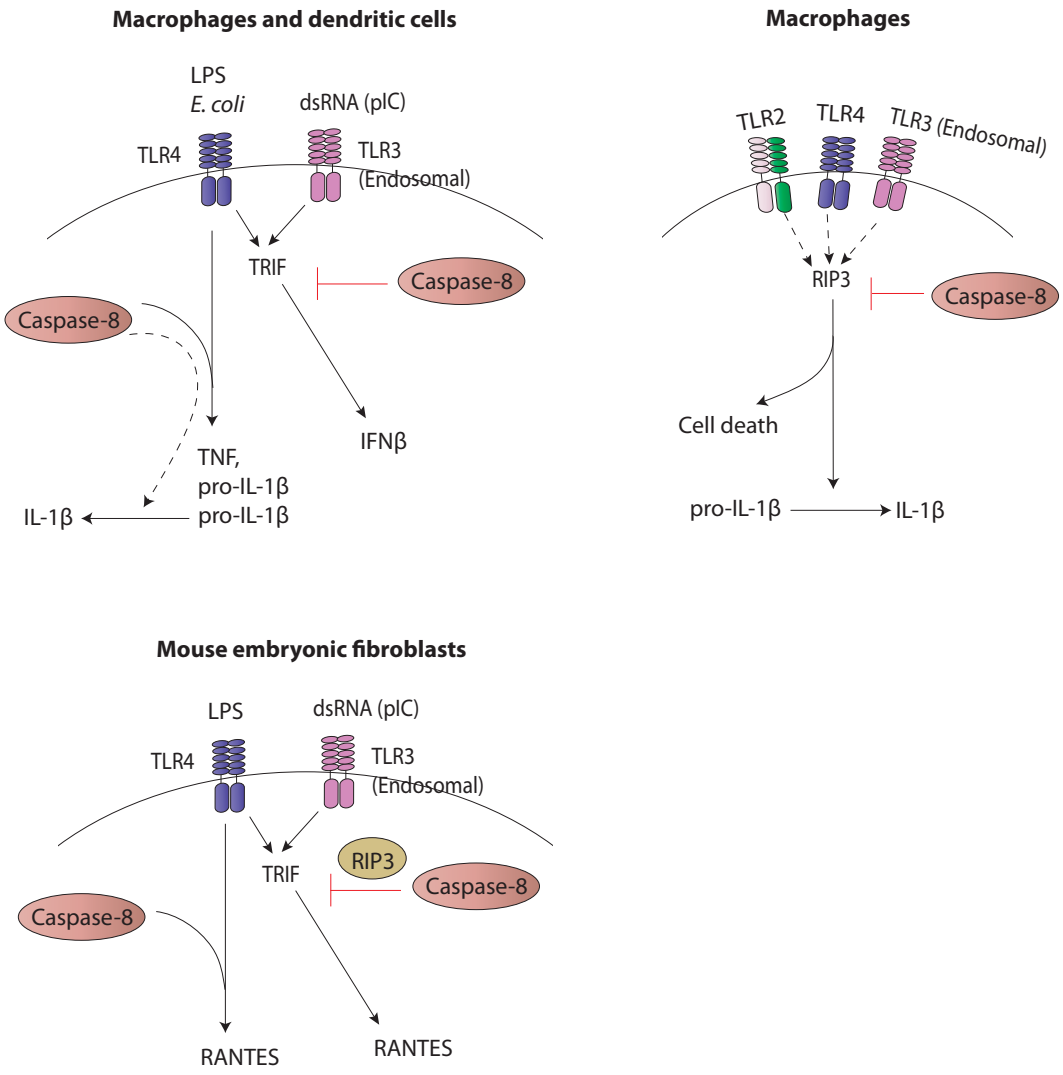


Figure 11. TLR induced IL-1β and cell death is higher in caspase-8 deficient macrophages. Bone marrow derived macrophages from WT and LysMcre *casp8*$^{-/-}$ mice were left untreated and probed for caspase-8 (A). A non-specific band indicates equal loading (A). WT and LysMcre *Casp8*$^{-/-}$ BMDM (B, D) or WT, *Rip3*$^{-/-}$ and *casp8*$^{-/-}$*Rip3*$^{-/-}$ BMDM (C) were stimulated with LPS, pIC or Pam2CSK4 for 6h (B, D) or 24h (C). Amount of IL-1β released in the supernatants was measured by ELISA (B, C). Viability of cells was measured by cell-titer glo assay (D).



Schematic model 8. Caspase-8 and RIP3 differentially regulate TLR signaling pathways and cytokine production. Caspase-8 promotes TLR4 (LPS, *E. coli*) induced TNF, pro-IL-1β synthesis, likely IL-1β maturation and release in macrophages and dendritic cells. Similarly, caspase-8 is required for LPS induced NF-κB activation and RANTES production in MEFs. In contrast, caspase-8 down regulates TRIF mediated IFNβ production in macrophages and dendritic cells. Caspase-8 also blocks RIP3 triggered IL-1β maturation and cell death downstream of many TLRs in macrophages. A similar phenomenon is observed in MEFs where enzymatically active caspase-8 in collaboration with RHIM domain- sufficient RIP3 suppresses TLR3, but not TLR4 induced RANTES production.

Discussion

The results shown in this chapter demonstrate that caspase-8 differentially regulates various innate immune pathways. While caspase-8 promotes NF-κB dependent cytokine production, it negatively impacts pathways leading to IFNβ production. Specifically, caspase-8 is required for optimal production of LPS induced TNF, pro-IL-1β in macrophages, dendritic cells and RANTES in MEFs. On the other hand, caspase-8 suppresses TRIF induced IFNβ production in macrophages, dendritic cells and TLR3 induced RANTES in MEFs (Schematic model 8). These findings have broad implications since caspase-8 and the cytokines studied here such as TNF, type I IFN and RANTES are expressed not only in myeloid innate immune cells, but also other cells such as T cells, B cells, NK cells, epithelial cells and fibroblasts. The quality and amplitude of cytokine regulation may be customized depending on the cell type. And the fact that these cells are equipped with the molecular machinery for death receptor triggered apoptosis and necrosis indicates that the role of caspase-8 may co-regulate TLR signaling and cell death in these cells. Importantly, IL-1β production in response to *E. coli* was also severely impaired in *casp8*$^{-/-}$ *Rip3*$^{-/-}$ indicating that the role of caspase-8 in TLR signaling has actual significance in anti-microbial immune responses (Schematic model 8). Consistent with our studies, many studies published recently show a strong requirement for caspase-8 in induction of NF-κB dependent genes such as pro-IL-1β, NLRP3, TNF, IL-6 in response to LPS or gram-negative bacteria such as *Escherichia coli*, *Citrobacter rodentium* [304,305].

