Different Journeys, Same Destination: Exploring the Role of a PYHIN Protein and Involvement of Caspase-8 in the Regulation and Activation of Inflammasomes

A Dissertation Presented

By

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Immunology and Microbiology Program

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In the loving memory of my grandmother, Anjana Mitra, my staunchest ally

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"*There are those that look at things the way they are, and ask why? I dream of things that never were, and ask why not?*" – R. F. Kennedy

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The journey has just begun.

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Abstract

Interferon-inducible PYHIN protein family includes the DNA-binding proteins, AIM2 and IFI16, which form ASC-caspase 1 dependent inflammasomes, important in immunity against cytosolic bacteria, DNA viruses and HIV. The role of other members of this family in the recognition of DNA and/or regulation of immune responses is unclear. We identified an immune regulatory function of p205, another member of the PYHIN family, in the transcriptional control of immune genes. Knockdown of p205 in macrophages revealed that inflammasome activation due to dsDNA and ligands that engage the NLRP3 inflammasome were severely compromised. Detailed mechanistic analysis showed that loss of p205 was associated with a decrease in Asc mRNA and protein levels. p205 knockdown resulted in reduced RNA Polymerase II-mediated endogenous *Asc* gene transcription and mRNA processing, suggesting a co-transcriptional control of *Asc* gene expression. Ectopically expressed p205 induced expression of an *Asc* gene-luciferase reporter and collaborated with other transcription factors, such as c/EBPβ, p65/RelA, to further enhance expression. p205 knockdown also affected the expression of the immune genes *Cd86, Cox2, Cxcl2, Il1α, Il10, Il12α, Il6 and Ifnα* in LPS-stimulated macrophages. Together these findings suggest that p205 regulates inflammation through control of *Asc* gene expression, and other immune genes.

Fungal infections activate both caspase 1-dependent and -independent inflammasomes. In an independent study, we show that *Paracoccidioides brasiliensis* fungal infection also induces caspase 8-dependent non-canonical inflammasome. Caspase 8-dependent IL-1β processing required dectin-1, Syk and Asc. *Rip3-/- Casp8-/-* mice infected with *P. brasiliensis* displayed increased fungal load and showed worse disease progression compared to wild type and *Rip3-/-* mice. These results revealed the importance of caspase 8 in activating and regulating inflammasome responses during fungal infection *in vivo*.

List of Publications

- **Ghosh S**, Wallerath C, Covarrubias S, Hornung V, Carpenter S, Fitzgerald KA. *The PYHIN protein p205 regulates the inflammasome by controlling ASC expression*. Journal of Immunology, 2017; 199 (9) 3249–3260

- Ketelut-Carneiro N, **Ghosh S**, Levitz SM, Fitzgerald KA*, Silva JS*. *A Dectin-1-caspase-8 pathway licenses canonical caspase-1 inflammasome activation and IL-1β release in response to a pathogenic fungus.* Journal of Infectious Diseases, 2017; doi: 10.1093/infdis/jix568 (published ahead of print)

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List of Abbreviations

AIM2	Absent in melanoma-2
ALR	AIM2-like receptors
AP1	Activator protein 1
ASC	Apoptosis-associated speck-like protein containing CARD
BMDC	Bone marrow derived dendritic cell
BMDM	Bone marrow derived macrophage
bp	base-pairs
CARD	Caspase activation and recruitment domain
Caspase	cysteinyl aspartate protease
CBV	Coxsackie B virus
c-di-AMP	Cyclic diadenosine monophosphate
c-di-GMP	Cyclic diguanosine monophosphate
CD	Cluster of differentiation
CDN	Cyclic-dinucleotide
c/EBP	CCAAT-enhancer-binding proteins
cGAS	Cyclic GMP-AMP Synthase
cGAMP	Cyclic guanosine monophosphate–adenosine monophosphate
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DD	Death domain
DDX	DEAD (Asp-Glu-Ala-Asp) box polypeptide
DED	Death Effector domain
DHX	DEAH (Asp-Glu-Ala-His) box polypeptide
DMXAA	5,6-Dimethylxanthenone-4-acetic acid
DNA	Deoxyribonucleic acid
EAE	Experimental Autoimmune Encephalitis
EBV	Epstein-Barr virus
ECMV	Encephalomyocarditis virus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FADD	Fas-associated protein with death domain
Fas (FasL)	First apoptosis signal (First apoptosis signal Ligand)
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HEK	Human embryonic kidney
HIN-200	Hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats
HIV	Human Immunodeficiency Virus
HSV	Herpes simplex virus
IAV	Influenza A Virus
ICP0	HSV infected cell polypeptide 0
IFI	Interferon-inducible protein
IFN	Interferon

IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISD	Interferon stimulatory DNA
ISG	Interferon stimulated gene
ISRE	Interferon-sensitive response element
JAK	Janus Kinase
JNK	c-Jun N-terminal kinases
KSHV	Kaposi sarcoma-associated herpesvirus
LCMV	Lymphocytic choriomeningitis
LGP2	laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
LRRFIP1	Leucine-rich repeat flightless-interacting protein 1
Mal	MyD88-adapter-like
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signaling protein
MCMV	Murine cytomegalovirus
MD2	Lymphocyte antigen 96
MDA5	Melanoma differentiation-associated gene-5
MLKL	Mixed lineage kinase domain-like protein
MMTV	Mouse mammary tumor virus
MNDA	Myeloid nuclear differentiation antigen
MyD88	Myeloid differentiation primary response gene 88
NALP	NACHT, LRR and PYD domain-containing protein
NBD	nucleotide binding domain
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NK cells	Natural Killer cells
NLRP	NOD-like receptor proteins
NO	Nitric oxide
NOD	nucleotide-binding oligomerization domain
NP	nucleoprotein
NTase	nucleotidyltransferase
PAMP	Pathogen-associated molecular pattern
PDC	Plasmacytoid dendritic cell
PML	Promyelocytic leukemia protein
Poly(dA:dT)	Polydeoxyadenylic acid : polythymidylic acid
Poly(I:C)	Polyriboinosinic acid : polyribocytidylic acid
PRR	Pattern Recognition Receptor
PYD	Pyrin domain
PYHIN	Pyrin and HIN domain-containing protein
RIG-I	Retinoic acid-inducible gene-I
RIP/RIPK1	Receptor-interacting serine/threonine-protein kinase 1
RIP/RIPK3	Receptor-interacting serine/threonine-protein kinase 3
RLR	Rig-I-like receptors

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
SLE	Systemic Lupus Erythematosus
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
SV	Sendai virus
Tab	TAK1-binding proteins
TAK	Tat-associated kinase
TANK	TRAF family member-associated NF-κB activator kinase
TBK1	TANK-binding kinase 1
TGF	Transforming growth factor
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
VACV	Vaccinia Virus
VSV	Vesicular stomatitis virus
ZBP1	Z-DNA Binding Protein 1

Chapter 1: Introduction

Innate Immunity, Inflammasomes & PYHIN proteins

1.1 What is Innate Immunity?

The innate immune system consists of genetically coded receptors that present an impressive ‘first line of defense’ to threats from outside or within the host system. These receptors known as the pattern recognition receptors (PRR) recognize foreign particles that are associated with pathogens such as bacteria, virus, fungus or protozoa called Pathogen-associated Molecular Patterns (PAMP) as well as self-derived molecules that may pose dangerous and harmful to the host called Danger-associated Molecular Patterns (DAMP) (1). Upon recognition and engagement by these molecules resulting from pathogenic infection or cellular damage, the receptors initiate a rapid immune response that triggers the production of cytokines and chemokines to contain and eliminate the threats, before the slower adaptive immune system kicks in to eradicate them and form a long-term memory response. These cytokines and chemokines also act as a herald that informs the system of imminent intrinsic or extrinsic danger, which is initiated within the infected cells to attract immune cells, also turns to protect the surrounding cells from being infected. Cells like macrophages, dendritic cells, fibroblasts, mast cells, monocytes, neutrophils and natural killer (NK) cells express these PRR and are the sentinels for the host immune system, while the adaptive immune system that is engaged by the innate immune system, consists primarily of B-cells and T-cells.

The innate immune system is evolutionarily the original host defense system, which is the dominant immune system in plants, fungi, insects and primitive multicellular organisms. The primary functions of the innate immunity include immune cells recruitment to sites of infection and cellular damage, activation of the complement cascade to identify pathogens and clear antibody complexes or dead cells, identification

and removal of foreign particles present in organs, tissues, blood and lymph, activating the adaptive immune system by antigen presenting cells (APC) and acting as a physical and chemical barrier to infectious agents.

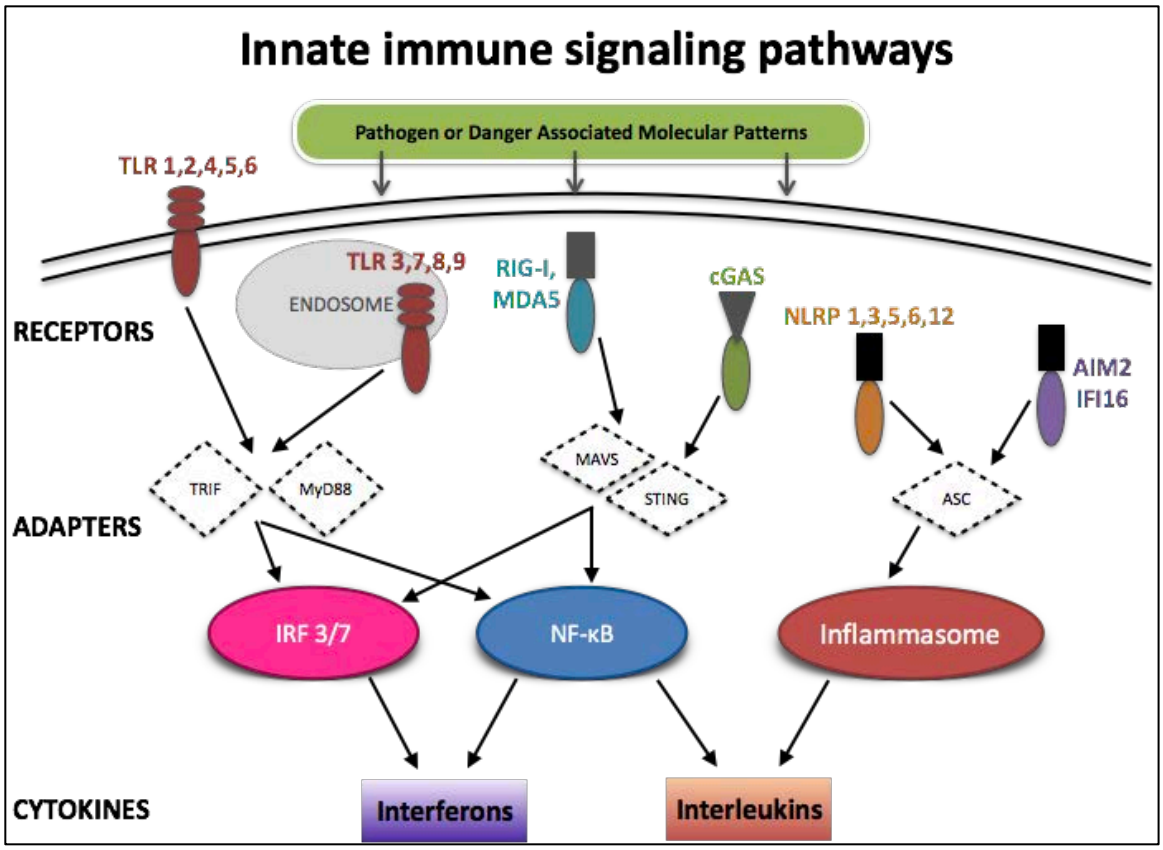

Figure 1.1: Pattern Recognition Receptors and Innate Immune pathways. Innate immunity consists of genetically coded receptors called Pattern Recognition Receptors (PRR) such as Toll-like receptors (TLR), RIG-I like receptors (RLR), NOD-like receptor (NLR) proteins, AIM2-like receptors (ALR) and cGAS. Upon binding their cognate pathogen-derived or danger-associated molecules, the PRRs activates multiple immune pathways through adapter proteins and transcription factors leading to immune responses including NF-κB, Interferons and Inflammasome pathways.

1.2 Pattern Recognition Receptors and Their Ligands

The Pattern Recognition Receptors or PRRs are classified into different families principally according to their structures as well as by ligand-specificity and the signaling pathways that they activate upon stimulation.

1.2.1 Toll-like receptors (TLR)

Toll-like receptors or TLR are the most comprehensively studied innate immune receptors that recognize a wide range of PAMP and DAMP. Initially, Toll was described in *Drosophila melanogaster* as a protein involved in embryonic development. It was later discovered to function in fungal pathogen detection and response (2). TLRs are type-I transmembrane (TM) proteins, found in the cell membrane or in the endosomes, primarily composed of multiple extracellular N-terminal leucine-rich repeats (LRR), followed by a cysteine-rich region, a TM domain and an intracellular Toll/IL-1 R (TIR) motif (3, 4). The LRR domain is responsible for ligand binding and detecting foreign particles while signal transduction occurs through the TIR domain that is capable of protein-protein interactions. Bacterial cell wall components in the extracellular compartment are detected by TLR present in the plasma membrane. TLR2 forms heterodimers with TLR1, TLR6 and possibly human TLR10 to detect triacylated or diacylated lipopeptides, peptidoglycans, lipoglycans, lipoteichoic acid (LTA) present in the cell walls of both Gram-positive and Gram-negative bacteria, while TLR5 recognizes the bacterial flagellin. Human TLR8 and mouse TLR13 respond to viral and bacterial RNA. TLR11 and TLR12, found only in mouse, recognize profilin from *Toxoplasma gondii*. TLR4, with the help of MD2 and CD14, shuttle between the plasma membrane

and the endosome, and recognizes lipoglycans like lipopolysaccharide (LPS) derived from Gram-negative bacteria (5). In contrast, the endosomal TLRs sense bacterial or viral nucleic acids within the cytosol – TLR3 senses double-stranded (ds) RNA (6), TLR7/8 sense single-stranded (ss) RNA (7) and TLR9 recognizes unmethylated CpG DNA (8). Upon activation, the TLR dimerize and stimulate MAPK, IFN or NF-κB pathways by recruiting the adapters myeloid differentiation primary response protein 88 (MyD88) or TIR-domain containing adapter inducing IFN (TRIF; also known as TICAM1) to induce proinflammatory cytokine production (9, 10). The downstream signaling proceeds through distinct pathways, and deletion of both MyD88 and TRIF completely blocks TLR signaling.