The molecular mechanism underlying the differential cytokine induction is not currently clear. Our preliminary experiments mutating the catalytic cysteine of caspase-8 indicated that the enzymatic activity is important for at least some of its functions such as promoting LPS induced RANTES production in MEFs (Figure 9C). Caspase-8 may directly target specific substrates to exert its effect on NF-κB and IRF3 activation. One potential candidate is Cylindromatosis (CYLD), a de-ubiquitinating enzyme that is important for type I IFN mediated anti-viral immunity, but dampens NF-κB and JNK activation downstream of certain TLRs [306-308]. K63 ubiquitinated proteins in the TLR and RIG-I pathways such as TRAF2, NEMO or RIP1 can serve as substrates for CYLD's de-ubiquitinating activity under different conditions [306,309]. Strikingly, caspase-8 has been shown to cleave CYLD at a conserved site, Asp215, the neighboring sequence being LESD^{215}FAGPG, which resembles that of the IMD cleavage site, LEKD^{30}AAPV [259,310]. In fact, CYLD appears to have a conserved IAP binding motif [A(G/A/S)P(V/A/G)] following its cleavage site, similar to IMD [AAPV] and to many other IAP binding proteins [259,310,311]. Caspase-8 targeting of CYLD may explain its opposing roles, namely the positive and negative regulatory roles in the activation of NF-κB and type I IFN, respectively. Interestingly, *Drosophila* CYLD (dCYLD) regulates the IMD signaling to control *Diptericin* expression and anti-bacterial immunity [312,313]. However, the precise function of dCYLD in IMD pathway, whether it can de-ubiquitinate IMD; is cleaved by DREDD are yet to be determined and will provide additional insights about the interplay between caspase-8, CYLD, RIP1 and NF-κB.

Another characterized target of caspase-8 is RIP1 that is an integral component of TRIF and RIG-I signaling. It has been shown that cleavage of RIP1 at D324, the same site at which cleavage occurs during apoptosis, inhibits RIG-I triggered IFN production [276]. In addition, caspase-8 has also been shown to cleave IRF3 and target IRF3 for proteasomal degradation downstream of TLR3 and RIG-I signaling [314]. These mechanisms could explain the negative regulatory effect on TRIF mediated IFN and RANTES production. Although the molecular specifics of a variety of cytosolic nucleic acid sensing pathways leading to type I IFN are just emerging [315], it is reasonable to hypothesize that RIP1 may be an important part of many of these pathways considering its role in DAI, RIG-I and TRIF dependent IRF3 activation [251,276,287,316]. This opens another category of pathways that could be regulated by caspase-8, which in turn will have an immense impact on the normal homeostasis, anti-viral immunity and autoimmune disorders.

It was very surprising that opposite phenotypes were observed between TLR3 and TLR4 induced RANTES production in caspase-8 deficient MEFs. TRIF signaling is usually considered to be similar downstream of TLR3 and TLR4 pathways. One possible explanation for this differential RANTES phenotype is that the positive effect of caspase-8 is in the MyD88 pathway while its negative regulatory effect arises from its engagement in TRIF signaling. And in the scenarios where both MyD88 and TRIF signaling are in effect, like that of the

TLR4 pathway, maybe one gains dominance over the other. Such a differential function of caspase-8 in TLR signaling can be direct consequence of caspase-8 targeting certain molecules that have opposing effects on MyD88 and TRIF signaling. For example, TRAF3 a common signaling component of TLR4 and TLR3 pathways, is degraded by K48 ubiquitination by cIAPs for the propagation of MyD88-AP1 signaling while it is self-K63 ubiquitinated to sustain TRIF-IRF3 signaling [317]. On a similar note, an E3 ubiquitin ligase NRDP1 which is known to promote cspase-8 mediated apoptosis [318], augments NF-κB and AP-1 activation while down regulating IRF3 and type I IFN production [319]. Finding other molecules with similar properties of differentially regulating TLR-cytokine pathways may provide clues to how caspase-8 regulates innate immune functions. It is not currently clear if these potential caspase mediated proteolytic events are specific or by-product reaction of caspase-8-cFLIP down-modulating necrosis. However, studies on caspase-8 will also help us understand the nature of intricate cross regulation in TLR signaling.

In the context of IL-1β, caspase-8 operates at the level of pro-IL-1β as well as IL-1β processing. While caspase-8 may regulate NF-κB activation and pro-IL-1β induction by any of the aforementioned mechanisms, caspase-8 may directly cleave pro-IL-1β at the same site as caspase-1, cleave or modulate the activity of caspase-1 following inflammasome activation for optimal processing of IL-1β and IL-18 (Schematic model 8) [305,320].

Highly conserved molecular partners of caspase-8 such as FADD and cFLIP have also been implicated in innate immune pathways. c-FLIP and FADD deficient mice are also embryonic lethal around day 9 or 10.5, exhibiting similar defects as caspase-8 KO indicating these proteins may act together in a complex during embryonic development[321,322]. The embryonic lethality of *Fadd*$^{-/-}$ mice is also protected by RIP3 deficiency similar to that of caspase-8 [321,323]. cFLIP and FADD mediate homeostatic immune cell functions such proliferation, survival and antibody responses similar to caspase-8 in T and B cells [324-328]. *Rip3*$^{-/-}$ *Fadd*$^{-/-}$ macrophages are also deficient in pro-inflammatory cytokine production in response to LPS and certain gram-negative bacteria similar to *Rip3*$^{-/-}$*Casp8*$^{-/-}$ cells [305]. Further, defective caspase-8 processing was observed in *Rip3*$^{-/-}$*Fadd*$^{-/-}$ macrophages in response to gram-negative bacteria and LPS priming [305]. Although the physiological relevance of caspase-8 processing is not quite clear, since caspase-8 can be active both under cleaved/uncleavable circumstances [301], it implicates FADD upstream of caspase-8 in the TLR4 signaling pathway.

FLIP is generated as 2 proteins through alternative splicing namely, $cFLIP_L$ (long form containing two DED domains and the catalytically inactive p10 and p20 segments) and $cFLIP_S$ (short form containing just the two 2 DED domains). $cFLIP_S$ and its viral homolog $vFLIP_S$ are considered to operate as dominant negative partners of caspase-8 and inhibit its apoptotic functions. It is compelling to speculate that caspase-8 may heterodimerize with c-$FLIP_L$ but retain its catalytic activity, as has been observed in other protective functions [301],

to control innate immune pathways. Thus, caspase-8 may operate in a complex with FADD and $cFLIP_L$ in PRR signaling.

Further, it should be noted that although “NF-κB” in the chapters refers to RelA-p50 complex, NF-κB family comprises of 5 members, RelA, RelB, c-Rel, p50 and p52. Caspases and RIP kinases may play a role in regulating the activation of any of these other proteins as well depending on the cell type. For example, the paracaspase MALT1 cleaves RelB at Arg85, not at Asp, which enhances the induction of RelA and c-Rel dependent genes in lymphoid cells [329]. Although not similar, this proteolytic event is reminiscent of DREDD directly targeting Relish in the *Drosophila* IMD pathway. Inflammasome activated caspase-1 can cleave caspase-7 which can in turn cleave nuclear PARP1 to relieve its suppression on certain NF-κB target genes in human monocytes [330]. Intriguingly, caspase-3 cleaves MyD88 and p65, in the final stages of apoptosis to block NF-κB activation [331,332]. Thus other apoptotic caspases can also indirectly regulate NF-κB dependent responses. It would also be exciting to investigate the role of caspases-3, -7 and -9 in innate immunity to expand our understanding in this context.