1.2.2 RIG-I like receptors (RLR)

RIG-I or retinoic acid inducible gene-1 encoded by the gene *DDX58* was the first DExD/H box RNA helicase to be identified as an intracellular receptor for RNA (11). The other members of this RIG-I like receptors or RLR superfamily include melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP-2) (12). These proteins contain a DExD/H box RNA helicase domain (DEAD domain) with ATPase activity and a C-terminal repressor domain (CTD). RIG-I and MDA5 also contain two tandem N-terminal caspase activation and recruitment domains (CARDs) that are absent in LGP-2. Although RIG-I and MDA-5 share similar domain structure, they recognize distinct RNA ligands in a sequence-independent, length-dependent manner as established by their crystal structures. RIG-I detects smaller 5’ triphosphate RNA molecules and ssRNA. MDA5 preferentially binds to the

phosphodiester backbone of longer dsRNA and oligomerizes to form filamentous structures on the RNA. RIG-I is involved in sensing Sendai Virus (SeV), Influenza A (IAV) and B (IBV) Virus, Hepatitis C Virus (HCV), Vesicular Stomatitis Virus (VSV) and Respiratory Syncytial Virus (RSV) (11). MDA-5 recognizes encephalomyocarditis virus (EMCV), coxsackie B virus (CBV) and Polio as well as the transfected synthetic viral RNA-mimic, polyinosinic:polycytidylic (poly I:C) (13). Upon detection of viral RNA particles, RIG-I and MDA5 oligomerize and activate type-I interferon (IFN) production via adapter protein mitochondrial antiviral signaling (MAVS) protein and transcription factors, Interferon Regulatory Factor (IRF) 3 and 7 (Pathway described in more detail in Section 1.3.1). Since 5'-triphosphate modified RNA are predominantly found in certain viral genomes, and not in mammalian system, a highly specific and strong immune response is initiated upon RIG-I and/or MDA5 induction upon RNA virus infection. The other member of the RLR family, LPG-2 was initially shown to negatively regulate RLR activity (14) as the LGP-2 overexpression decreased the capacity of RNA virus to induce type I IFNs. However, later Satoh et al. showed that LGP-2 deficient mice had increased interferon signaling upon infection contrary to its suggested function as a negative regulator (15). The RNA-binding PRRs are described in more detail in Section 1.5.

1.2.3 NOD-like receptors (NLR)

Multiple PAMP (e.g. *Salmonella* sp. type-III secretion, bacterial toxins, bacteria-derived RNA:DNA Hybrids etc) system as well as DAMP (e.g. mitochondrial damage, β-amyloids, K^+ efflux, alum etc) activate the intracellular nucleotide

oligomerization domain-like receptors (NLR). In contrast to other receptor family members, the NLRs have not been shown to definitively bind or receive any specific microbial product. The NLR family includes NLRP1, NLRP3, NLRC4, NLRP6 and NLRP12 that activate the inflammasomes to regulate caspase activity and induce an inflammatory response. There are 22 human and 34 mouse NLRs that have a common central nucleotide binding and oligomerization (NACHT) domain and a C-terminal leucine-rich repeat (LRR) responsible for immune activities of the NLR (16). (More details about NLR Inflammasomes in Section 1.4.1)

The NOD1 and NOD2 proteins were the first NLRs found to regulate NF-κB and MAPK pathways. Another NLR, NLRC5, expressed widely in various tissues and cell types in both human and mouse, acts as a negative regulator in both NF-κB and type I IFN signaling pathways (171). NLRX1 is specifically located at the mitochondria and interacts with the MAVS to activate the transcription factors IRF3 and NF-κB signaling pathways, leading to the production of IFNα/β and proinflammatory cytokines. RIG-I-MAVS interaction is impeded by NLRX1, thus negatively regulating of IFN-α/β induction during RNA virus infection. In contrast, NOD2 uses MAVS to activate an anti-viral IRF3 response, leading to enhanced IFN-α/β induction (175).

1.2.4 C-type lectin receptors (CLR)

The CLR family includes the receptors DC-SIGN, dectin-1, dectin-2, Mincle. They are soluble or transmembrane proteins, containing a distinct C-type lectin-like domain (Carbohydrate Recognition Domain or CRD), that detect various fungal, bacterial and viral sugar-based derivatives such as zymosan, β-glucan, N-linked mannan,

a-mannose etc (17) The CLR activates NF-κB signaling pathway via a kinase, Syk (spleen tyrosine kinase) through direct interaction with the immunoreceptor tyrosine based activation (ITAM) motif present in their intracellular region (e.g. Dectin-1, DC-SIGN) or indirectly through other molecules containing the ITAM motif (e.g. Dectin-2 or Mincle via FcRγ), and regulates TLR-induced gene transcription as well.

1.2.5 AIM-2 like receptors (ALR) and cGAS

Of all the ALRs, so far, only absent in melanoma (AIM) 2 and Interferon Gamma Inducible protein (IFI) 16 have been characterized as PRRs. cGAS (*c*ytosolic *GA*MP *s*ynthase; also known as MB21D1) is an enzyme that belongs to the nucleotidyltransferase family (18-20). AIM2 and cGAS localize in the cytoplasm and senses DNA in a sequence-independent manner to activate ASC-dependent inflammasome and STING-dependent type-I IFN respectively. IFI16 is a nuclear protein, which shuttles to the cytoplasm as well on post-translational modification or during specific infections, and activates the inflammasome upon Kaposi's sarcoma-associated herpesvirus (KSHV) infection, and also in CD4+ T-cells during Human Immunodeficiency Virus (HIV) infection (21, 22). The nucleic acid sensors AIM2 and IFI16 belong to the family of proteins called PYHIN proteins or as AIM2-like receptors (or ALRs) and are described in detail in Section 1.6. Other DNA sensors include DAI/ZBP1, MRE11, DDX41, LRRFIP1 and RNA Polymerase III that are described in detail in Section 1.5.

1.3 Innate Immune Signaling pathways

Activation of the PRRs upon detection of DAMPs and PAMPs within the host system triggers multiple distinct and parallel downstream signaling pathways that control the cellular processes, e.g. inflammation, autophagy, apoptosis, necrosis, cell survival and proliferation, that determines the fate of the infected or damaged host cells as well as that of the neighboring bystander cells. The Type-I Interferon pathway elicits the synthesis and secretion of IFN-α and IFN-β that initiates another cascade of antiviral and anti-tumor gene expression. The NF-κB/MAPK pathway, also known as the MyD88-dependent pathway, activates multiple proinflammatory cytokine genes, including tumor necrosis factor (TNF) α, IL-6 and pro-IL-1β. Inflammasome activation causes a robust pro-inflammatory response through secreted cytokines such as IL-1β, IL-18 and leads to an inflammatory cell death.

1.3.1 The Complement System

The complement system is comprised of a subset of proteins circulating in the blood that enhances the ability of antibodies and phagocytic cells to clear microbial pathogens and damaged cells, promotes opsonization and disrupts the plasma membrane of microbes (membrane attack complex or MAC) to kill the organism. The distinct biochemical pathways that activate the complement system are (i) the classical complement pathway, (ii) the alternative complement pathway and (iii) the lectin pathway (23). These reactions amplify the innate anti-microbial mechanisms, recruit leukocytes, initiate phagocytosis of pathogens and clear immune complexes. While un-opsonized pathogens are eventually sensed and phagocytosed by other PRRs,

opsonization by complement provides an immediate and effective mechanism of pathogen clearance.

The classical pathway is triggered by activation of the C1-complex composed of C1q, C1r and C1s (C1qr2s2), when C1q binds to IgM or IgG antibodies bound to antigens. The complex is also activated when C1q binds directly to the surface of the pathogen. The C1r2s2 is a serine protease complex that cleaves C4 and then C2, producing C4a, C4b, C2a, and C2b. C4b and C2b bind to form the classical pathway C3-convertase (C4b2b complex), which promotes cleavage of C3 into C3a and C3b. C3b later joins with C4b2b to make C5 convertase (C4b2b3b complex). It initiates the later events of complement activation comprising of a sequence of polymerization reactions in which the terminal complement components, C6, 7, 8 and 9 interact to form a membrane-attack complex, which creates pores in the plasma membranes of pathogens leading to their death.

In the alternative pathway, a similar C3 convertase is formed from membrane-bound C3b complexed with Bb, from the component Factor B. The alternative pathway can act as an amplification loop for all three pathways, as it is initiated by the binding of C3b, and spontaneous C3 hydrolysis due to the breakdown of the internal thioester bond. The lectin pathway is similar to the classical pathway, except that it is initiated by mannose-binding lectin (MBL) instead of C1q. It is activated by the binding of MBL to mannose residues on microbial surfaces, which activates the MBL-associated serine proteases, MASP-1, and MASP-2 (similar to C1r and C1s in the classical pathway).

1.3.2 Type-I & Type-II Interferons

The type-I Interferon family consists of multiple cytokines including IFN-α (13 human subtypes, 14 mouse subtypes), IFN-β and other less characterized cytokines such as IFN-ε, IFN-τ, IFN-κ, IFN-ω, IFN-δ and IFN-ζ. IFN-α/β are the most well defined, broadly expressed type-I IFN that can induce an anti-pathogenic state via changes in host gene transcription in infected as well as surrounding uninfected cells (24). Dependent on infections, different conditions arise that determine how type-I IFN are induced as well as the downstream signaling pathways that are initiated through type I IFN receptor (IFNAR), which trigger IFN-stimulated genes (ISG) activation or repression. The primary ligands that can activate type-I IFN are lipopolysaccharides (LPS), RNA and DNA. Hence, among the TLRs, TLR4 on the cell surface and TLR 3/7/8/9 in the endosomes are potent activators of IFN. Similarly, the RNA helicases, RIG-I, MDA5 and the DNA sensors, cGAS, IFI16 can activate the type-I IFN pathways.

The multiple receptor signaling pathways converge upon the recruitment of the cytosolic TIR domain activating the tank binding kinase 1 (TBK1) complex that phosphorylates the transcription factors, IRF3 and IRF7 leading to their dimerization. The IRF3 and IRF7 homodimers translocate to the nucleus and activates downstream type-I IFN pathway including IFNα/β and other ISGs.

Upon activation, the receptors employ different adapter proteins to initiate IFN signaling. TLR3 activates TRIF to recruit TRAF3, IKKι and NAK-associated protein (NAP) 1 to activate TBK1 kinase activity while the other TLRs recruit MyD88 and IL-1 receptor associated kinases (IRAK) to activate type-I IFN. TLR4 dimers use Myd88 and IRAKs as well as TRIF and TRIF-related adapter molecule (TRAM) to activate IRF3.

The RNA helicases, upon detection of viral RNA, signal through the adapter, MAVS also known as VISA (virus-induced signaling adapter), IPS-1 and Cardiff located on the outer membrane of mitochondria and peroxisomes via homotypic interactions between their CARD (Caspase-recruitment domains) domains. On binding of activated RIG-I or MDA-5 filaments, MAVS aggregates on the on the surface of the mitochondria and recruits TNF receptor associated factor (TRAF) 3 and 6, caspase-8, RIP1, FAS-associated death domain (FADD), and TNF receptor-associated death domain (TRADD) are recruited to activated MAVS, forming a signaling complex for activation of the kinases IKKα/β and TBK1, resulting in downstream NF-κB and IRF3/7-dependent transcription of antiviral genes (Kawasaki et al., 2011). The cytosolic DNA sensors initiate IFN signaling through yet another adapter protein, STING. Overexpression of STING drives IFN expression. STING localizes to the outer membrane of the endoplasmic reticulum (ER) but relocalizes, upon activation, to the Golgi forming large aggregates (Ishikawa et al., 2009). The activated STING complex recruits TBK-1 that leads to the phosphorylation and dimerization of IRF3 and IRF7.

IFNα/β activates a second wave of IFN-dependent gene expression by engaging its extracellular receptor, IFNAR. Binding of IFNα/β, IFNAR1 and IFNAR2 recruits the tyrosine phosphorylases, JAK1 and TYK2 that phosphorylates and activates STAT1, STAT2 and STAT3 that form STAT1 or STAT3 homodimers and/or STAT1/2 heterodimers. These dimers translocate to the nucleus, bind the chromatin (ISRE; IFN-Stimulatory Response Element) and activate transcription of several ISGs (e.g. IRF7, IRF9, Cxcl10, Cxcl9, RANTES, Viperin) in the damaged cell (autocrine) as well as in the surrounding cells (paracrine).

Type II interferon or IFN-γ is mainly secreted by T cells, natural killer (NK) cells and macrophages. IFN-γ is c ritical for innate antiviral and anti-tumor immunity. While mice lacking IFN-γ or its receptor, IFNGR1 are able to manage the viral burden during VSV or Semliki Forest virus (SFV) infections, they are more susceptible to Vaccinia Virus (VACV) or lymphocytic choriomeningitis virus (LCMV) infections (14, 19, 20). Interestingly, mice lacking both *Ifnar1* and *Ifngr1* gene expression show higher mortality rate to VV and LCMV (21), which suggests that the two IFN types are often complementary with respect to immune resistance to different viral pathogens.

IFN-γ–activated macrophages are better equipped to kill bacterial or protozoan pathogens. IFN-γ activates the factors, NADPH oxidase subunits, NOS2, lysosomal enzymes, and tryptophan-metabolizing enzymes that kill of microbes by reactive oxygen species (ROS), NO radicals, degradation, and tryptophan depletion respectively. Some of these factors are also regulated by STAT1. Additionally, IFN-γ enhances the synthesis of cytokines (e.g. IL-12 p40 subunit) that enhance antimicrobial immunity *in vivo*. IFN-γ is particularly effective against pathogens within the cells such as *Listeria*, *Mycobacterium*, *Salmonella*, *Chlamydia*, and *Leishmania*. Treatment with recombinant IFN-γ or genetic depletion of IFN-γ demonstrates the efficacy of IFN-γ as factor regulating pathogen survival or clearance.

1.3.2 NF-κB and MAPK

The NF-κB (Nuclear Factor- κB) and MAPK (Mitogen-activated protein kinase) or ERK (extracellular protein kinase) pathways are the downstream signaling

pathways of most PRRs and are crucial for immune responses to cellular stress and infection. In mammals, the NF-κB family consists of five members, NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel while the MAPK family contains 14 kinases, of which extracellular signal-regulated kinase 1 (ERK1), ERK2, p38α, Jun N-terminal kinase 1 (JNK1) and JNK2 have been studied extensively in innate immunity (25, 26).

All the TLRs induce a NF-κB dependent inflammatory gene signature in the host cells. Most of the TLRs signal through MyD88, with the exceptions of TLR3 that uses TRIF, and TLR4 that uses both MYD88 and TRIF. Activated TLRs bind MyD88 and TIR domain containing adaptor protein (TIRAP) in the cytosol, which recruits IRAK-1, IRAK-4 and TRAF-6. IRAK-4 and TRAF6, in turn, associate with another complex consisting of transforming growth factor (TGF)-β-activated kinase (TAK1; also called MAP3K7) and TAK1-binding proteins (TAB) 1 and 2 that activates TAK1 and consequently, the transcription factors NF-κB through the IκB kinase (IKK) complex and activator protein 1 (AP-1) through the MAPK pathway. The TLR3-mediated NF-κB is activated through the homotypic binding of Receptor-interacting protein (RIP1) to the Rip motif in TRIF C-terminus and cooperative binding of TRAF6 to the N-terminus of TRIF. Although NF-κB pathway, upon TLR3 activation, in TRAF6-deficient fibroblasts is impaired, TRAF6 is not necessary for NF-κB activation in macrophages. Similar to TLR3, TLR4 can also activate TRIF/RIP1/TRAF6 dependent NF-κB signaling. Cells deficient for TRAF6 and MyD88 can activate NF-κB in response to LPS suggesting that TRIF could activate the pathway in TRAF6-independent RIP-1-dependent manner. The

NF-κB pathway remains active in LPS-stimulated, RIP1-deficient cells, but LPS-stimulation in cells deficient for both MyD88 and RIP1 show no NF-κB activation.