Overall, we have shown that caspase-8 promotes NF-κB activation, and induction of proinflammatory genes such as TNF, pro-IL-1β, while on the other hand negatively regulates TRIF mediated IFNβ and RANTES production depending on the cell type. In addition, caspase-8 protects cells from RIP3

mediated necrosis and de-regulated processing and maturation of IL-1β. Additional research is warranted to gain a comprehensive understanding of the novel role of proteins classically involved in different cell death processes such as apoptosis, necrosis, and pyroptosis, in innate immune signaling. Efforts in this direction may identify targets that can be manipulated for therapeutic purposes.

Materials and Methods

Reagents: Sendai virus was obtained from Charles River Laboratories (Boston, MA). Long synthetic pIC (High Molecular Weight poly I:C) is from Invivogen (tlrl-pic). Curdlan, R848, Pam2CSK4 and Pam3CSK4 were also from Invivogen. *E. coli* J5 (Rc) LPS was purchased from Sigma.

Mice and femurs: Mice were maintained and bred at UMASS Medical School in accordance with the Institutional Animal Care and Use Committee (IACUC). *Casp8*$^{+/-}$*Rip3*$^{-/-}$ mice were kindly provided by Dr. Douglas Green (St. Jude Children's Research Hospital) and in some cases *Casp8*$^{-/-}$ *Rip3*$^{-/-}$, *Casp8*$^{+/+}$ *Rip3*$^{-/-}$ femurs were from Dr. Bill Kaiser and Dr. Edward S. Mocarski (Emory University School of Medicine) and *Rip3*$^{-/-}$ mice were from Dr. Francis Chan (UMASS Medical School). *Casp8*$^{+/-}$*Rip3*$^{-/-}$ mice were on a mixed C57/BL6-129 background and were intercrossed to generate *Casp8*$^{-/-}$*Rip3*$^{-/-}$ and *Casp8*$^{+/+}$*Rip3*$^{-/-}$ mice, which were used as littermate controls in all of our experiments. All other mice were on a C57/BL6 background. Femurs of caspase-8 fl/fl, LysMcre+/+ mice were kindly provided by Dmitry. M. Shayakhmetov. Dr. Razq Hakem (University of Toronto) kindly provided *Casp8*$^{+/+}$ MEFs, which are MEFs derived from caspase-8 fl/fl mice and then E1A/Hras transformed. *Casp8*$^{-/-}$ MEFs are referred to the MEFs which were prepared by the same procedure and then made to express Cre to delete caspase-8.

EHEC culture conditions: EHEC (Enterohemorrhagic *Escherichia coli*, strain

O157:H7, EDL 933) was maintained as glycerol stocks and cultured overnight in LB media, at 37°C, 250 rpm and used in stationary phase at MOI 25 for experiments as previously described [174].

BMDC and BMDM culture: BMDM and BMDC were cultures as described in 'Materials and methods' section in chapter II. For ELISAs, BMDC or BMDM were plated in 96 well plates at 1 x 10^6 cells/ml. Cells were stimulated with 100ng/ml Pam2CSK4, 10 or 100ng/ml LPS, 10µM Pam3CSK4, 25µg/ml pIC, 40 or 200HAU/ml SeV, 1mg/ml Curdlan (I) for 6h, 16h or 24h as indicated. In the case of EHEC, media was replaced with fresh gentamicin containing media after 1h of infection. Cytokine levels were measured in triplicate and data are representative of two or three independent experiments.

For western blot analysis, cells were plated at 2 x 10^6 cells/ well in 12 well plates in serum free, antibiotic free medium, stimulated as described above. Cells were lysed with 1% NP-40 lysis buffer and Bradford assay was performed to normalize the amount of protein. Samples were mixed with 4X SDS loading dye with β-ME, boiled for 10 mins at 100°C and then run on 12% SDS-PAGE and transferred to nitrocellulose membrane. Western blotting was performed with the antibodies to caspase-1 p10 (sc-514, Santa Cruz Biotechnology), caspase-1 p20 (Casper1 clone; Adipogen), IL-1β (AF-401-NA, R&D Systems), caspase-8 (1G12, Enzo Life Sciences), RIP3 (2283, Pro-Sci) and α-tubulin (2125, Cell signaling).

ELISA: BD Biosciences (Cat # 559603) or eBioscience (Cat # 88-7013-77, 88-7324-77) kits were used to measure the amount of IL-1β and TNF present in cell culture supernatants according to manufacturers' instructions. RANTES in the supernatants was measured by a kit from R&D systems (DY478). For IFNβ, a sandwich ELISA that has been previously described was used [226].

Plasmids and cloning: Plasmids encoding mouse caspase-8, RIP3 and their mutants were kindly provided by Dr. Bill Kaiser (Emory University School of Medicine) and Dr. Douglas Green (St. Jude Children's Research Hospital). We obtained pBABE_2a caspase-8 encoding either full-length caspase-8, caspase-8 mutated at its catalytic cysteine C362A (TGC>GCC) and self-processing mutant of caspase-8 D387A (GAT>GCT). Clontech vector pQCXIH expressing full-length RIP3 or RIP3 with its core RHIM motif mutated to 4 alanines were also obtained from them.

Caspase-8 was digested out from these plasmids and cloned into the retroviral vector pMSCV which has a neomycin selection marker with the protocol described below. Ampicillin was used to select for the growth of bacteria containing plasmids pBABE, pMSCV and pQCXIH.

Cloning of caspase-8:

The following primers were used to clone caspase-8 from the pBABE plasmid.

5'-TTGACAGAATTCATGGATTTCCAGAGTTGT-3' (F)

5'-ATCCACCTCGAGGGGAGGGAAGAAGAGCTT-3' (R)

50µL PCR reaction mix:

2.5µl of F and R primers (10µM final)

10µl of 10X enzyme buffer

1µl of 10mM dNTP

1µl Phusion enzyme

100ng DNA

Make up to 50µl with MilliQ water

PCR conditions:

Preheat 95°C 2min

94°C 45sec

55°C 45sec

72°C 1min 10sec

35 cycles

72°C 10mins

4°C~

5µl of the PCR product was run out on a 2% gel to verify the product size and the rest was run on a 1% gel and extracted (Qiaquick gel extraction kit) to 50µl final volume.

Insert digestion:

The eluted PCR product was digested using XhoI and EcoRI.

A 50µl reaction mix containing

5µl of 10X NEB buffer IV

All of the eluted PCR product

1.5µl each of EcoRI and XhoI

0.5µl BSA (100X)

and the rest MilliQ water was incubated at 37°C for 3hrs, ran on a 1% gel and eluted to 20µl volume.

Vector digestion:

pMSCV vector was digested with EcoRI and BglII.

A 50µl reaction mix containing

5µl of 10X NEB buffer II

5µg vector

1.5µl each of EcoRI and BglII

and the rest MilliQ water was incubated at 37°C for 3hrs, ran on a 1% gel and eluted to 40µl volume.