Various PRRs that can activate the MAPK pathway include the TLRs among others. MAPK activation in innate immune cells has been mostly studied in the context of TLR agonists. Following ligand binding to TLRs, IRAK4 is recruited to MYD88to form a complex with the related kinases IRAK1 and IRAK2, TRAF6 and the E2 ubiquitin-conjugating enzyme 13 (UBC13; also known as UBE2N). TRAF6 and UBC13 catalyzes K63-linked polyubiquitination on TRAF6 and IRAK1, which leads to the activation of the MAPK pathway. TRAF3 is also recruited to MYD88 and its degradation by IAPs (inhibitor of apoptosis proteins) is required for the activation of MAPKs and for the induction of pro-inflammatory cytokines, but not for TLR-dependent of type I IFN production. The activated MAP3K TAK1 directly activates the MAPK kinases (MAP2K, MAPK) for both p38 and JNK that induce AP1-mediated gene activation. However, depending on the stimuli (e.g. TNF or IL-1) TAK1 can induce parallel pathways that may or may not require regulation by TAB1 and TAB2.

1.3.3 Inflammasomes

Cellular stress, bacterial, fungal or viral infections also trigger a distinct set of sensors that activates the inflammasome complex. The receptors that activate the inflammasome are the NLRs and AIM2, IFI16 (ALRs). The inflammasome is a large multimeric complex consisting of the receptor, the adaptor protein Apoptosis-Associated Speck-Like Protein Containing a CARD domain (ASC; also known as PYCARD), and its

effector protein, caspase 1 (cysteine aspartate proteases 1) (27, 28) that cleave the inactive forms of IL-1β and IL18 to their mature, bioactive, secreted forms leading to a robust inflammatory response. The Inflammasomes are described in more detail in the following Section 1.4.

1.4 What are Inflammasomes?

Inflammasomes are cytosolic multi-protein complexes that are assembled upon detection of pathogenic or self-derived danger molecules, and are responsible for the activation and induction of pro-inflammatory cytokines Interleukin-1β (IL-1β) and Interleukin-18 (IL-18), and an inflammatory form of programmed cell death known as pyroptosis via caspase 1 (27, 29) and Gasdermin-D (GSDMD) (30, 31). Inflammasomes are critical for resisting infection upon detection of pathogen-associated molecular patterns (PAMP) such as microbial nucleic acids, lipoproteins and carbohydrates by pattern recognition receptors (PRR). Similarly, inflammasomes also are activated upon recognition of endogenous triggers or danger-associated molecular patterns (DAMP) like mitochondrial damage, ionic imbalance, free ATP that are typical markers of injured or dying cells. Innate immune cells like macrophages, dendritic cells, fibroblasts, mast cells, monocytes and neutrophils express the PRRs that are responsible for launching inflammatory responses. However, deregulated inflammasome activation can lead to aggravated disease symptoms, autoimmune disorders and inflammatory diseases such as Alzheimer's disease, atherosclerosis, diabetes and cancer.

Over the years, multiple distinct PRRs have been identified that can activate inflammasomes. The classical inflammasome complex contains a cytosolic receptor (e.g. a nucleotide-binding domain and leucine-rich-repeat containing protein or NLR or, an AIM2-like receptor or ALR) that gets activated upon microbial or endogenous insults and oligomerizes to recruit an adapter protein called ASC (*a*poptosis-associated *s*peck-like protein containing a *C*ARD domain) and an effector, inflammatory cysteine aspartate protease, caspase to form a multimeric structure that promotes a

signaling cascade that elicits the maturation and production of inflammatory cytokines and chemokines as well as pyroptosis.

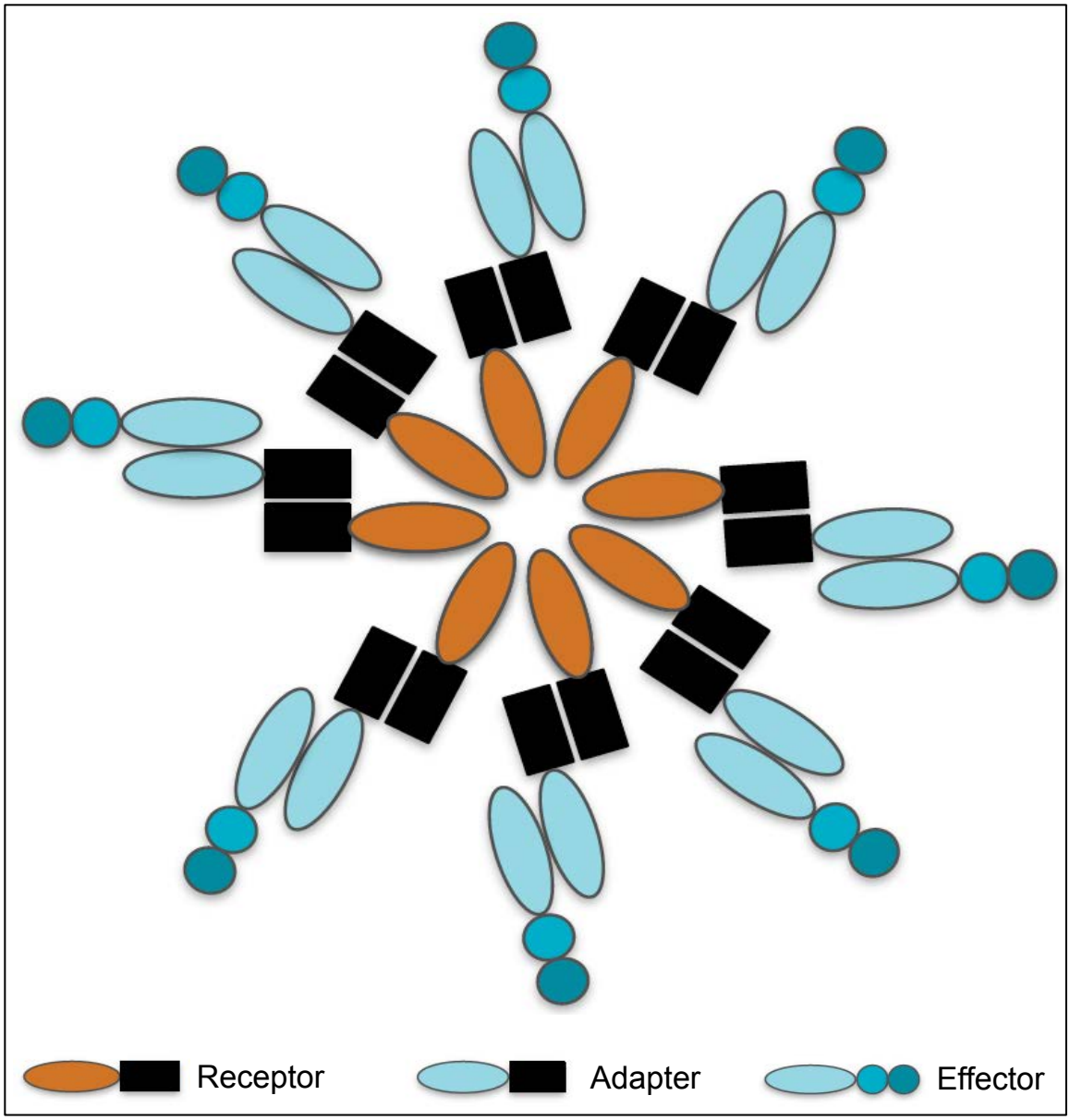

Figure 1.2: Structure of an Inflammasome. The inflammasome is a multimeric protein complex, which forms a speck-like structure, composed of a ligand-activated receptor protein (e.g. NLRP3, NLRP1, AIM2), an adapter, ASC and an effector protease such as Caspase 1. The inflammasome is required for the maturation of inflammatory cytokines such as IL-1β and IL-18 as well as in promoting an inflammatory form of cell death called pyroptosis.

1.4.1 Caspase-1 dependent canonical inflammasomes

The term “inflammasomes” was initially coined to describe the multimeric complex composed of a receptor that recruits ASC, which enhances the nucleation and polymerization of the effector protease, caspase 1 that cleaves pro-IL-1β and pro-IL-18 into their mature, secreted forms. Caspase 1 was initially called Interleukin-1 converting enzyme (ICE) owing to its role in converting the 31 kD pro-IL-1β into an active 17.5 kD fragment in 1989 (32, 33). In 1992, it was characterized as a heterodimeric cysteine protease composed of two subunits, p10 and p20 (34). The macromolecular protein complex comprising caspase 1, caspase 5, ASC and an NLR, NLRP1 was described *in vitro* for the first time in 2002 (27). The most well characterized caspase 1-dependent inflammasomes are NLRP1b, NLRP3, NLRC4 and AIM2. Other inflammasomes include NLRP12, NLRP6 and pyrin.

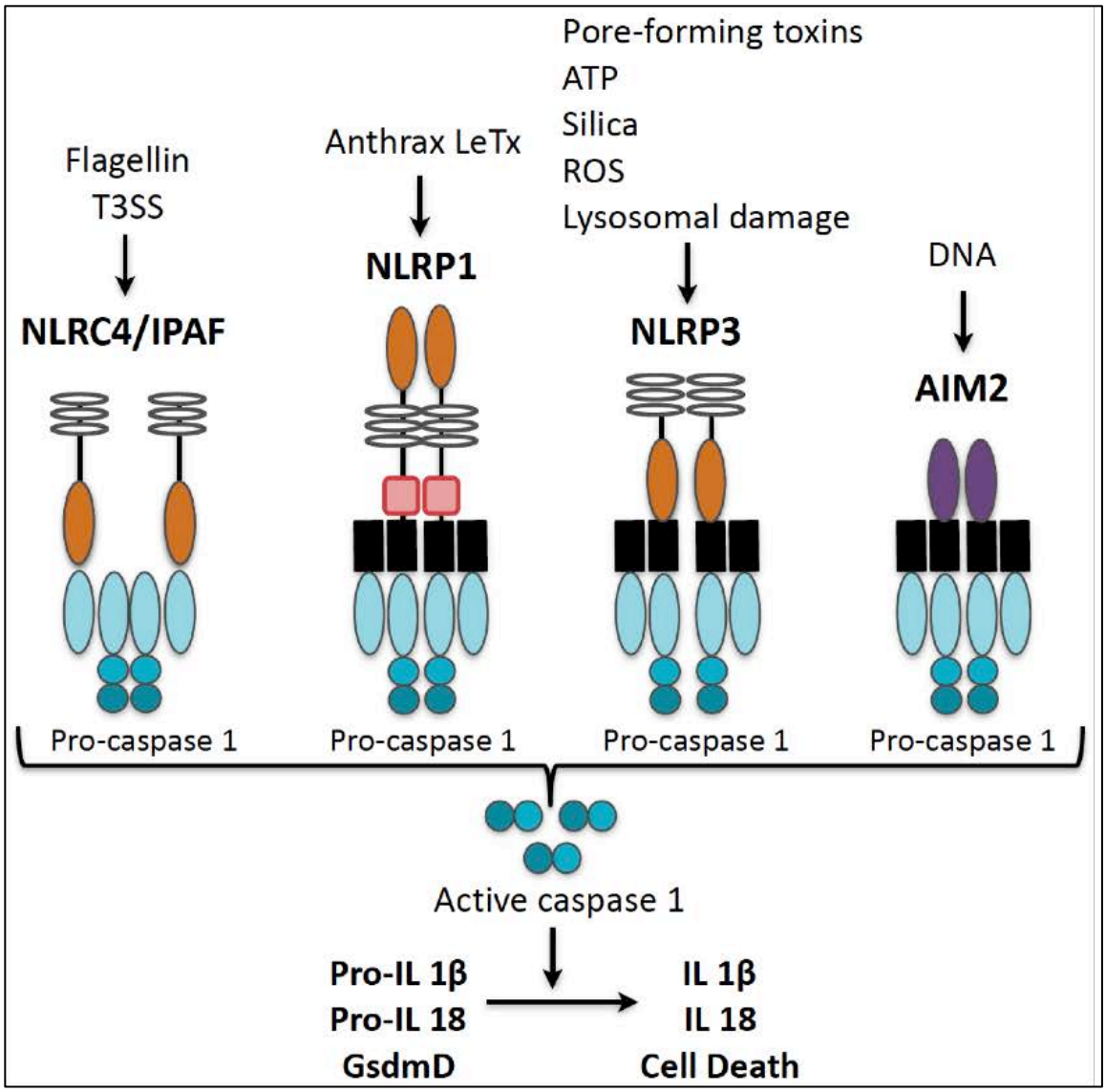

Figure 1.3: Canonical caspase 1-dependent inflammasomes. Canonical inflammasomes receptors include NLRC4 (activated by bacterial flagellin) NLRP1b (activated by anthrax lethal toxin), AIM2 (activated by cytosolic dsDNA) and NLRP3 (activated by a wide variety of signals e.g. pore-forming cytotoxins, ATP, ROS or reactive oxygen species). On activation, the receptors form an inflammasome complex with or without the adaptor, ASC, and recruit procaspase-1, which is subsequently cleaved into active caspase-1, which in turn cleaves pro-forms of IL-1b and IL-18 into their active forms as well as Gasdermin D (GsdmD) to induce cell death. (Adapted from Vanaja, Rathinam and Fitzgerald, *Trends in Cell Biol.,* 2015)

NLR inflammasomes

NLRs are generally composed of three separate domains, all of which are found throughout metazoan evolution. At the N-terminus, NLRs either have a pyrin (PYD) domain, a caspase recruitment domain (CARD), or a baculovirus inhibitory repeat (BIR; also called IAP repeat) domain (2, 5). The central NBD (nucleotide-binding domain; also called NACHT, NAIP, CIITA, HET-E and TP1) domain is responsible for nucleoside-triphosphatase (NTPase) activity and oligomerization. The NBD, in the presence of nucleotides, especially ATP, is thought to maintain a ligand-receptive state. The C terminus of NLR proteins is composed of a manifold series of leucine-rich repeats (LRR). All these domains have been implicated in protein-protein interactions and downstream signal activation, through association with large macromolecular complexes called inflammasomes (28).

NLRP1b The first NLR that was shown to form an inflammasome complex was NLRP1. Humans only have one NLRP1 protein compared to the multiple paralogues found in mice, such as Nlrp1a, Nlrp1b and Nlrp1c. NLRP1 is expressed in adaptive immune cells and tissues as well as in non-hematopoietic tissues. Human NLRP1 is structurally unique among NLRs- it has an N-terminal CARD, central NACHT, LRR, function-to-find (FIIND) domains, and another C-terminal CARD.

Mouse Nlrp1b recognizes the anthrax lethal toxin (LeTx) from *Bacillus anthracis* in the cytoplasm to activate inflammasomes that is crucial for defense against *B. anthracis* spores in mice. The *B. anthracis* LeTx is a zinc metalloprotease lethal factor that enters the host cytosol and cleaves mitogen-activated protein kinase (MAPK) kinases

to block immune signaling. The LeTx also cleaves Nlrp1b close to its N-terminus that likely releases the receptor from its autoinhibitory state and/or induces structural changes that is more conducive to oligomerization. The lethal-toxin responsive Nlrp1b also undergoes an autoproteolytic processing in its FIIND domain, which is required for its function (35).

In vitro, NLRP1 interacts with caspases 1 and 5 to form a macromolecular complex that is necessary for IL-1β/IL-18 processing and pyroptosis. NLRP1 also interacts with caspases 2 and 9 to facilitate cell death via the apoptosome (36). Additionally, the Nlrp1b inflammasomes are functional independent of ASC, as caspase 1 is activated via ubiquitination, and not cleavage, to induce IL-1β release and cell death.

NLRP3 The NLRP3 (also called as cryopyrin and NALP3) inflammasome is the most well characterized inflammasome to date. NLRP3 is expressed by myeloid cells and is upregulated in response to the stimulation PAMPs (13). The gene encodes a pyrin domain, NBD and C-terminal LRR (14). NLRP3 lacks the CARD domain and thus, recruits procaspase-1 only in the presence of the adaptor molecule ASC through homophilic interactions between pyrin domains. It is triggered by various distinct DAMPs and PAMPs like K^+ efflux, mitochondrial reactive oxygen species (ROS), mitochondrial or lysosomal damage, ATP, β-amyloids, hyaluronan, cholesterol crystals, crystalline particles (e.g. silica, alum), RNA:DNA hybrids, pore-forming toxins (e.g. hemolysin, pneumolysin) and several microbial pathogens. It is likely that NLRP3 is activated in response to a common cellular event (e.g. disruption in the cellular homoeostasis, pore formation in plasma membrane, lysosomal damage releasing

cathepsin B, ROS generation), which is downstream of all the aforementioned triggers. Activation of NLRP3 requires priming of the innate immune cells that induces *NLRP3* gene expression. NLRP3 activates IL-1β release via the classical ASC and caspase 1-dependent inflammasome pathway. However, caspase 8 and FADD have also been implicated in NLRP3 inflammasomes in bacterial (e.g. *Staphylococcus aureus* (37), *Listeria monocytogenes* (38), *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Escherichia coli*, *Porphyromonas gingivalis*, *Shigella flexneri* and *Chlamydia* spp.), fungal (e.g. *Paracoccidioides brasiliensis* (39), *Cryptococcus neoformans* (40), *Aspergillus fumigatus* (41) *Candida albicans* (42)) and viral (e.g. adenovirus, IAV, SeV, VACV) infections, though the mechanism has not been entirely elucidated.

In addition, nucleic acids like bacterial RNA (43), mitochondrial DNA (44) as well as RNA:DNA hybrids (45) derived from bacterial replication intermediates also stimulate the NLRP3 inflammasomes. Although the nucleic acids co-localize with active NLRP3 inflammasome specks, it is unclear if they interact directly with NLRP3 or through an intermediate nucleic acid binding component.

It is not completely understood how NLRP3 gets activated though some recent insights have defined a role for NEK7 (NIMA-related protein kinase 7) that acts downstream of K^+ efflux, binds to NLRP3 and controls NLRP3 oligomerization via its catalytic domain. HSP90 and SGT1 have been shown to interact with NLRP3 LRRs to maintain an inactive but stabilized structure (46). Further, deubiquitination of NLRP3 is required for its activity. So far there has been no direct evidence of ligand binding to NLRP3, which led to the general hypothesis that NLRP3 senses changes in the cellular milieu initiated by activators both self-derived and foreign.

Genetic mutations that activate or predispose towards activation of the NLRP3 inflammasome are associated with cryopyrinopathies or cryopyrin-associated periodic fever syndromes (CAPS) such as FCAS (familial cold-induced autoinflammatory syndrome), MWS (Muckle-Wells syndrome), and NOMID/CINCA (neonatal onset multisystem inflammatory disorder or chronic infantile neurologic cutaneous and articular syndrome) (47). All these disorders are associated with periodic fevers and rashes, arthralgia, and conjunctivitis associated with neutrophil-dependent inflammation as well as neurological involvement, including aseptic meningitis and deafness as seen in NOMID/CINCA.

NLRC4 NLRC4 is expressed predominantly in hematopoietic tissues and cells. The gene encodes a CARD, NBD and C-terminal LRRs. It can interact directly with procaspase-1 via homotypic CARD interactions as well as through the adapter ASC, which leads to the processing of caspase-1 (48). Intracellular flagellin is an activator of the NLRC4 inflammasome (49, 50). These responses to flagellin depend on an intact type III or IV secretion system (T3SS or T4SS) in the bacteria by which the flagellin enters the host cytosol (51). The rod proteins, which are the components of the bacterial secretion system, also act as activators of NLRC4 inflammasome (51). Using NLRC4-deficient system demonstrated that NLRC4 is critical for host defense against *Salmonella*, *Shigella*, *Pseudomonas*, and *Legionella*. Moreover, it has been shown that *Salmonella* and *Legionella* mutant lacking flagellin are incapable of activating the inflammasome.

Similar to NLRP3, there has been no direct evidence of a ligand-receptor relationship between NLRC4 and flagellin or rod proteins. NLRC4 also co-operates with

other NLRs such as NLRP3 in *Salmonella, Shigella* infections to activate the inflammasome (52, 53). In contrast to other inflammasomes, NLRC4 requires another NLR protein that functions as a co-receptor. There are four NAIP proteins in mice - NAIP1 binds to needle proteins of the T3SS, NAIP2 binds to the *Salmonella* SPI-1 basal rod component PrgJ and Shigella T3SS protein MxiI, and NAIP5/6 sense flagellin (54-58). Humans express only one NAIP protein, which binds the *Chromobacterium violaceum* needle protein, Cprl (and not flagellin) (58). NAIP5 had been genetically linked to *Legionella* replication in mice (83) and humans (84) and was shown to sense an additional epitope found within flagellin (85, 86) which led to the discovery of association of NLRC4 with NAIP5 that restricts *Legionella* replication (87).

NLRP12 Infection with the plague-causing bacteria, *Yersinia pestis* induces NLRP12 inflammasome activation leading to production of IL-18, which is critical for the pathogen clearance (59). Further, NLRP12-deficient mice are highly susceptible to *Y. pestis* infection. The NLRP12 as well as NLRP3 inflammasome complexes are also present in monocytes of malaria patients, suggesting that, together with the NLRP3 inflammasome, the NLRP12 inflammasome may be important in IL-1β production and hypersensitivity to secondary bacterial infections in infected patients (60). However, the specific ligand that activates the NLRP12 inflammasome is not known, and further elucidation of the role of NLRP12 in infectious diseases is required.

NLRP6 The NLRP6 inflammasome induces IL-1β and IL-18 production and simultaneously regulates autophagosome formation that is required for

mucin granule exocytosis from the goblet cells in the intestine that maintains intestinal barrier integrity. NLRP6 depletion in mice alters the composition of colonic microbiota due to expansion of colitogenic bacteria, *Bacterioidetes* and TM7 (61-63). However, the mechanism of how NLRP6 modifies the intestinal microbiota, and the ligand that drives NLRP6 activation are still unknown.

Pyrin inflammasomes

Pyrin (also called marenostrin and TRIM20) is a non-NLR protein expressed primarily in immune cells such as monocytes and dendritic cells (64). Encoded by the gene, *MEFV*, the protein contains an N-terminal pyrin, two B-box zinc-fingers, a coiled coil, and C-terminal B30.2 (also called SPRY) domains (65). Mutations in the MEFV gene cause an autoinflammatory disease, familial Mediterranean fever (FMF) (66). Pyrin had been shown to form an inflammasome complex with ASC and activate caspase-1 in *in vitro* reconstitution assays, but the biological relevance of this inflammasome had remained unknown for a while (65, 66). The physiological activation of pyrin inflammasome was only recently characterized in macrophages and DCs that occurred in response to bacterial toxins of *Clostridium difficile* Toxin B, of *Clostridium botulinum* C3 toxin as well as by effector proteins VopsS from *Vibrio parahaemolyticus* and IBpA from *Histophilus somni* (64). Intriguingly, pyrin is activated by pathogen-mediated modifications of the host proteins, Rho GTPases. Rho glycosylation by *C. difficile* Toxin B, FIC-domain adenylylation by VopS and IbpA, ADP-ribosylation by *C. botulinum* C3 toxin, and deamination by *Burkholderia cenocepacia* act as ligands for pyrin and activate inflammasomes. Pertussis toxin also induces the pyrin inflammasome

through its ADP-ribosyltransferase activity (67). All of these inactivating modifications occur in the switch-I residue of RHOA proteins and inactivation of other members (e.g. RAC and CDC42) does not trigger pyrin inflammasomes. This indicates that pyrin may not directly interact with RHOA but senses a downstream event such as changes in cytoskeletal dynamics, since pyrin is known to interact with actin (68). Further, *Wdr*$^{-/-}$ mice that lacks a protein involved in actin depolymerization has been found to be susceptible to pyrin-dependent autoinflammation and thrombocytopenia (69). Taken together, the pyrin inflammasome represents a novel inflammatory pathway that detects pathogen-induced cellular changes to induce fitting immune responses.

AIM2 and IFI16 inflammasomes

AIM2 detects cytosolic DNA derived from DNA viruses such as murine cytomegalovirus (MCMV) and Vaccinia Virus (VACV) as well as from cytosolic bacteria like *Francisella tularensis* and *Listeria monocytogenes* to induce an ASC-caspase 1-dependent inflammasome that leads to IL-1β, IL-18 processing and cell death. IFI16, on the other hand, activates an ASC-dependent inflammasome in the nucleus in cells infected with KSHV. IFI16 also detects the RNA virus, HIV to initiate inflammasome activation. (Detailed descriptions of AIM2 and IFI16 in Sections 1.5 and 1.6)

1.4.2 Caspase-11/Caspase-4/5 dependent non-canonical inflammasomes

The non-canonical inflammasome pathway involves the activation of mouse caspase 11 and the human caspases 4 and 5 in response to the Gram-negative bacterial endotoxin, LPS in the host cytoplasm (70-73). Unlike caspase 1, caspase 11, 4 and 5 activation does not process IL-1β or IL18 (71) but initiates GSDMD-mediated pyroptotic cell death (30) and release of endogenous alarmin molecules such as HMGB-1 in a caspase-1 independent manner (72). Activation of caspase-11 by intracellular LPS cleaves gasdermin D to release an active N-terminal fragment of gasdermin D that controls NLRP3-dependent activation of caspase-1 and cell death via pore formation in the plasma membrane that promotes pyroptosis. Again, in contrast, caspase 11 activation seems to progress independently of all known inflammasomes including NLRP3, NLRC4 and NLRP6 (72).

In comparison to canonical inflammasomes, it was expected that a CARD-containing upstream of caspase 11 should be responsible for intracellular LPS-mediated caspase 11-dependent inflammasome activation. However, interestingly, an alternative multiprotein complex assembly was proposed that suggests that caspase 11 (caspase 4/5 in humans) itself may be acting as the direct receptor for cytosolic LPS. This conclusion was drawn from the observations that caspase 11 purifies from E. coli as an oligomer of higher order. Further, biochemical studies showed that caspase 11 bound directly to LPS through the positively-charged lysine residues in the caspase 11 CARD domain (73). Interestingly, this interaction was specific to caspase 11 as the closely related CARD domain of caspase 1 was unable to bind LPS (73). However, it still remains to be shown

whether there are other factors that facilitate the detection of cytosolic LPS, binding and subsequent activation of caspase 11.

Other studies have demonstrated the roles of TRIF and type-I IFN in causing pyroptosis and caspase 11- dependent caspase 1 activation in Gram-negative bacterial infections. Type-I IFN, via TLR3 and/or MAVS, induces caspase 11 expression through STAT1-IRF9 activation though it is partially upregulated by NF-κB as well. Furthermore, IFNα/β regulates caspase 11 pathway by inducing guanylate-binding proteins (GBPs) that are involved in host immunity and antimicrobial defenses (74, 75). The precise mechanism by which GBPs control caspase-11 remains unresolved. The GBPs are though to mediate optimal caspase 11 activation by disrupting the pathogen-containing vacuoles that releases bacteria into the cytosol and thus activating caspase 11 (74). Conversely, another study claims that instead of regulating the integrity of the bacteria-containing vacuole but the GBPs act downstream to activate caspase 11 (75).

Moreover, while it is known that caspase-11 synchronizes pyroptotic cell death and caspase 1 activation downstream of NLRP3 inflammasomes, the mechanism still remains unclear. While the assembly of NLRP3-ASC specks does not require caspase 11, caspase 11 does interact with caspase 1 during Gram-negative bacterial infections. However, the discovery that induction if caspase 11 by poly (I:C) (TLR3 ligand) conferred *Tlr4-/-* mice susceptible to LPS-induced lethality indicates that caspase 11 drives LPS- dependent endotoxemia (in contrast to TLR4 that only primes the cells in response to extracellular LPS) (70, 72). Thus, the host system has evolved with two distinct pathways that respond differentially to extracellular and intracellular Gram-

negative bacteria. Together these findings establish the essential role of caspase 11 dependent immune responses in controlling bacterial infections.

1.4.3 Caspase-8 dependent non-canonical inflammasomes

Initially, the inflammasomes induction and processing of IL-1β was attributed solely to caspase 1 activity. However, recently it has come to light that there are alternative pathways, involving other caspases, which can process IL-1β release and regulate inflammatory responses.

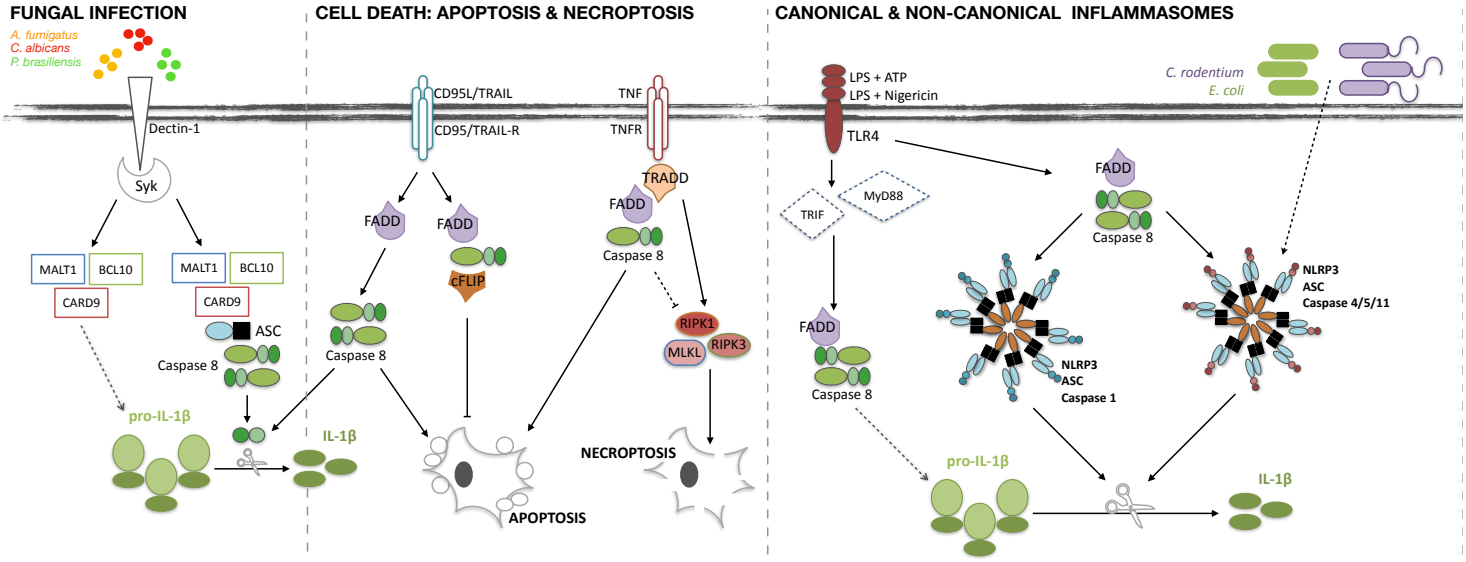

Figure 1.4: Role of caspase 8 in fungal infection, cell death and regulation of inflammasomes. Caspase 8 activates IL-1β activation during fungal infections in dectin-1/Syk/MALT1/CARD9/Asc dependent manner. The dectin-1 complex also regulates pro-IL-1β gene expression. Signaling through CD95/Fas (e.g. LPS stimulation) or with TRAIL induces apoptosis by recruitment of FADD and caspase 8 homodimers, which is blocked by cFLIP-Caspase 8 heterodimers. Caspase 8 also controls the decision between apoptosis and necroptosis (through RIPK1, RIPK3 and MLKL) upon TNFα stimulation. Caspase 8 is also required for canonical and non-canonical NLRP3 inflammasome activation. Stimulation of TLR results in NF-κB activation and up-regulation of pro–IL-1β as well as NLRP3 mRNA that is partially dependent on caspase-8. Active caspase-8 is required for activation of the canonical caspase 1 (e.g. LPS+ATP, LPS+nigericin) and non-canonical caspase 11 (e.g. *C. rodentium*, *E. coli*) inflammasome through assembly and activation of the NLRP3 complex. However, caspase 8 can also negatively regulate NLRP3 inflammasome activation in a RIPK1/RIPK3 dependent manner (not shown).