Annealing the oligos:

The following oligos were used to add His and HA tags and a specific BgIII site to the caspase-8 sequence to facilitate its insertion into the EcorI-BglII cloning site in the vector:

5'-

TCGAGTACCCCTACGACGTTCCCGACTACGCCGCACATCATCACCACCATC

ACTAGA-3'

5'-

GATCTCTAGTGATGGTGGTGATGATGTGCGGCGTAGTCGGGAACGTCGTAG

GGGTAC-3'

2.5µl each of 100µM oligos was mixed with 94µl TE buffer and 1µl of 5M NaCl, heated at 95°C for 10mins and allowed to cool slowly to RT after turning off the heat.

Ligation:

A 10µl reaction mix containing

1µl 10X T4 DNA ligase buffer

1µl ligase

1.5µl vector (pMSCV vector)

1µl annealed oligo

5.5µl insert (Caspase-8 sequence)

was prepared, left on bench O/N and transformed the next day.

Transformation:

The ligated mix was mixed with 20µl competent bacteria, incubated on ice for 10 mins followed by a heat shock of 42°C for 45 seconds and left to rest on ice. This mix was then added to 50µl of LB/SOC media and incubated at 37°C for 1h in 200rpm shaker. 20µl of this mix was then plated on LB agar plates with ampicillin and left overnight in 37°C. Colonies were inoculated in 5ml of LB media with ampicillin for overnight for miniprep.

Plasmids were examined for excision of insert by double digesting them with EcorI and BglII and confirmed by sequencing.

Retroviral transduction

Immortalized MEFs were transduced with retroviral pMSCV and pQCXIH plasmids using the following protocol. For each of the plasmids, HEK293T cells

were grown in DMEM with 10% HI FBS and Penstrep, were first transfected with a mix of

4µg of the corresponding plasmid,

1µg VSV

1µg gag pol

using 18µl Genejuice (Novagen) and 300µl Optimem (Gibco) per transfection. Media was collected after 48h and replaced with fresh media, which was again collected after 96h. The retrovirus containing supernatants (48 and 96h collection) were pooled, filtered through 0.45µM, mixed with complete DMEM culture media at a ratio of 1:1 and used to transduce MEFs. This mix was added on days 1 and 3 to the cells and on days 2 and 4, cells were placed in complete DMEM to recover. After day 4, cells were cultured in complete DMEM in the presence of neomycin selection (200-500µg/ml) for pMSCV transductions or hygromycin (200-400µg/ml) for pQCXIH transductions.

Quantitative RT-PCR: RNA was extracted from cells using RNeasy kit (QIAGEN). iScript Select cDNA synthesis kit (Bio-Rad) was used to synthesize cDNA from 1µg total RNA from each sample. Quantitative RT-PCR for RIP3 and β-actin was performed using iQ SYBR green supermix (Bio-Rad). Primers used for RIP3 were 5'-GGGACCTCAAGCCCTCTAAC-3' (F) and 5'-CTGGGTCCAAGTACGCTAGG-3' (R) and β -actin were 5'-TTGAACATGGCATTGTTACCAA-3' (F), 5'-TGGCATAGAGGTCTTTACGGA-3' (R). Levels of RIP3 mRNA were normalized to that of β-actin.

RIP3 genotyping:

RIP3F1 CGCTTTAGAAGCCTTCAGGTTGAC

RIP3F2 GCAGGCTCTGGTGACAAGATTCATGG

RIP3R CCAGAGGCCACTTGTGTAGCG

94°C 2min

94°C 30s

67°C 30s

72°C 1min

35 cycles

72°C 5min

4°C~

WT allele band: 700bp

KO allele band: 450bp

Statistics: Data were analyzed by the two-way analysis of variance followed by Bonferroni test using PRISM software. P value less than 0.05 was considered significant. For cytokine measurements, experiments were performed in triplicates and the graphs depicted are representative of atleast two or three independent experiments. The triplicates of the representative experiment shown were analyzed using two-way ANOVA. The significant comparisons are marked by an asterisk (p value < 0.05) and the unmarked groups in the same graph can be assumed to not be significant. In the case of experiments with *Casp8*$^{+/+}$

Rip3$^{+/+}$, *Casp8*$^{+/+}$ *Rip3*$^{-/-}$ and *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ dendritic cells, the groups *Casp8*$^{+/+}$ *Rip3*$^{-/-}$ and *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ were compared to assess significance.

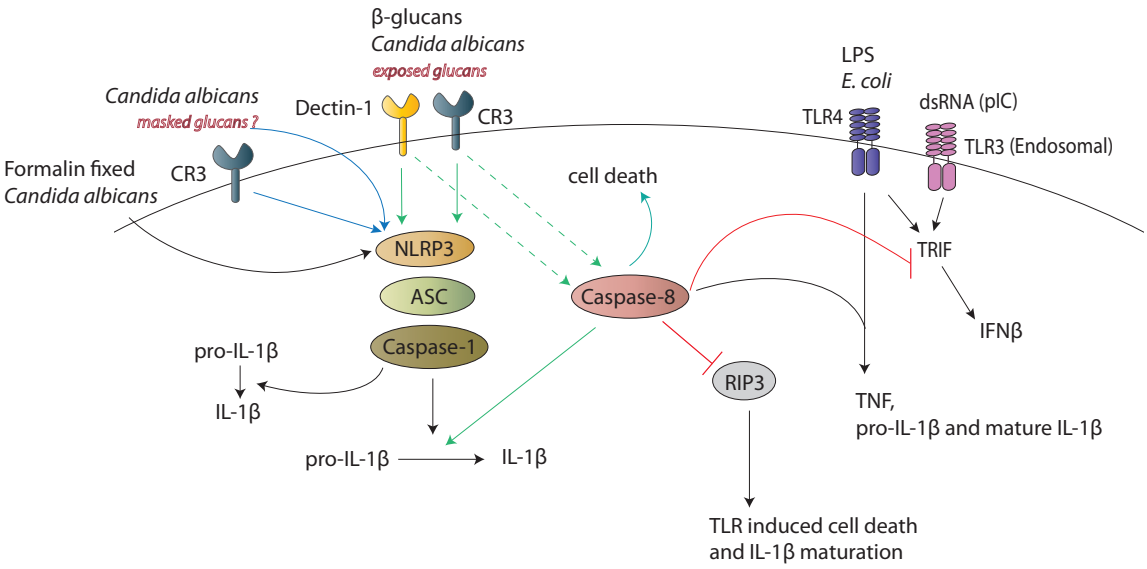
Chapter V:

Overall Perspectives

Chapter V: Overall Perspectives

NLRP3, NLRC4 inflammasomes and anti-fungal responses

This doctoral research work aimed towards better understanding the innate immune responses against the fungal pathogen *C. albicans*, with a specific focus on inflammasomes. Inflammasomes mediate the maturation of the pro-inflammatory cytokine IL-1β in response to a broad range of microbial infections, foreign as well as self-danger molecules. We observed that *C. albicans* triggers IL-1β production in a variety of mouse and human immune cells. Using formalin fixed preparations of different morphological forms of *C. albicans* such as yeast and hyphae; or live and heat-killed *C. albicans*, the mechanisms underlying the production of IL-1β were investigated. NLRP3-ASC-caspase-1 inflammasome was found to be critical in triggering IL-1β production in response to all tested preparations of *C. albicans* (Schematic model 9). This strongly indicated that irrespective of the morphological stage of *C. albicans*, exposed/masked state of the polysaccharide structures in its cell wall and the virulence or invasiveness, NLRP3 inflammasome is activated and is crucial for IL-1β maturation and the ensuing host immune responses. The fact that NLRP3, but not NLRC4 is required for IL-1β production in hematopoietic innate immune cells (Chapter I) highlights the specificity and the importance of understanding this central NLRP3 inflammasome pathway in the context of *C. albicans*.