Involvement of caspase 8 in cell death through mitochondrial apoptotic signaling is well characterized. Caspase 8 is expressed in its zymogen form that is activated by its autoactivation and proteolytic cleavage. Activation of death receptors (e.g. Fas, tumor necrosis factor receptor (TNFR) 1 and death receptor 3) promotes the association of the adapter protein Fas-associated death domain (FADD) with caspase 8. Activated caspase-8 propagates the apoptotic signal either by directly cleaving and activating downstream apoptotic caspases such caspase 3, or by cleaving the pro-apoptotic proteins, BH3 Bcl2-interacting protein, which leads to the release of cytochrome c from mitochondria, that activates caspase-9, which promotes apoptosis.

Recent studies have shown that activated caspase 8 acts as a protease that can cleave IL-1β, independently or as a part of inflammasomes, at the Asp117 site, the same site that is targeted by caspase 1 but not by caspase 3, caspase 7, caspase 9 and catalytically inactive caspase 8 mutant (76-79). Caspase 8 is a critical protease that that activates pro-IL-1β during fungal pathogenesis such as *Candida albicans* and *Aspergillus fumigatus* infections (80). The dectin-1 receptor signaling pathway is activated by fungal components such as β-glucans and induces a non-canonical inflammasome complex composed of Caspase recruitment domain-containing protein (CARD) 9, B-cell lymphoma/leukemia (BCL) 10 and Mucosa-associated lymphoid tissue lymphoma translocation protein (MALT) 1 that interact with ASC and caspase 8. The CARD9-BCL10-MALT1 (CBM) complex activates the kinase, Syk that induces NF-κB-dependent transcription of pro-IL-1β, through the phosphorylation and degradation of the IKK complex. The recruitment of ASC-caspase 8 to this complex activates caspase 8 that cleaves pro-IL-1β in a caspase 1 –independent manner. This mechanism of caspase 8-

dependent IL-1β activation was is important during infections with *Mycobacterium bovis* and *Mycobacterium leprae* (80).

Blocking or depletion of IAPs (inhibitors of apoptosis proteins) in macrophages resulted in LPS-induced IL-1β secretion that was dependent on both caspase 8 and caspase 1- NLRP3 inflammasome. Blocking of the IAPs, XIAP (X-linked IAP; also called BIRC4), cellular IAP (cIAP) 1 (also called BIRC2) and 2 (also called BIRC3), resulted in the release of receptor-interacting serine/threonine-protein kinase (RIPK) 3 that triggers the caspase 1 and/or caspase 8 dependent pathways (81, 82). Further, a later study confirmed that RIPK3 is required for LPS-induced caspase 1- or caspase 8-dependent secretion of IL-1β in dendritic cells. The caspase 8-containing pro-IL-1β processing complex contains RIPK1, RIPK3 and FADD, and requires signaling through interaction with the C-terminal receptor-interacting protein homotypic interaction motif (RHIM) of TRIF (83).

Pro-apoptotic chemotherapeutic drugs (e.g. doxorubicin) that induce ER stress promotes caspase 8-TRIF dependent, caspase 1 independent secretion of IL-1β in LPS-primed macrophages or dendritic cells. It is hypothesized that the drugs dysregulate mitochondrial functions that induce the release of second mitochondrial-derived activator of caspases (SMAC), which inhibits IAPs, and thus averting IAP-mediated degradation of RIPK3 and inhibition of caspase 8-dependent processing of IL-1β (77, 84).

Another pathway employs TLR7-MyD88-primed FAS and FAS ligand (FASL; also called TNFSF6 and CD95L) interactions to drive caspase 8- and FADD-dependent release of IL-1β and IL-18, and is independent of RIPK3.

Recent studies have implicated caspase 8 involvement in the activation of caspase 1 that leads to IL-1β maturation. Caspase 8 have been identified to co-localize with ASC or caspase 1 inflammasome specks in macrophages and DCs infected with *S. typhimurium*, *F.novicida*, *C. rodentium* and *A. fumigatus*. Recombinant caspase 8 has been shown to cleave and activate caspase 1 *in vitro* upon stimulation with LPS and ATP or infection with *Yersinia pestis*, *Yersinia psuedotuberculosis*, *C. rodentium* and *A. fumigatus*. Activation of caspase 1 by caspase 8 in *Yersinia* infection requires RIPK1 and FADD and partly dependent on RIPK3 and TRIF. This is in contrast to *C. albicans* infection where caspase 1 and caspase 8 regulate IL-1β maturation independently of each other. Also, a delayed pathway of caspase 8-dependent IL-1β release occurs in absence of caspase 1 upon prolonged stimulation with LPS or nigericin, or infection with *S. typhimurium*. Hence, the caspases 1 and 8 can act synergistically as well as independently dependent on the stimulations to activate canonical and non-canonical inflammasomes and further studies are required to tease apart the mechanics of this relationship.

1.4.4 Regulation of Inflammasome Activation

The inflammasomes are tightly modulated by multiple mechanisms including transcriptional and post-transcriptional control, post-translational modifications and through other factors and pathways that regulate the expression of the inflammasome components like the receptors, the adapter ASC or the caspases as well as fine-tune the intensity and efficacy of inflammasome activation.

Regulation by Host Proteins Interactions

Pyrin-containing regulators

A major class of inflammasome regulators includes proteins that contain only the pyrin domain. The PYD-only proteins (POPs) are found primarily in humans and higher primates but not in mouse. There are three POP proteins, POP1, POP2 and POP3 that blocks the PYD-PYD interactions and subsequent inflammasome multimerization.

POP1 (also called PYDC1, ASC2) shares sequence similarity with the pyrin domain of ASC and its overexpression blocks IL-1β activation. It competitively binds to ASC, making the latter unavailable to bind NLRP3, thus dysregulating NLRP3-mediated, canonical inflammasome activation. POP1 expression is induced by TLR-NF-κB signaling, suggesting that it provides a regulatory feedback loop that may block further assembling of the inflammasome structure (85, 86).

Though POP2 is more similar to the pyrin domains of NLRP3, its mode of action is comparable to POP1. Recently Stehlik and group showed that POP2 inhibits inflammasome assembly by binding ASC and interfering with the recruitment of ASC to upstream sensors. Using transgenic mice expressing POP2, they showed that POP2 also reduces macrophage priming by inhibiting the activation of non-canonical kinase, IκBε, and IκBα and thus, protects the mice from excessive inflammation and acute septic shock (87).

The third pyrin-only protein gene, POP3 is present in the human PYHIN locus between PYHIN1 and IFI16, and is often considered as the fifth PYHIN protein in humans. Similar to the PYHINs, type-I IFN controls the expression of POP3 and shows sequence similarity with AIM2 and other PYHIN family members. POP3 interacts with

AIM2 and IFI16, and regulates DNA-induced cytokine responses. Knockdown of POP3 expression in human macrophages resulted in increased AIM2 inflammasomes. Mouse macrophages expressing ectopic POP3 reduced AIM2-mediated inflammation during infections with the DNA virus, MCMV or modified vaccinia virus Ankara but did not alter NLRP1, NLRP3 or NLRC4 activation (88).

Interestingly, a murine IFN-inducible, PYHIN protein, p202 (also called ifi202) contains a single pyrin-domain and negatively regulates AIM2 activation. But instead of PYD-PYD interactions, it blocks AIM2 activation through DNA binding and interaction with AIM2 that prevents ASC clustering (89).

CARD-containing regulators

Another class of proteins that modulate inflammasome signaling are the caspase recruitment domain or CARD-only proteins (COPs), whose modes of action are similar to that of the POPs. There are three COPs in humans, CARD16 (also called COP, PSEUDO-ICE), CARD17 (also called INCA) and CARD 18 (also called ICEBERG), all of which share high sequence homology with caspase 1. Evidence from biochemical interactions in overexpression systems suggests that they may negatively regulate caspase 1 activity possibly by sequestration or blocking recruitment to ASC. Alternatively, a recent study showed that CARD16 could promote caspase 1 oligomerization to enhance activity. Other studies have shown that CARD16 and 18 can also interact with RIPK2 indicating they may regulate other pathways as well.

A less-characterized caspase, caspase 12 has also been shown to regulate caspase 1 activity. Multiple polymorphisms are found in the *CASP12* gene, which can

generate a full-length caspase (Caspase12L) or an inactive truncated form (Caspase12S). Caspase12L is found amongst populations of African descent that makes them hyporesponsive to LPS and protects from sepsis. Overexpression of caspase 12 in mouse negatively regulates caspase 1 activity.

Post-translational modifications

Phosphorylation

Modifications of the receptors and the adapter ASC by phosphorylation or ubiquitination regulate activity of the inflammasome. The phosphorylation of NLRC4 by protein kinase Cδ (PKCδ) was essential against *Salmonella* infections but not *Shigella flexineri*-induced responses in macrophages (90, 91). Dixit and group showed that a Serine residue (Ser533) in the helical domain 2 (HD2) of the NLRC4 NOD motif requires phosphorylation in unprimed macrophages to induce caspase 1 activation (90). Conversely, in primed cells, the need for phosphorylation is bypassed, with NLRP3 expression that associates with NLRC4, independently of its Ser533 phosphorylation, to activate caspase 1 (91). Similarly, the dsRNA-dependent protein kinase (PKR or EIF1AK2) has been suggested to regulate the activities of NLRP3, NLRP1, NLRC4 and AIM2, though a role for PKR in the phosphorylation of these PRRs in inflammasome activation is yet to be elucidated (92, 93). Phosphorylation of the inflammasome adapter protein, ASC at multiple sites in its CARD domain by the kinases, Syk and JUN N-terminal Kinase (JNK) are essential for caspase 1 activity.

Ubiquitination

Activation of NF-κB pathways that prime the immune cells is required for inflammasome activation. Priming has been known upregulate NLRP3 expression transcriptionally. In addition, it was demonstrated that it also enhanced NLRP3 inflammasome signaling by eliciting the deubiquitination of NLRP3 by a Lys63-specific deubiquitinase, BRC33.

The multi-protein linear ubiquitin chain assembly complex (LUBAC) consists of three proteins, HOIL-1, HOIP and SHARPIN, of which HOIL-1 and SHARPIN are essential for TLR-dependent for NF-κB activation and inflammasome activation. LUBAC activates the inflammasome by directly interacting ASC, but not NLRP3 or AIM2, catalyzing its linear ubiquitination, through the E3 ligase activity of HOIL-1 and HOIP. However, SHARPIN inhibits inflammasome activation, likely through ubiquitination and degradation of the inflammasome.

K63-ubiquitination can provide either inhibitory or activating signal for the ASC-dependent inflammasomes. In macrophages, activation of autophagy results in decreased inflammasome activity with ASC displaying increased K63-linked ubiquitination and autophagosome recruitment in the NLRP3 or AIM2 inflammasome (described later in the section). In contrast, in response to viral RNAs or VSV infection, MAVS engages TRAF3, an E3 ligase and ASC. K63-linked ubiquitination of ASC, at Lys^{174}, by TRAF3 was essential for inflammasome activation and IL-1β release, but also promoted autophagosome recruitment. Activation of the inflammasome and autophagy by the same signal provides a possible mechanism by which the host immune responses are self-limiting and controlled.

Levels of IL-1β are also regulated by ubiquitination. TLR-induced pro-IL-1β (Signal 1) that does not receive an inflammatory signal (Signal 2) is ubiquitinated and degraded at the proteasome. A high-risk strain of human papillomavirus (HPV16) uses an ubiquitin ligase E6-AP that promotes ubiquitination and proteasomal degradation of pro-IL-1β and thereby evading host immune responses.

Transcriptional and Post-transcriptional Regulation

Priming, through NF-κB activation, is the critical first step in the two-step activation process of inflammasomes. Priming (e.g. LPS-TLR4, Pam3-TLR2) constitutes the Signal 1 that induces a subset of proinflammatory genes including NLRP3, pro-IL-1β. Signal 2 (e.g. NLRP3 triggers such as K+ efflux, mitochondrial DNA or AIM2 triggers such as DNA viruses) are provided by the ligands that engage the receptors that activate the inflammasome formation, caspase 1 activity and IL-1β secretion.

Fungal PAMPs are recognized by several CLRs and TLRs that can prime cells (94). In addition to inducing caspase 1-independent, caspase 8-dependent IL-1β, dectin-1 signaling is also involved in fungus-induced priming for NLRP3 inflammasomes (95). As mentioned earlier, dectin-1 uses an immunoreceptor tyrosine-based activation motif (ITAM) to couple itself to Syk kinase for downstream signaling to NF-κB via the CBM scaffold resulting in cytokine production. Additionally, phagocytosis and reactive oxygen species (ROS) production result from dectin-1 engagement (96). *Dectin-1*$^{-/-}$ mice orally infected with *C. albicans* presented significantly reduced serum IL-1β levels (97). Similarly, mice lacking CARD9 as well as humans with mutations in dectin-1 and CARD9 show susceptibility to candidiasis and other fungal infections (97-101). The Syk

kinase-dependent NLRP3 priming also occurs in infections with *Paracoccidioides brasiliensis* (102), *Cryptococcus neoformans* (40) and *Aspergillus fumigatus* (41). Intriguingly, glucuronoxylomannan, the main capsule component of C. neoformans, inhibits Syk signaling and subsequently NLRP3 inflammasomes, which may assist in the infectivity of the fungus (40). Studies from our group showed that caspase-8, dectin-1 and CR3 (another receptor capable of detecting β-glucan) are necessary for IL-1β processing by in dendritic cells infected with *C. albicans* (103). It also opened up new questions about *C. albicans* -induced, caspase 8 -dependent programmed cell death pathways (104).

Another pathway that has been implicated to enhance NLRP3 activation is the TAK1-JNK pathway, which is activated by lysosomal rupture (an NLRP3 trigger), and is essential for the priming of NLRP3-ASC pathway.

Regulation by Autophagy

Autophagy is a cell-intrinsic, homeostatic process during which cellular components are recycled through the autophagosomes and lysosomes to benefit cell survival, and is triggered by starvation, damaged organelles and infection. Further, autophagy can regulate both cell death and inflammasome assembly, and depending on the conditions, can be either pro- or anti-apoptotic (105). Autophagy negatively regulates inflammasome activation and IL-1β release, such that mice that lack ATG16L1 (autophagy-related protein 16-like 1), an essential component of the autophagy machinery, show increased caspase 1 activity and higher IL-1β, IL-18 levels in LPS-treated macrophages (106). The mechanisms by which autophagy regulates the

inflammasome is not entirely elucidated. One theory suggests that autophagy removes ubiquitinated inflammasomes (107) or pro-IL-1β (108).