Schematic model 9. *C. albicans* and β-glucan induced inflammasome responses and caspase-8 mediated regulation of TLR and inflammasome signaling. NLRP3-ASC-caspase-1 inflammasome pathway is critically required for *C. albicans* and β-glucan induced IL-1β maturation. Dectin-1 and CR3 mediate inflammasome activation and IL-1β maturation in response to β-glucans, while only CR3 is required to different extent for live or heat-killed *C. albicans* induced IL-1β. Caspase-8 promotes β-glucan and heat-killed *C. albicans*, but not live *C. albicans* induced IL-1β production. Dectin-1, CR3 and caspase-8 also mediate a cell death response induced by β-glucan and heat-killed *C. albicans*, but not live *C. albicans*. The the axes specific for β-glucans and HK *C. albicans* are highlighted in green and the pathway for live *C. albicans* is marked in blue. Dotted arrows indicate inferred functional associations. Caspase-8 also regulates TLR4 and TLR3 induced pro-inflammatory cytokine production. Caspase-8 promotes TLR4 induced TNF, pro-IL-1β synthesis and IL-1β production while negatively regulating TRIF mediated IFNβ in macrophages and dendritic cells. Caspase-8 also protects macrophages from RIP3 mediated cell death and pre-mature activation of inflammasome responses in response to TLR signals.

Our studies on the inflammasome responses to *C. albicans*, along with important *in vivo* data from the Dr. Hise's group, present a model for the overall mobilization of the immune system against the pathogen. In the mouse OPC model of infection, NLRP3 and NLRC4 inflammasomes are both required for anti-fungal immune responses [135]. While NLRP3 inflammasome responses are critical both in hematopoietic as well as the stromal compartments for protecting the host, NLRC4 plays an important role in the stromal compartment at the site of infection [135]. The NLRP3 and NLRC4 inflammasomes control cytokine production (IL-1β), anti-microbial mechanisms (defensins and other peptides), leukocyte (neutrophils) recruitment to the site of infection, and development of protective Th17 adaptive immunity, all of which come together to efficiently restrict the dissemination of the pathogen and promote the survival of infected mice [66,135]. It is not currently clear if NLRP3 and NLRC4 directly interact. However, such co-operativity among different NLR and PYHIN inflammasomes has been witnessed during infection of intracellular pathogens such as *Salmonella* and *Listeria* [152,333]. It is yet to be determined if NAIP proteins or NLRC4 can directly be activated by *C. albicans*. Mucosal biofilms in a mouse model of OPC reveals the presence of commensal bacterial species in addition to yeasts, hyphae and inflammatory cells [139]. This raises the possibility of NLRC4 responding to other commensal bacterial species during *C. albicans* infection, which can potentially arise due to the anti-microbiotic treatment given to mice, prior to infection.

IL-1 family of cytokines plays an essential role in anti-*Candida* responses. While our primary focus in these studies was on IL-1β, other IL-1 members such as IL-1α, IL-33 and IL-18 also play important roles in protecting the host from disseminated candidiasis (chapter II discussion)[122,123,334]. Further studies are also required to identify the role of NLRP3 and NLRC4 in the context of other IL-1 cytokines.

Dectin-1, CR3 in inflammasome and anti-fungal responses

Upstream phagocytic receptors dectin-1 and CR3 are critical for fungal recognition and subsequent innate immune responses. Previous studies have shown that dectin-1 deficient mice fail to adequately clear *C. albicans* in oral or systemic infection or *A. fumigatus* in pulmonary infection models respectively [38,39,84,335]. This indicates that sensing of the fungus by dectin-1 is important to tackle the infection *in vivo*. Further, a polymorphism Y238X identified in the extracellular CRD domain of human dectin-1 has been associated with susceptibility to mucocutaneous candidiasis [40]. Importantly, this mutant dectin-1 does not localize to the cell surface and has impaired β-glucan binding and IL-1β inducing capacity, [40,121,336]. Similarly, patients who had a Q295X polymorphism in the adaptor molecule CARD9 suffered from recurrent fungal infections [337]. This polymorphism introduces an early stop codon and impairs CARD9 of its ability to mediate TNF production in response to dectin-1 ligands [337]. These findings point out the conserved nature of these pathways, and that dectin-1-CARD9 expression and signaling are key components of anti-fungal defenses in humans as well. CR3 deficiency in mice also results in impaired anti-fungal responses

and patients with leukocyte adhesion deficiency (who lack CD18 and hence CR3 expression) suffer from *Candida* and *Aspergillus* infections [81,338]. Together, these findings paint a picture of an essential role for the dectin-1 and CR3 signaling pathways in anti-fungal host defenses in humans and whole animal models.

We observed that dectin-1 and CR3 are required for the activation of inflammasomes against β-glucan particles. CR3 is also required for *C. albicans* induced IL-1β, more so when the fungus is heat-killed, presumably exposing its glucans (Schematic model 9). In the case of live fungus, maybe dectin-1 and CR3 function redundantly. This possibility should be tested with dectin-1 and CR3 double deficient conditions. Viable *C. albicans* induced IL-1β and cell death did require the uptake of the fungus. However, minimal contribution was observed for β-glucan sensing in both these responses. One possibility is that live fungi do not expose cell wall β -glucans that well in the yeast form. Alternatively, glucans may be exposed, but other phagocytic fungal fungal receptors may be involved. Some candidates include, mannose receptor, dectin-2, dectin-3, DC-SIGN or Mincle as described in chapter I. However, *in vitro* studies may not always recapitulate changes in fungal cell wall architecture and remodeling during replication, germination and invasion which dictate the extent of β-glucan exposure and hence, dectin-1 or CR3 requirement *in vivo* [335]. Hence, it is important to examine if the requirement for dectin-1 and CR3 *in vivo* stems from their particular ability to couple β -glucan sensing to inflammasome activation, IL-1β and cell death. In order to draw broader conclusions about the

role of β -glucan sensing in immune responses against *C. albicans*, other parameters such as induction of cytokines other than IL-1β, anti-microbial peptides; reactive oxidative species and phagolysosomal fusion/ killing mechanisms should all be examined using soluble glucan antagonists, dectin-1 and CR3 knockouts.

Our studies indicated that, CR3 is required for inflammasome activation and cell death in response to β-glucans and unopsonized *C. albicans* (Schematic model 9). This adds to its conventional role of recognition and signaling in response to complement-opsonized pathogens. A previous report showed that phagocytosis of serum opsonized *C. albicans* yeast by neutrophils through CR3 triggers caspase-8 mediated cell death [194]. Now, our studies add to these observations and claim that CR3 activates inflammasome and induces caspase-8 dependent cell death in response to unopsonized heat-killed yeast as well (Schematic model 9). It will be curious to see if this specific CR3-inflammasome and CR3-caspase-8 signaling axes contribute towards heightened oxidative killing of opsonized as well as non-opsonized fungal pathogens by neutrophils. Considering the role of CR3 in recognizing serum opsonized bacterial pathogens, the relevance of these signaling pathways are expected to go beyond anti-fungal responses.

Lessons on fungal recognition and inflammasome responses

Remarkably, β-glucan-sensing is a pattern detection system that has been highly conserved evolutionarily from CED-1 and C03F11.3 (orthologues of

mammalian scavenger receptors SCARF1 and CD36) in *C. elegans* and GNBP3 in *D. melanogaster* to name a few [339,340]. The *D. melanogaster* Toll pathway can be triggered by GNBP3 (a β-glucan sensor) or Persephone protease (activated by virulence factors of entomopathogenic fungi) upon detection of fungal pathogens [340]. In fact GNBP3 bound curdlan, suggesting the highly conserved nature of *Drosophila* and mammalian β-glucan sensors [340]. The requirement of GNBP3 in the induction of the anti-microbial peptide Drosomycin, becomes strikingly prominent, when *Candida albicans* and *Beauveria bassiana* are heat-killed compared to their live forms [340]. These studies strongly suggest that while β-glucan-sensing is an evolutionary preserved fungal recognition system, live fungi are often not readily detected by this pathway. This implies that pathogens may have evolved mechanisms to hide their β-glucans. This is reminiscent of the evolutionary conservation of peptidoglycan and LPS sensing pathways and the evasion of these pathways by bacteria such as *Yersinia pestis* which synthesizes tetraacetylated LPS to avoid TLR4 detection [341]. Hence, it is all the more important to understand the β-glucan-sensing pathways in detail to harness their potential.

C. albicans and β-glucan system also provides us a valuable model to learn about the functioning of the NLRP3 inflammasome itself. A live microbial infection activating the inflammasome may not be surprising as it entails the firing of many inflammatory signals and metabolic perturbances. Here, we observe even heat-killed *C. albicans* to activate the NLRP3 inflammasome in contrast to

the case of many other pathogens such as *Salmonella, EHEC, Listeria* or *Shigella* [92]*.* Previous reports have shown inflammasomes to respond to signature microbial molecules such as LPS, prokaryotic mRNA, DNA and flagellin which breach the cytosolic compartment signifying active infection/danger [92,107,342,343]. Uniquely, adding to that list is β-glucan whose recognition at the cell surface of phagocytes is able to activate NLRP3 activation without the necessity for priming. Thus, the findings outlined in chapters II and III expand our knowledge on how β-glucan recognition through dectin-1, CR3 and possibly other receptors can directly be coupled to NLRP3 activation.

It is important for the host innate immune system to not only detect the structural features of a pathogen but also sense its infectivity. Pathogenic fungi possess several virulence mechanisms including the production of hydrolytic enzymes such as lipases, phospholipases and proteases (*C. albicans, A. fumigatus*), dimorphism, biofilm formation (*C. albicans*), the presence of a capsular polysaccharide (*C. neoformans*) [24]. *C. albicans* is also capable of forming mature biofilms (highly heterogeneous structures with increased antibiotic resistance) in infected hosts and on medical/surgical devices [24,344]. These biofilms are capable of secreting more aspartic proteases (SAPs), which are hydrolytic enzymes[344,345]. Interestingly, secreted aspartic proteases Sap2 and Sap6 have been shown to induce NLRP3 activation and caspase-1-dependent pro-IL-1β maturation in human monocytes[144]. Similarly, the transition of *C. albicans* from its yeast form to the highly virulent and filamentous hyphal form,

but not necessarily the hyphal forms themselves, have been shown to activate inflammasomes in macrophages [134]. This indicates that macrophages sense the germination of the fungus and mount responses accordingly. A unique trait of some *C. albicans* strains, which is also considered to be a virulent feature, is the ability to switch between the formations of white or opaque colonies. Interestingly, the "white phase" which is associated with the ability to form pseudohyphae and hyphae is also the form that triggers more IL-1β production in macrophages[134]. In the case of *A. fumigatus*, it is the germ tube and hyphal forms that induce higher cytokine production (e.g. IL-18) compared to that of the conidial forms that are frequently inhaled by humans [346]. Capsular polysaccharide, a major virulence factor of *C. neoformans*, is associated with evasion of its uptake by phagocytic cells. Interestingly, capsulated *C. neoformans* induces more IL-1β and other pro-inflammatory cytokines in neutrophils compared to the acapsular forms of the fungus[347]. These studies show that inflammasomes tune their responses depending on the level of threat so that an appropriate immune response is mounted. This is similar to the concept of inflammasomes sensing not only structural or cell surface patterns, but also "viability-associated PAMPs" ("vita-PAMPs") such as mRNA, in the case of gram negative bacteria [92]. Similarly, there may be yet undiscovered, "vital" properties to β-glucans that directs the inflammasome responses to discriminate between live infection and sterile particles.

Thus, the inflammasomes, with their strategic location in the cytosol, hold additional functional capacities compared to other pathogen detecting receptors such as TLRs and CLRs. Uncovering mechanisms by which inflammasomes sense the progression of infection will be key to understanding their roles in customizing the immune response.

β-glucans as Immunomodulators

Immune modulation by β-glucan sensing may also have an important role in the success of anti-fungal therapy. Currently available anti-fungal drugs include azoles and polyenes (which act on the ergosterols on fungal cell membrane) and echinocandins (which act on the β (1,3) glucan synthase)[196]. They can all disrupt the fungal cell wall integrity leading to membrane permeability, cell lysis and leakage, expose cell surface glucans and other PAMPs among other functions [196]. In fact, treatment of mice with caspofungin, an echinocandin, has been shown to expose *Candida* and *Aspergillus* hyphal β (1,3) glucans and promote dectin-1-mediated cytokine responses[195,348]. Interestingly, fungal overload in kidneys caused by a dectin-1-unresponsive strain of *C. albicans* became dectin-1 responsive after exposure to caspofungin[335]. Thus, even though dectin-1, CR3 and caspase-8 are not strictly required for IL-1β in the case of live *C. albicans,* these pathways may be engaged during anti-fungal drug treatment, which may contribute towards host protective responses. Further the ability β-glucans to augment the cytotoxicity of CR3 expressing phagocytes and NK cells specifically towards iC3b coated tumor cells [220,349] raise the potential of

harnessing of the CR3 pathway not only for anti-microbial but also for anti-tumor applications.