Induction of AIM2 or NLRP3 inflammasomes in macrophages triggers the activation of a Ras-like small G protein, RalB that promotes autophagosome formation, and was dependent on the presence of the inflammasome receptor, not ASC or caspase 1. In the assembled inflammasomes, ASC was Lys63 (K63)-linked polyubiquitinated that recruited the autophagic adaptor p62, which in turn delivered the inflammasome to the autophagosomes for degradation (107). Activation of autophagy with rapamycin, in macrophages, induces the degradation of TLR-induced pro-IL-1β in autophagosomes and blocked secretion of the mature IL-1β. Similarly, in vivo, treatment with rapamycin decreased levels of IL-1β in the serum upon LPS stimulation. Inhibition of autophagy promoted the processing and secretion of IL-1β in an NLRP3- and TRIF-dependent manner and was dependent on ROS, but not NOX2 (108).

Another mechanism suggests that autophagy removes damaged mitochondria, which limits the availability of NLRP3 inflammasome triggers (109). ROS-generating mitochondria are constantly removed by mitophagy, a specialized process of autophagy. Treatment THP1 macrophages with a mitophagy inhibitor to, which, as expected, resulted in the accumulation of damaged mitochondria and increased concentrations of mitochondrial ROS that correlated with the dose-dependent secretion of IL-1β. Processing of proIL-1β caused by the blockade of mitophagy, that prevents the release of mitochondrial ROS and DNA into the cytoplasm, was NLRP3- and ASC-dependent and not reliant on NLRC4. In contrast, caspase 1 activation in NLRP3 and AIM2 inflammasomes leads to blockade of mitophagy through multiple that allow

mitochondrial disassembly, ROS production, loss of membrane potential, to amplify mitochondrial damage, partly by the cleavage of a mitophagy regulator, Parkin. Hence, these studies show that inflammasomes and autophagy are tightly coordinated processes that control each other. The pathways are regulated at multiple steps to achieve an optimal level of inflammasome activation.

Regulation through Crosstalk with Other Pathways

Regulation by Interferons

Both type I (IFN-α/β) and type II (IFN-γ) are known to control inflammasome signaling. Type I IFN reduces the expression of pro-IL-1β and pro-IL-18 as well as represses NLRP1b and NLRP3 inflammasomes in a STAT3- (through anti-inflammatory cytokine, IL-10) and STAT1- dependent manners respectively. Similarly, type I IFN induction *in vivo* by poly (I:C) treatment reduces cell recruitment after injection with a NLRP3 activator, alum as well as increases susceptibility during *C. albicans* infection. In mycobacterium infection, T-cell derived IFN-γ was shown to inhibit NLRP3 inflammasome *in vivo*, through nitric oxide synthase that promotes nitrosylation and inactivation of NLRP3, and $CD4^+$ T cell-derived IFN-γ suppresses IL-1 in inflammatory monocytes. IL-1 production by macrophages and dendritic cells is also inhibited by type I IFN in infection with *Mycobacterium tuberculosis*.

Interferons can also activate inflammasome signaling. For example, AIM2 activation during *F. novicida* needs STING-dependent production of type I IFN. As described before, the activation of caspase 11 in bacterial infections requires TLR4-TRIF-IRF3 mediated IFN signaling. Both *AIM2* and *caspase 11* gene expressions are

stimulated by IFN signaling. Further, a family of IFN-iducible GTPases, GBPs upregulated by IFNAR-IRF1, control inflammasome activation. GBPs restrict the replication of intracellular pathogens and control antimicrobial processes such as oxidation, autophagy, to the destabilization of pathogen-containing vacuoles and direct killing of the pathogens. GBP2 and GBP5 were shown to be required for *F. novicida*-dependent AIM2 activation and GBP2 is required for caspase 11 activation but not for inflammasome activation in response to poly (dA:dT) and LPS. In another study, GBPs were shown to enhance inflammasome signaling by promoting NLRP3–ASC oligomerization or by inducing pyroptosis.

Conversely, our group and others have observed that inflammasome activation can also dampen IFN responses. For example, AIM2 activation by DNA viruses or purified DNA ligands, such as poly (dA:dT) that activate the inflammasome represses the STING-dependent type-I IFN induction in response to DNA. Together, these observations begin to explain how different pathways, which are activated during infections or cellular stress, interact and how they may regulate each other, and the efficacy and intensity of immune responses.

Regulation by Caspase 8, RIPK1/RIPK3 and Necroptosis

In addition to being able to form non-canonical inflammasomes dependent and independent of caspase 1, caspase 8 has multiple regulatory roles that can activate or inhibit the inflammasome as well as modulate signaling and priming of antigen-presenting cells. Caspase 8 has been shown to be involved in modulating NF-κB signaling and production of pro-inflammatory cytokines in mouse macrophages and B

cells (78, 110-112). When mouse macrophages lacking both caspase 8 and the kinase, RIPK3 were infected with *S. typhimurium*, *Citrobacter rodentium* or *E. coli* or were stimulated with LPS (TLR agonist), Pam3CSK4 (a TLR2 agonist), muramyl dipepetide (MDP; a TLR2/4 agonist) or poly (I:C) (a TLR3 agonist), the macrophages failed to induce robust expression of pro-IL-1β, IL-6 and TNFα compared with wild-type macrophages or macrophages lacking RIPK3 alone. Further studies confirmed that the caspase 8 adapter, FADD (Fas-associated protein with death domain) is required for the NF-κB activation. It is known that the genomic deletions of *Casp8* or *Fadd* confer embryonic lethality in mice, which is rescued by a parallel genomic deletion of *Ripk3* (113-115). Hence, the macrophages deficient in caspase 8 or FADD also lack RIPK3. Conditional deletion of caspase 8 in B cells exhibit impaired phosphorylation and nuclear translocation of the NF-κB transcription factor, p65/RelA and concomitant decrease in expression of IL-6, TNFα, IFNβ and CXC-chemokine ligand 10 (CXCL10) in response to LPS stimulation.

The molecular mechanism by which caspase 8 mediates NF-κB signaling is unclear. One study found that full-length caspase 8 and its catalytic activity were crucial for driving NF-κB activities in humans (116), while another study in cancer patients showed that the DEDs of caspase 8 are sufficient (117). Additional studies have reported that the catalytic activity of caspase 8 is dispensable for NF-κB signaling (118). A two-pronged role for caspase 8 has also been proposed, where pro-caspase 8 contributes to TNFR1-induced activation of NF-κB and the activated caspase 8 engages cleavage of NF-κB-inducing kinase (NIK; also called MAP3K14) to prevent the same (118)Caspase 8 also targets and cleaves the N-terminal fragment of c-FLIP (FLIP(L);

FLICE-like inhibitory protein), which generates a p43 fragment that forms a tripartite complex with TRAF2 and caspase 8 to activate the NF-κB signaling (119). Although the specific mechanism of caspase 8 involvement in NF-κB signalling is still unclear, it is evident that caspase 8 is critical for NF-κB-dependent priming and production of pro-inflammatory cytokines.

In addition to its pro-inflammatory function, caspase 8 has anti-inflammatory roles including inhibition of inflammasome activity. Caspase 8 deficient dendritic cells have increased inflammation that can be blocked by deletion of the TLR adaptor, MYD88, which suggests a role for caspase 8 in modulating TLR signaling (120). In skin, loss of caspase 8 expression promotes IL-1α secretion and the transcriptional activation of NF-κB-responsive genes during wound healing (121). Dendritic cells lacking caspase 8 can activate the NLRP3 inflammasome and release of IL-1β in response to LPS or Pam3CSK4 (a TLR2 agonist) alone, without the requirement of an inflammasome activator (122). This LPS-induced production of IL-1β in conditionally deleted DCs required RIPK1, RIPK3, the effector MLKL and the serine/threonine protein phosphatase, PGAM5 and hence could be blocked by genetic deletion of RIPK3 and necrostatin (an inhibitor of RIPK1). Further siRNA-mediated ablation of MLKL also rescued the *Casp8*$^{-/-}$ DCs. This suggests that LPS stimulation promotes an association of caspase 8 with FADD that inhibits RIPK1–RIPK3-MLKL complex and its downstream effector functions including the NLRP3 inflammasome activation (122). It has also been proposed that caspase 8 and FADD bind NLRP3 to directly inhibit the inflammasome (122).

Caspase 8 indirectly inhibits the inflammasomes is by its ability to prevent necroptosis. Necroptosis is an inflammatory and necrotic form of programmed cell death mediated by RIPK1 and RIPK3 independently of caspases (123, 124). Caspase 8 can bind and inhibit RIPK1-RIPK3-mediated inflammasome activation (122), as well as cleave RIPK1, RIPK3 and the deubiquitinating enzyme CYLD, which activates RIPK1 by removing its ubiquitin chains (125-127). Intestinal epithelial cells lacking caspase 8 display spontaneous ileitis, loss of Paneth and goblet cells, elevated infiltration of granulocytes and $CD4^+$ T cells into the lamina propria, and increased expression of inflammatory markers. Inhibition of RIPK1 and subsequently necroptosis, by necrostatin 1 prevents spontaneous inflammation and TNF-induced lethality in mice epithelial cells lacking caspase 8 (128). Another study reported that caspase 8 prevents RIPK3-mediated necroptosis, death of enterocytes and immune cell infiltration of the colon (129). Similarly, mice intestinal epithelium lacking FADD are more susceptible to necrosis of epithelial cells, loss of Paneth cells and develop colitis, a phenotype that is rescued by deletion of RIPK3 (130). Further in-depth studies are required to discern the varied roles of caspase 8 as a regulator of inflammasomes.

1.4.5 Effector functions of Inflammasomes

Beyond controlling infection in the host system, recent discoveries have revealed a range of effector functions of the inflammasome that include cell viability, induction of eicosanoids, phagosome maturation and roles in metabolism, aging and tumorigenesis. The inflammasomes have also been implicated in disorders such as

inflammatory bowel diseases, vitiligo, gouty arthritis, type 1 and type 2 diabetes (131-137).

Eicosanoids are lipid molecules that include the prostaglandins, thromboxane and leukotrienes, and are derived from the membrane lipids. They are important for various homeostatic and pathological processes, such as leukocyte recruitment, increasing vascular permeability. The resultant active caspase 1 from inflammasome activation generates arachidonic acid that is converted to prostaglandins and thromboxane by the enzymes, cyclooxygenases. Macrophages also synthesize and secrete prostaglandins and leukotrienes upon inflammasome activation. This “eicosanoid storm” can also be activated by caspase 11.

As described earlier, autophagy is a mechanism of regulation for inflammasome activation but interestingly, the inflammasome itself has an inhibitory effect on autophagy. AIM2, NLRP3 and NLRC4 inflammasomes suppresses autophagy and autophagosome formation. Autophagy is pro-cell survival process versus inflammasome that is pro-cell death, the balance between the two processes decides the cell’s decision to survive or die. However, the NLRP6 inflammasome uses autophagy as a downstream effector mechanism to maintain intestinal homeostasis. Phagosome acidification and maturation are critical anti-microbial defenses that are dependent on the NLPR3 inflammasome. Acidification of phagosomes into lysosomes that carry Gram positive bacteria such as *Staphylococcus aureus* or Group B streptococcus require ROS production and accumulation of active caspase 1 on the phagosome double-membrane carrying the bacteria. Additionally, caspase 11 or caspases 4/5 promotes the fusion of

phagocytic vacuoles containing specific pathogenic bacteria such as *Legionella pneumophila* with lysosomes by manipulating the cytoskeletal assembly.

Exciting recent developments have linked inflammasomes to metabolism. While metabolism within the host system has been found to dictate the kind and intensity of inflammatory responses, many metabolic disorders (e.g. obesity, diabetes) are a result of deregulated, chronic inflammation. Recently, proteomic analyses revealed proteins in the glycolysis pathway (e.g. aldolase, triose-phosphate isomerase, glyceraldehyde-3-phosphate hydrogenase, pyruvate kinase) as substrates of active caspase 1. Hence, the glycolytic rates are dampened in cells with active inflammasomes. Additionally, inflammasomes regulate lipid homeostasis as well. Caspase 1 activation by a pore-forming, bacterial toxin called aerolysin activates the sterol regulatory element-binding proteins (SREBPs), that upregulate the expressions of cholesterol and fatty acid biosynthesis proteins. Caspase 1 is also involved in the metabolism of triglycerides, by enhancing its absorption in intestine, production in liver as well as clearance from blood during fasting conditions or uptake of excess lipid.

Inflammasome-independent functions of AIM2 include control of tumor progression in various tissues (described in detail in Section 1.6) while NLRP3 regulates T-cell immunity. NLRP3 is expressed in differentiated T helper cells, but do not form inflammasomes and controls T_h2 cell differentiation instead via IRF4-dependent T_h2 cytokines induction. NLRP6 negatively regulates MAPK and NF-κB pathways in response to TLRs while NLRP12 blunts NF-κB and ERK activation, which is protective against colon inflammation and cancer. NLRP12 also functions as a negative regulator of cell migration in neutrophils, and blocks T cell-mediated IL-4 production.

All these effector mechanisms possibly function together to mount a fast and effective innate immune response against an invading pathogen, and might manipulate the host immune system to function in a manner detrimental to the pathogen, and beneficial to the host while alerting the adaptive immune system to respond to the risks present.

1.5 Nucleic acids (NA) as triggers for immune responses

Amongst the multiple PAMP or DAMP that the host immune system is equipped to identify, the appreciation for nucleic acids (NA) as a trigger for immune responses has escalated only in the last two decades. Foreign DNA or RNA as well as aberrantly expressed or differentially modified nucleic acids can elicit strong immune reactions within the host cells. Most receptors differentiate between the sugars, ribose and deoxyribose, that enable them to specifically recognize either RNA or DNA respectively. However, several sensors termed as Universal NA Sensors can recognize both RNA and DNA while there are other receptors that detect RNA:DNA hybrids which are often a by-product of pathogen replication.

Introduction of nucleic acid into the host cytoplasm as well as in the nucleus via viral infection, viral and bacterial replication or transfection triggers multiple distinct, parallel and often redundant pathways that culminate in interferon production, inflammation, autophagy as well as cell death through pyroptosis, apoptosis or necroptosis. The currently known NA sensors include TLR3, TLR7/8, RIG-I/MDA5 (6, 11, 138) and TLR9, cGAS, AIM2, IFI16 (8, 18-20) that can sense RNA and DNA respectively. Universal NA sensors include LRRFIP1, HMGBs, LSm14A and RNA:DNA hybrids trigger TLR9, NLRP3 and cGAS/STING pathways.

1.5.1 RNA sensors

The RNA immune receptors can recognize extracellular and intracellular, pathogen-derived single-stranded or double-stranded RNA. In order to avoid responding

to host cytosolic RNA, often these receptors distinguish foreign RNA by recognizing distinct modifications and specific lengths.

RIG-I

The RLR, RIG-I recognizes short blunt-ended, 5'-triphosphorylated (5'-ppp) dsRNA, or short hairpin, "panhandle" structured RNA found in negative-strand RNA viruses such as IAV, VSV, modifications that are absent in host mRNA (139, 140). Composition of the viral RNA ligand also play a role in RIG-I activation such as, poly (U/UC) motifs combined with a 5'-ppp, found in the genomic RNA of HCV. In addition to RNA viruses, RIG-I contribute to the detection of DNA viruses, such as Epstein-Barr virus (EBV), that detects 5'-ppp small RNA species generated through transcription of viral AT-rich DNA by RNA Polymerase III.