Recent studies have illustrated the importance of MyD88 signaling and gut microbiota in priming the overall immune response and neutrophil recruitment [350]. It will also be very informative to study the interaction between commensal *Candida spp.,* and the innate immune system. Incidentally, gut commensal *Candida* species were found to overgrow and invade intestinal tissues in dectin-1 deficient mice making them more susceptible to DSS induced colitis [351]. Consequently, dectin-1 polymorphism has also been associated with increased tendency towards ulcerative colitis [351]. This indicates that dectin-1 pathway is important for maintaining the commensal status and host "tolerance" towards fungal commensal population. This brings up the possibility of constant "tasting" of fungal and bacterial β-glucans by intestinal cells and potential priming effects of these molecules on the immune system. In fact, *C. albicans* and β-glucans have been shown to prime and train the NK cells and macrophages by epigenetic changes to induce higher cytokine production to subsequent TLR stimulation in a concept called "training the innate immunity" [222,223,352]. Thus, it appears that the β- glucan sensing pathways serve as a rheostat in determining the quality and scale of innate immune responses depending on whether the fungal infection is acute or commensal.

Cell death processes in fungal infections

The understanding of host cell death processes in the fight against fungal infections is still at its infancy. We found the apoptotic cysteine protease, caspase-8, to modulate cytokine production and cell-death in response to β-glucans, *C. albicans* and TLRs. Caspase-8 was required for IL-1β production and cell death in dendritic cells specifically in response to β-glucans or heat-killed *C. albicans*, but not the live fungus (Schematic model 9). Neither caspase-8 nor RIP3 was required for live *C. albicans* induced dendritic cell death, which rules out extrinsic apoptosis and necrosis. Dectin-1, CR3 and the NLRP3 inflammasome were also not strictly required for live *C. albicans* induced host cell death. However, uptake of the live fungus is required for this process. One possibility is that the germination and hypheation of live *C. albicans* inside cells eventually leads to mechanical injury and lysis of host cells. The pathogen may also compete for nutrients and starve the host cells to death. It remains to be seen if dendritic cells engage a different programmed death pathway such as intrinsic apoptosis (caspase-9 mediated) in response to live *C. albicans*.

Conventionally, caspase-8 mediated, apoptotic death is considered to be immunologically "silent" which will not attract the attention of other cells. However, studies from our group and others show that, in certain conditions, even when the cell-death genetically requires caspase-8, abundant production of pro-inflammatory cytokines, importantly IL-1β, is maintained. Some examples include treatment of cells with β -glucans/HK *C. albicans*, drugs such as

doxorubicin and staurosporine [177], smac mimetics [353,354] or infection with the gram-negative bacterium *Y. pestis* (unpublished data, Dr. Lien lab). In the case of β-glucans and HK *C. albicans*, intracellular ATP and LDH release were measured. For *Yersinia pestis,* DNA fragmentation, electron microscopic imaging and caspase-3 cleavage assays suggest that the cell death process observed is indeed, apoptosis (unpublished data, Dr. Lien lab). This is in line with previous findings that Fas induced apoptosis is accompanied by the release of chemokines MCP-1 and IL-8 which serve as “find me” or “eat me” signals for phagocytes [355]. From these data, it appears that apoptotic cell death can go hand in hand with the controlled release of certain cytokines to promote inflammation and neutrophil recruitment. Currently it is not clear how aforementioned apoptosis or other forms of cell death such as necrosis, pyroptosis or autophagy affects the inflammatory environment and anti-fungal responses.

Many bacterial pathogens are known to actively down regulate apoptosis to develop an intracellular niche for growth or when necessary, promote necrosis to spread the infection [356,357]. *C. albicans* and *A. fumigatus* have been reported to suppress TNF induced apoptosis which presents an attractive hypothesis that fungal pathogens may actively suppress host cell apoptosis [358,359]. TNF and Lymphotoxin-α have been reported to be essential for efficient recruitment and phagocytic ability of neutrophils and production of cytokines such as IL-1 and IL-6 in a mouse model of systemic candidiasis [360,361]. In contrast, Fas deficiency makes mice resistant to systemic candidiasis and this was attributed to enhanced

neutrophil recruitment in Mrl/lpr mice and higher TNF, IL-1β and IL-1α in Mrl/lpr macrophages [362]. However, Fas signaling induced chemokine production seems to follow a RIP1/FADD/caspase-8 axis similar to that of TNF [355]. Hence, a difference in TNF and Fas phenotypes in the context of candidiasis may potentially reflect their ability to trigger different types of cell death (apoptosis, necrosis or as the case may be) and it will be worth re-visiting those models to dissect this further.

It is also important to keep in mind that, *C. albicans* is a eukaryotic pathogen and has been observed to undergo cell death with characteristic features very similar to apoptosis or necrosis under conditions of acid, oxidative stress or treatment with the anti-fungal agent Amphotericin B [363]. At least one caspase with proteolytic ability has been observed in *C. albicans* and *S. cerevisiae* which can be inhibited by mammalian caspase inhibitors such as zVAD-fmk [364]. Hence, manipulation of mammalian host cell processes with zVAD-fmk, necrostatins and other cytotoxic drugs etc., should also consider the effect of these molecules on fungal-programmed death pathways, their survival and virulence.

It is also conceivable that the dendritic cell death may not only be caused by Candida outgrowth and germination but also due to toxic secondary metabolites produced by the fungus. Many virulent and filamentous fungi are capable of producing toxins or harmful enzymes to paralyze host defenses.

“Mycotoxins” can be toxic to vertebrates as well as plants and other microorganisms and can be encountered during infections and as contaminants in food. A number of these toxins particularly derived from *Aspergillus* and *Fusarium spp*, have been characterized. These include, but are not limited to gliotoxin (*Aspergillus fumigatus*), aflatoxins (*A. flavus* and *A. parasiticus*), citrinin (*Aspergillus* and *Penicillium spp.*) and fumonisins (Fusarium spp) (reviewed in Bennett, J.W. and Klich M.) [365]. They can target many organs such as liver and kidneys and can also be carcinogenic in certain cases. Some of them, including aflatoxins and gliotoxin have been described to be immune suppressive with inhibitory effects on phagocytosis, T cell development and host oxidative responses. They also appear to exert their roles by inhibiting RNA and protein synthesis, altering gene expression, membrane integrity and causing the death of host cells [365]. Purified gliotoxin induces plasmatoid DC (pDC) death in a dose dependent manner and deficiency in gliotoxin decreases the cell death caused by *A. fumigatus* [366]. However, the precise mechanism of action for many of these molecules requires extensive investigation.

In the case of live *C. albicans*, a critical virulence mechanism of *C. albicans* is its ability to synthesize and secrete enzymes such as phospholipases, lipases and aspartyl proteinases that may facilitate not only their invasion but also cytotoxicity [24,367]. Metallopeptidases and serine peptidases can cleave several extracellular matrix proteins and SAPs can hydrolyze both intracellular and extracellular host substrates [24]. Interestingly, most of these enzymes are

active in a broad pH range (say 2.0-7.0), which could allow them to digest host proteins even in lysosomes [24]. Infact, *C. albicans* SAP2 and SAP6 have been shown to induce lysosomal damage [144]. Further, the high pressure exerted by the hyphal tips and the hydrolytic enzymes and phospholipases present at these sites aids penetration of tissues and digestion of host cell membranes [368,369]. Some reports suggest that *C. albicans* can also produce gliotoxin [365]. Transwell experiments will help us determine if any of the above discussed toxic enzymes or molecules secreted by the fungus triggers dendritic cell death and if the death phenotype can be correlated with specific molecules of *Candida albicans*. The temperature requirements and the physiological concentration of these broad-spectrum mycotoxins and fungal enzymes is also not entirely clear. Hence it is all the more important to assess the effect of a spectrum of doses of their recombinant/purified version on dendritic cell viability. The availability of genomic sequence of many fungal pathogens also provides the tools to create genetic mutants lacking one or cluster of these molecules to evaluate their pathogenicity *in vitro* and *in vivo*. It is also possible that metabolic by-products produced by the fungus such as acetaldehyde or ammonia contribute towards the cytotoxic properties of *Candida spp.* The field of mycotoxicology holds tremendous promise in terms of identifying not only cytotoxic factors during infection, but also potential antibiotics, anticancer drugs and in the protection of food products and crops.

Balancing act of caspase-8: Significance and Relevance

Caspase-8 is the only member of the family that appears to balance many roles such as promoting NF-κB activation, apoptosis, prevention from necrosis and immune cell homeostasis. Our findings show that caspase-8 is required for TLR4 induced NF-κB activation and cytokine production in macrophages, dendritic cells and embryonic fibroblasts (Schematic model 9) in addition to previously reported T, B and NK cells. Consistent with a role in TLR4 signaling, enterohaemorrhagic *E. coli* induced IL-1β production strongly required caspase-8, suggesting its role in defenses against gram-negative bacterial infections. An important thing to consider is, usually, a TLR based "signal 1" that is separately provided in *in vitro* inflammasome experiments to up regulate pro-IL-1β and NLRP3 is not required in whole animal models to induce IL-1β production. One mechanism proposed is that the LPS from the commensal microbiota can substitute for signal 1. In that case, caspase-8, through its role in LPS induced pro-IL-1β synthesis (Schematic model 9) will be important for mature IL-1β production not only in response to gram-negative bacteria, but likely also other microbial pathogens, even though they themselves may not engage TLR4.

We also observe that caspase-8 alone or in conjunction with RIP3 negatively regulate IFNβ and RANTES production in TLR3 and TLR4 signaling pathways (Schematic models 8 and 9). This role of caspase-8 may impact anti-viral responses. Some of the proteins that interact with caspase-8 such as RIP1, FADD, CYLD and TRADD have also been shown to modulate various anti-viral

signaling pathways such as TLR3, TLR4, RIG-I and DAI [370]. This indicates that caspase-8 may operate in a similar molecular platform as in the death signaling pathways and may also play a role in other intracellular DNA and RNA sensing pathways. In that context, caspase-8 deficient epidermis was shown to exhibit spontaneous as well as DNA-induced IRF3 activation, ISG expression and the mice developed chronic skin inflammation [302]. Exacerbated type I IFN and ISG signature in caspase-8 deficient conditions; deficiency in cell death pathways in *casp8*$^{-/-}$*Rip3*$^{-/-}$ mice, could also contribute to the development of autoimmune attributes such as lymphocyte accumulation and splenomegaly.

The role of caspase-8 in the negative regulation of IFNβ production can also affect anti-fungal immune responses. Recently, *C. albicans* was also found to induce type I IFN in myeloid cells. Many receptors such as dectin-1, CD11b, TLR7 have been proposed to be responsible [73,371] and the protective versus pathological role of IFNAR signaling in candidiasis is under debate [371,372]. However, identification of a robust type I IFN signature in candidiasis patients indicates the relevance of this pathway [373]. The fact that IFN can suppress the transcription of pro-IL-1β[374] further intertwines inflammasome and IFN pathways and places caspase-8 in a web of cytokine regulation. Caspase-8 through its regulation of IL-1β, TNF, IFNβ cytokine production during primary bacterial and viral infections, can also indirectly affect immune responses against secondary fungal infections.

Intestinal bowel disorders including Crohn's disease and ulcerative colitis can be caused by the breakdown of intestinal barriers and excessive inflammation driven by intestinal microbiota. Caspase-8 and FADD protect intestinal cells from TNF, RIP3 and CYLD mediated necrosis leading to Crohn's disease [375,376]. Crohn's patients also exhibit higher RIP3 expression and necrotic intestinal cells [375]. Interestingly, TLR4, which is normally expressed low in intestinal epithelial cells, is up regulated in conditions of IBD and TLR4 deficiency/TLR4 antagonists have been shown to ameliorate the inflammation [377,378]. Based on our findings, it will be curious to determine how caspase-8 may balance its function of maintaining intestinal homeostasis by suppressing necrosis while also promoting TLR4 induced TNF (which can trigger necrosis).

Many caspases including caspase-8 have been found to be mutated/silenced in human cancers [379-381]. Naturally, some of the anti-cancer therapies are aimed at boosting caspase-8 expression through IFNγ treatment and demethylating agents to better prime the cancer cells for apoptosis induced by molecules such as TRAIL, doxorubicin or staurosporine [382]. However, caution should be exercised since this can also increase NF-κB activation and possibly, proliferation of these tumor cells. Hence it is critical to understand the function of caspase-8 in an all-inclusive manner. Caspase-8 appears to heterodimerize with cFLIP to mediate certain functions such as cleavage of RIP3, facilitating T cell survival and NF-κB activation while the homodimerized version cleaves itself to potentiate apoptosis [383]. Thus, caspase-8 represents an important compass for

cell fate decisions. Peptide drugs are under development to activate/inhibit caspases directly or block cIAPs and other inhibitors of apoptosis by targeted delivery to inflammatory sites or tumor cells [384-386]. Interestingly, a pox-virus protein CrmA inhibits caspase-8 homodimers more efficiently compared to heterodimers [301], raising the possibility of developing drugs that can uncouple these functions for increased specificity.

In conclusion, we illustrate the critical role of the NLRP3-ASC-caspase-1 inflammasome in IL-1β production and in host immune responses to the fungal pathogen *C. albicans*. We identified an essential role for the β-glucan sensors dectin-1 and CR3 and the apoptotic protease caspase-8, in mediating β-glucan and *C. albicans* triggered IL-1β production and cell death when its cell wall glucans are exposed. Similar to the role of DREDD in the *Drosophila* NF-κB pathway (IMD pathway), we found an important role for caspase-8 in modulating pro-inflammatory cytokine production specifically in TLR4 and TLR3 pathways. Overall, these findings add to our current understanding of how the host immune system integrates innate immune as well as death signaling pathways and mounts a specific response against fungal pathogens and molecular signatures representing fungal, bacterial and viral infections.

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