MDA5

MDA5 also identifies viral RNA, however, the distinct RNA ligands that activate MDA5 signaling is largely unknown. It is widely considered that MDA5 detects long molecules of dsRNA, organized in web-like structures as found in the *Picornaviridae* and *Caliciviridae* virus families, other positive-sense RNA viruses as well as DNA viruses that transcribes long dsRNA. Further, MDA5 is activated by poly (I:C), a synthetic RNA that is equivalent to long dsRNA. Kato et al. recently showed that MDA5 can be activated by long dsRNA from reoviruses or by annealed sense and antisense strands of *in vitro* transcribed, synthetic RNA (141). Detection of RNA virus through its packaged, genomic RNA may occur upon its entry into the host cell. However, the receptors are

also activated by other RNA species, internalized with the virion as well as those produced during viral replication (e.g. SeV, IAV) during different times after infection.

TLR3

Endosomal TLR3 is a critical receptor that senses dsRNA, a common intermediate of viral replication but is also triggered by dsRNA of non-viral origin, especially endogenous mRNA and RNA released from necrotic cells. The TLR3 pathway can sense and potentially control infections by flaviviruses, hepatitis B and C viruses, herpesvirus, rotavirus, retroviruses, orthomyxoviruses and poxviruses as well as extracellular poly (I:C) that is internalized within endosomes (6).

TLR7/8

TLR7 and TLR8 also initiate immune responses to viral infection. Specifically, they recognize ssRNAs as their natural ligand as well as small synthetic molecules such as imidazoquinolines and nucleoside analogs. TLR7/8 senses GU-rich, ssRNA viral replication intermediates from SeV, IAV, Coxsackie B that are released the endosomes (142).

1.5.2 DNA sensors

Unlike RNA, changes in subcellular localization of DNA in cell, which is generally within organelles like nucleus, mitochondria or peroxisomes in a healthy cell, serves as an indicator of infection or cellular stress. DNA in the cytosol is associated with viral infection or tumorigenesis where the DNA acts either as PAMP or DAMP

respectively. Hence, when any DNA, pathogenic or host-derived, appears in the cytosol, it can trigger strong immune reactions in the form of type-I IFN production and/or inflammation. Any dsDNA longer than 25 bp with a phosphodiester backbone, present in the cytoplasm is capable of generating such immune responses.

TLR9

TLR9 was the first PRR discovered that initiates immune responses utilizes DNA as PAMP/DAMP. TLR9 senses single-stranded, unmethylated, CpG (deoxycytidylate-phosphate-deoxyguanylate)-rich viral and bacterial DNA or oligodeoxynucleotides (ODN), containing the core sequence “GACGTT”. The CpG motifs are less frequent in the vertebrate genomes compared to bacterial or viral genomes. The CpG DNA with a sugar-phosphate backbone interacts with TLR9 in a sequence specific manner whereas, DNA with phosphorothioate backbone (where the sugars have a sulfur atom in the backbone instead of phosphorus) bind to TLR9 much less specifically, but show a CpG sequence-dependent activation. Thus, this difference between binding of two distinct ODN suggests that activation of TLR9 requires discrete conformational changes that are governed by the structure of the ODN (143). TLR9 is expressed in dendritic cells, macrophages, natural killer (NK) cells, and other antigen presenting cells. It is expressed within endosomes where it detects phagocytosed extracellular DNA as well as DNA released in the cytosol and taken up by the endosomes. Hence, it follows that TLR9-CpG-DNA interaction occurs at the acidic pH (6.5–5.0) found in endosomes and lysosomes (143). TLR9 is critical for detection of multiple DNA viruses such as EBV, Human Papilloma Virus (HPV), Hepatitis B Virus (HBV), Herpes Simplex Virus (HSV) 1 and 2,

MCMV, Kaposi's sarcoma-associated herpesvirus (KSHV) and Murid herpesvirus 68 (MHV-68).

DAI/ZBP1

After TLR9, DNA-dependent activator of IFN-regulatory factors (DAI; also known as ZBP1 or DLM-1) had been reported as an intracellular dsDNA sensor (144), but *Zbp1*$^{-/-}$ mice are capable of producing type I IFN after intracellular DNA stimulation (145), suggesting that the roles of DAI are either redundant or limited to specific cell types. Recently, DAI/ZBP1 was shown to respond to dsDNA virus by positively regulating programmed necrosis or necroptosis through its interaction with RIP3 during MCMV infection (146).

cGAS and STING

It had been known for a long time that pathogen-derived DNA could activate fibroblasts to produce type I IFNs (147). The existence of DNA sensors, other than TLR9, became apparent with the observations that transfection of pathogenic dsDNA could activate a thyroid cell line lacking TLR9 to induce multiple immune genes (148). Following the discovery of TLR9 as a sensor for CpG DNA, Akira and group showed that *Tlr9*$^{-/-}$ MEFs could still activate type-I IFN in response to synthetic B-form dsDNA or genomic DNA isolated from bacteria, viruses and mammalian cells. Further, Medzhitov and group showed that transfection of Immunostimulatory DNA (ISD), a 45bp dsDNA region from the *Listeria monocytogenes* genome to the cytosol induced type-I IFN in mice lacking both the TLR adapters, MyD88 and TRIF. The discovery of DNA-induced type-I IFN

pathway was propelled forward with the discovery of the adapter molecule STING (*Sti*mulator of *I*nterferon *G*enes). STING is the critical adapter required to induce type I IFN and NF-κB dependent inflammatory responses to cytosolic DNA. *STING*$^{-/-}$ mice have impaired cytokine responses and are susceptible to HSV-1 infection (149). Interestingly, STING deficient mice are also susceptible to VSV infection alluding to an involvement of STING in RNA virus pathogenesis, possibly through the sensing of host DNA released into damaged tissues (149).

The C-terminal domain of STING directly recognizes bacterial cyclic-dinucleotides (CDN) through its C-terminal domain resulting in its dimerization and activation of TBK1/IRF3 and NF-κB signaling. Bacteria often use CDNs as secondary messengers in intracellular signaling pathways. For example, cyclic-adenosine monophosphate (cAMP) and cyclic-guanine monophosphate (cGMP) from *Vibrio cholerae* stimulates type I IFN in a STING-dependent manner (150).

Of all the cytosolic DNA receptors, so far, cGAS is the best-characterized DNA sensor with a clarified mechanism of STING activation and type-I IFN production. cGAS is an enzyme, identified in vertebrates, that catalyzes the production of endogenous cyclic-GMP-AMP (cGAMP) in a DNA-dependent manner. The secondary messenger cGAMP acts as a substrate for STING, which then activates the TBK1/IRF3-dependent IFNα/β pathway. cGAS belongs to the nucleotidyltransferase family of enzymes that catalyzes the conversion of mononucleotides into oligonucleotides and other higher order structures. cGAS directly binds to DNA using its N-terminal region, forms dimers that process 2’3’-cGAMP production from ATP and GTP, which is an intermediate capable of inducing STING-dependent IFN activation (151, 152). Structural studies have shown

direct binding of cGAMP to STING, stimulating its dimerization and activation (153, 154). cGAMP also behaves as an intercellular signal that activates type-I IFN signaling in neighboring cells, by moving through cellular gap junctions (151). cGAS deficient macrophages, dendritic cells, and fibroblasts have completely abrogated type-I IFN responses to transfected DNA and DNA virus (155). Further, *cGAS*$^{-/-}$ mice were found to be susceptible to HSV-1 and VACV infections, and had higher viral loads during latent infection with MHV-68 (155, 156).

DDX41

DEAD (Asp-Glu-Ala-Asp) box polypeptide 41 (DDX41), a member of the DEAD/H helicases family identified from a siRNA screen, was characterized as a putative cytosolic DNA sensor (157). Knockdown of DDX41 in BMDC and THP-1 leads to an impaired IFN production in response to cytosolic DNA and HSV-1 infection (157). DDX41 also senses bacterial CDNs, cyclic di-AMP (cAMP) and cyclic di-GMP (cGMP) by directly binding through its DEAD catalytic domain, leading to STING-dependent activation of type I IFN (158). However, further elucidation of DDX41-dependent IFN signaling pathway in response to cytosolic DNA remains to be uncovered.

DHX9/36

DHX9 and DHX36 are DEAH RNA Helicase A helicases that bind CpG-B and CpG-A DNA respectively in plasmacytoid dendritic cells (pDC). DHX9 activates IRF-7 and IFNα production, while DHX36 activates IL-6 and TNFα through NF-κB pathway. RNAi

knockdown of DHX9 and DHX36 abrogated cytokine responses in HSV-1 infection, while responses to the IAV (an RNA virus) were unaltered (159).

DNA-PK & Mre11

Host DNA damage also induces type I interferon production. DNA-dependent protein kinase DNA-PK, a holoenzyme with three subunits: the catalytic subunit DNA-PKc and the DNA binding proteins Ku70 and Ku80, elicit IFN-β in response to DNA transfection, HSV-1 and co-localizes with sites of viral DNA replication in VACV infection in MEFs in an IRF3-dependent, NK-κB-independent manner. DNA-PK-deficient mice showed attenuated IFN response to VACV (160). Meiotic recombination 11 homolog A (Mre11), a double-strand break repair protein, along with its binding protein Rad50, is a cytosolic sensor for transfected dsDNA that induces IFN-β production in BMDCs. However, Mre11 was found to be dispensable for type-I IFN production in HSV-1 and *Listeria monocytogenes* infection in BMDC (161). The identification of DNA Damage Response (DDR) proteins as regulators of immune response to cytosolic DNA allude to interdependence of DNA damage and DDR with generation of DAMPs and DNA sensing pathways.

RNA Polymerase III

Cytosolic RNA Polymerase III (Pol III) uses transfected AT-rich DNA (poly dA:dT) in the cytoplasm as a template to generate short ssRNA transcripts, which are then recognized by RIG-I to activate type-I IFN (162).

AIM2 and IFI16

The Aim2-like receptors or ALRs or the PYHIN proteins (e.g. AIM2, IFI16) have a conserved DNA sensing motif that make them good candidates for behaving as DNA receptors in immune sensing pathways. They are described in detail in the Section 1.6.

1.5.3 Sensors for both RNA and DNA

Some less-studied pattern recognition receptors, also called universal NA sensors (e.g. LRRFIP1, HMGBs, LSm14A), have been implicated in triggering innate immune responses, which do not differentiate between the sugar backbones of nucleic acids and can identify both RNA and DNA with similar affinities. Hence, they often respond equally effectively to infections with bacteria, RNA or DNA viruses.

LRRFIP1

LRRFIP1 (Leucine-rich repeat flightless-interacting protein 1) has recently been implicated as a regulator of DNA-driven innate immune signaling. LRRFIP1 was found to bind to the *Drosophila* homolog flightless I and play a role in cytoskeletal development during *Drosophila* embrogenesis. An RNAi screen designed for discovering potential cytosolic DNA sensors showed that LRRFIP1 inhibited type-I IFN induction upon *Listeria monocytogenes* infection. LRRFIP1 knockdown also affected IFN production upon VSV infection as well as in response to poly (I:C), and the synthetic DNA species, poly (dG:dC) and poly (dA:dT), implicating LRRFIP1 as a sensor for dsRNA as well as both B and Z forms of dsDNA. Intriguingly, this function is independent of RNA Pol III. However, LRRFIP1 regulates type-I IFN activation through

a β-catenin-dependent co-activator pathway. Upon binding RNA or DNA, LRRFIP1 mediates the phosphorylation of β-Catenin, which translocates to the nucleus and associates with the p300 acetyltransferase to induce increased IFNβ production (163). β-catenin also activates IRF3 to do the same. Though, LRRFIP1 has been shown to act as a sensor in *Listeria* and VSV infections, further studies are required to delineate the role of LRRFIP1 as a nucleic acid immune receptor.

HMGB1

HMGB1 (High Mobility Group Box protein 1) is released from cells during necrosis and acts as a DAMP. It interacts with RAGE, TLR2 and TLR4 to induce inflammatory responses. HMGB1 binds both DNA and RNA to activate RIG-I and other NA sensors (164).

LSm14A

LSm14A is a component of RNA processing bodies (P-bodies). It has been shown to bind both DNA and RNA and induce type I interferon production through IRF3 and requires STING, MAVS, and RIG I respectively. This suggests that viral recognition may take place within P-bodies and LSm14A plays a role in antiviral activation through nucleic acid sensors (165).

1.5.4 Sensors for RNA:DNA hybrids

Recent advances have classified RNA:DNA hybrids as a novel class of immunostimulatory molecules that behave as pathogen-associated or danger-associated ligands and can activate immune signaling pathways. Bacterial nucleic acids as well as retroviral replication intermediates form RNA:DNA hybrids in the infected host cell cytoplasm. In a healthy cell, the RNA:DNA hybrids are formed only within the nuclei and mitochondria as DNA replication intermediates (166) or transcription-induced R-loops (166) or G-quadruplexes (167). Inhibitory studies showed that RNA Polymerase III generates cytosolic RNA:DNA hybrids within virus-free, human cancer cell lines, including transformed cell lines that may act as a danger signal for activating immune pathways (168). Endosomal TLR9 and cytosolic cGAS and NLRP3 have been implicated in detecting RNA:DNA hybrids in different contexts (45, 169, 170).

TLR9

Retroviral replication intermediates form RNA:DNA hybrids in both cytoplasm and endosomes of the infected host cells and can activate the endosomal receptor TLR9. TLR9 was shown to bind the hybrid molecules with high affinity and induced MyD88-dependent pro-inflammatory cytokines and antiviral type I IFN in dendritic cells and human PBMCs (169).

NLRP3

Infection with an extracellular bacteria, enterohemorrhagic *Escherichia coli* (EHEC), generates bacterial RNA:DNA hybrids in the cytoplasm that was found to co-localize

with NLRP3 specks. Introduction of the hybrid molecules are sufficient to activate the NLRP3 inflammasome and this inflammatory response can be blocked by RNase H that degrades RNA:DNA hybrids (45).

cGAS-STING

cGAS knockout in THP-1 cells revealed that type-I IFN responses upon recognition of synthetic RNA:DNA hybrids of different lengths, produced enzymatically *in vitro* using transcribed RNA molecules (e.g. T7 RNA polymerase) as templates for reverse transcriptase, was found to be highly dependent on the cGAS–STING pathway. The *in vitro* studies showed that, similar to dsDNA recognition, a recombinant cGAS could produce cGAMP on detecting RNA:DNA hybrids (170).

1.6 Introduction to PYHIN proteins

AIM2 and IFI16 belong to the same family of proteins called the PYHIN (Pyrin and HIN domain containing proteins) or IFI200 (Interferon-inducible with a 200 amino acid repeat) proteins, classified by their defining structures, also known as ALR (Aim-2 like Receptors), following the discovery of AIM2 as an innate receptor that detects pathogen-derived DNA (171).

The *PYHIN* genes are found in a cluster in many mammals within a syntenic region, situated between *CADM3* (*cell adhesion molecule 3*) and a set of olfactory receptors and *SPTA1* (*spectrin alpha chain*) genes. The human *PYHIN* genes, *AIM2*, *IFI16*, *IFIX* and *MNDA* are encoded on chromosome 1q23 (172, 173). There is a fifth gene, *POP3* situated at the locus between IFIX and IFI16, which belongs to the Pyrin-only Protein family (POP) (174). Comparatively, other mammals express variable number of PYHIN proteins. There are 13 PYHIN genes found on chromosome 1q band H3 in C57BL/6 mice within a locus flanked by the *PYHIN* genes, *Aim2* and *p205*.

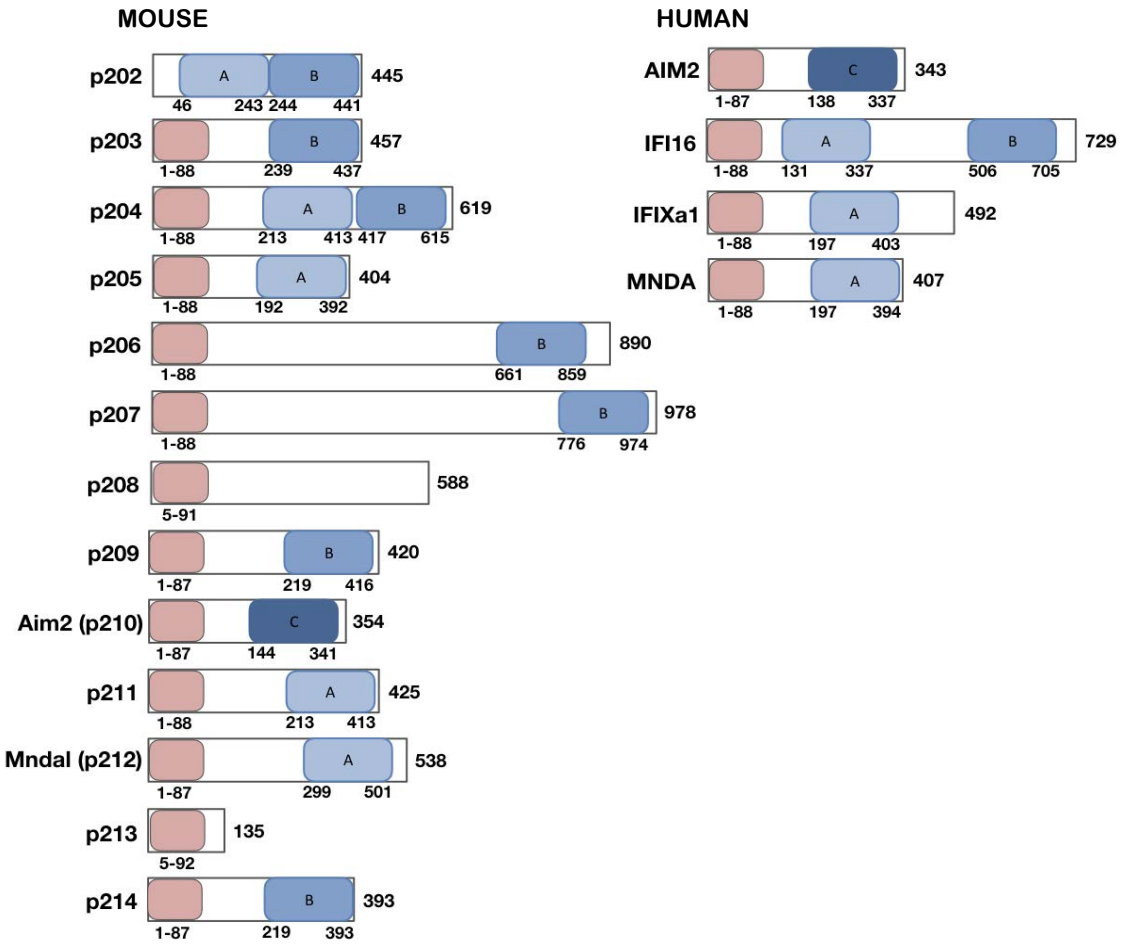

Figure 1.5: The PYHIN family of proteins in mice and humans. There are 13 PYHIN proteins in mice and 4 in humans. The pyrin (PYD) domains are indicated by pink boxes, and the HIN domain (in blue) subtypes (A, B or C) are denoted within boxes.

The interferon-inducible PYHIN proteins are expressed in hematopoietic cells as well as in non-hematopoietic cells (e.g. skin epithelium). Spleen is a major site opf expression for most of the proteins. *p204*, *p205* and *p207* show the strongest expression in the skin, but are also expressed in heart and muscle (172). They are primarily nuclear proteins with defined nuclear localization signal (NLS) that may be monopartite, bipartite or both, although some PYHINs like Ifi204/p204 in mouse have a characterized nuclear export sequence as well that allows them to move in between the cytosol and nucleus. Some of the PYHIN proteins also undergo relocalization upon stimulation or overexpression with other proteins e.g. STING (173), upon heterodimerization with other PYHIN and/or by modification of their nuclear localization signals (e.g. acetylation of IFI16 causes relocalization to the cytoplasm). The exceptions are AIM2/Aim2 and Ifi202/p202 that lack an NLS and are almost exclusively localized in the cytoplasm, though Aim2 has recently been reported to sense DNA damage in the nucleus (175).

The PYHIN proteins are characterized by the presence of a pyrin domain at the N-terminal and one or two HIN200 domain at the C-terminal, flanking a variable intermediate spacer region.

Pyrin/PAAD/DAPIN domain

Pyrin domain (PYD), also known as the PAAD or DAPIN domain, is a member of the death domain (DD) family of proteins which is a 80 amino acids motif that consists of five α-helices, and can mediate homotypic interactions with other DD-containing proteins. It forms higher complexes that have been implicated in

inflammation, apoptosis and cell cycle. The only PYHIN proteins that do not contain a pyrin domain are the p202 variants, p202a and p202b.

HIN200 domain

The HIN (*h*ematopoietic, *i*nterferon-inducible, *n*uclear) domain is a conserved 200 amino acid motif that consists of two-oligonucleotide/ oligosaccharide binding (OB) folds that form a β-barrel structure capable of binding NA, and are the characteristic DNA-binding domains found predominantly in the PYHIN proteins. The HIN200 (or HIN) domains are classified into three subtypes called HIN A, B or C depending on their consensus motifs. Structural analysis have shown that IFI16 HIN-A domain has a higher affinity to ssDNA that can wrap, stretch and form oligomers with ssDNA. In contrast, IFI16 HIN-B domain binds dsDNA with higher affinity but this binding was augmented by the presence of both HIN-A and HIN-B. Biochemical and structural studies using DNA-bound AIM2 revealed that in the absence of DNA, AIM2 is maintained in self-inhibited state, in a wrapped structure, where the region around the negatively charged α2- helix in the pyrin domain binds the DNA-binding interface of the HIN domain limiting the ability of the pyrin domain to initiate downstream signaling. Interestingly, work from Stetson's lab using a transfected DNA-dependent cell death assay in AIM2 deficient THP-1 cells expressing individual chimeric PYHINs, showed that the proteins containing PYHIN1/IFIX HIN domain or MNDA HIN domain did not bind DNA to induce cell death. Additionally, both IFI16 HIN domains were required to bind dsDNA and induce cell death in this assay (176).

The HIN domain has been implicated in protein-protein interactions and dimerization as well through a highly conserved motif MF/LHATVAT/S. The domain also contains a retinoblastoma (Rb)-binding site, LXCXE and putative Cyclin-dependent kinase (CDK)-2 phosphorylation sites (e.g. p204, p205, p203, p202, IFI16, MNDA) (177).

Intermediate domain

The intervening spacer regions, separating the two domains, that confer the maximum variability between proteins, are consistent in size and are rich in S/T/P residues. The NLS is located in this region, just after the pyrin domain and some PYHINs (e.g. p204, p205) have a common heptamer repeat sequence within the domain (177).

Before the discovery of the role of PYHIN proteins in innate immunity, they were characterized as regulators of cell growth, differentiation and proliferation in apoptosis, tumor progression and DNA damage responses. Much of the functions of PYHIN proteins have been linked with cellular differentiation as many of the family members are upregulated to varying degrees at different stages of the process in multiple cell lineages. IFI16, PYHIN1/IFIX, p202, and p204 regulate cell cycle transcription factors such as p53, p21, pRb, and E2F resulting in cell cycle arrest (178). p202 acts as a transcriptional repressor targeting NF-κB (179), AP-1 (180, 181), MYOD1 (182), and myogenin (181, 183). p204 also regulates gene expression during monocyte/macrophage differentiation and osteoblast differentiation. p205 acts as a putative tumor suppressor and controls

$p21^{CIP/WAF}$ gene expression via p53 in Saos2 cell lines as well as impacts gene expression in adipogenesis and osteogenesis via transcriptional mechanisms.

1.6.1 PYHINs as Regulators of Immune Responses

AIM2/Aim2

AIM2 (also called PYHIN4) was identified during a functional screen for tumor suppressor genes in melanoma (100). Though not an NLR protein, AIM2 activates the inflammasome similar to the NLRs. The gene encodes an N-terminal pyrin domain and HIN200 domain, sub-type C. The pyrin domain of AIM2 interacts with the pyrin domain of ASC to recruit caspase 1 and form the inflammasome.

Activation of the AIM2 inflammasome is mediated by DNA binding to form the DNA:protein heteroduplex. Structural study shows that the positively charged HIN200 domain binds to the negatively charged sugar phosphate backbone of dsDNA, making contacts across the major and minor grooves, which makes AIM2 unable to form stable complexes with ssDNA (184). Like other DNA sensors, AIM2 does not discriminate between the origins of DNA, synthetic, mammalian, or microbial, but relies on the misexpression of DNA in the cytosol to initiate an immune response. AIM2 binds DNA independent of the sequence, but the dsDNA must be more than 80bp in length.

The DNA from invading intracellular bacteria which escape into the cytoplasm and the cytosolic DNA viruses activate the AIM2 inflammasome leading to the processing and secretion of IL-1β and IL-18. *Francisella* is a facultative intracellular pathogen that infects macrophages through phagosomes but escape into the cytosol to

replicate. *Francisella novicida* infection was known to induce IL-1β and IL-18 in an NLRP3- and NLRC4- indepedent ASC-dependent manner. Subcutaneous *Francisella* infection of *Aim2*$^{-/-}$ mice showed decreased IL-1β/IL-18 secretion and increased bacterial burden and mortality (185, 186). AIM2 is required for IL-1β production by macrophages and dendritic cells in response to infection with the intracellular bacterium *Listeria monocytogenes,* as well as the DNA viruses VACV and murine cytomegalovirus (MCMV) (171, 187). MCMV infection in *Aim2*$^{-/-}$ mice led to increased viral titers due to decreased NK cell activation, as a likely consequence of result of decreased IL-18R signaling (171). However, infection with attenuated MCMV severely dampens the AIM2 inflammasome indicating that replication of the virus is required for AIM2 activation. In contrary to the hypothesis that AIM2 would respond to all DNA viruses, it was discovered that inflammasome activation during HSV-1 or MHV-68 infections is independent of AIM2, but require NLRP3, emphasizing the necessity of the cellular localization of the viral genomic DNA was critical in eliciting AIM2-dependent immune responses. In contrast, a recent study from Flavell and group observed that the *Aim2*$^{-/-}$ mice were protected against radiation-induced gastrointestinal as well as hematopoietic toxicity. They showed that AIM2 mediates the inflammasome-dependent death of intestinal epithelial cells and bone marrow cells in response to radiation-induced double-strand DNA breaks caused by ionizing radiation and chemotherapeutic agents, and interestingly AIM2 detected the damaged DNA in the nucleus (175).

IFI16

The human PYHIN family member, IFI16 contains one N-terminal Pyrin domain and two HIN200 domains (subtypes A and B) that can bind directly to dsDNA in a sequence-independent manner (183). IFI16 was first identified as a putative DNA sensor by its ability to pull down IFN-β-inducing VACV dsDNA motif in human monocytes cytosolic extracts (20). Knockdown of IFI16 in human THP-1 cells partly inhibited IRF3 activation and type-I IFN responses to transfected DNA and HSV-1 infection (20). Similarly, another study observed that viral DNA intermediates from the RNA virus, HIV-1 as well as synthetic ssDNA corresponding to DNA formed during lentivirus replication cycle stimulates the IFI16-dependent STING-TBK-1-IRF3/7 pathway, where IFI16 colocalizes with the lentivirus in the cytoplasm and is activated by stem regions in the single stranded DNA structure (188).

However, the role of IFI16 as a cytosolic DNA sensor has been controversial with debates about its necessity for type-I IFN production, which has been highly variable between studies. Recent studies from Stetson and group, using lentiCRISPR-mediated genetic disruption have demonstrated that cGAS-STING was the critical pathway for human cytomegalovirus (HCMV)-induced type-I IFN production in human primary and immortalized fibroblasts whereas IFI16 was dispensable for the same (176). However, work from other groups has established that IFI16 is a critical mediator that enhances the cGAS-cGAMP-STING axis of immune responses during infection (189-191). These studies used human fibroblasts, keratinocytes and macrophages to show that IFI16 is an essential component in type-I IFN induction, which co-operates with the cGAS-STING pathway during viral infections to enhance and control immune activation.

Orzalli et al., showed that nuclear cGAS interacted with and stabilized IFI16 in HSV-infected human fibroblasts or human fibroblasts with transfected plasmid DNA, and IFI16 served as the primary sensor in the nucleus (189). Unterholzner and group used keratinocytes lacking IFI16 or cGAS to show that the sensors were not redundant in sensing exogenous DNA but that IFI16 interacted with cGAS in a DNA-dependent manner and was essential for the full activation of STING (190). Similarly, work from Jakobsen's lab used CRISPR-Cas9 technology in human monocyte-derived macrophages, and phorbol myristate actetate (PMA) -treated THP1 cells, to show that IFI16 increased cGAMP production by enhancing cGAS activity, and is critical for downstream signaling by controlling the efficacy of STING dimerization, phosphorylation and recruitment of TBK1 to the STING complex (191). Additionally, our group also described IFI16 as a positive regulator involved in the transcription of critical ISGs in response to RNA virus infection (192).

Cell-specific, cellular distribution of IFI16 that varies between the nucleus and cytoplasm depending on cell types, determines the function of IFI16 as a pathogen sensor (193). IFI16 recognizes DNA from the nuclear-replicating herpesvirus, KSHV in infected endothelial cells, in the nucleus (21). It also sensed HSV-1 genomic DNA in infected human foreskin fibroblasts in the nucleus, and relocalized to the cytoplasm (194). These studies have also highlighted the role of IFI16 as an inflammasome activator. IFI16 co-localized with ASC and pro-caspase-1 in the KSHV-infected endothelial cells, first in the nucleus and then relocalized to the perinucleus. In the HSV-1 infected human fibroblast, IFI16 recognized the virus in the nucleus, re-localized and co-localized with ASC in the cytoplasm. Knockdown of IFI16 was found to block

processing of caspase-1 and IL-1β in response to KSHV and HSV-1, but not VACV infection (194). Additionally, cytosolic viral DNA derived from of incomplete HIV reverse transcripts, that lead to abortive HIV-infection of CD4+ T cells stimulates the IFI16 that triggers caspase-1-dependent pyroptosis (22). Conversely, other reports have shown IFI16 being unable to form inflammasome in other cell types indicating that further work is necessary to define the cellular contexts in which IFI16 activates the inflammasome (18).

IFIX/PYHIN1

The human PYHIN, IFIX (also called PYHIN1) was originally discovered as a tumor suppressor that promotes ubiquitination and subsequent degradation of pro-survival proteins (195-197). IFIX contains the N-terminal pyrin domain and a C-terminal conserved HIN-A domain. Recently it was reported that IFIX detects herpesvirus DNA in both the nucleus and cytoplasm, binding foreign DNA via its HIN domain in a sequence-independent manner, and leads to interferon responses (198). Further study from the group reported the IFIX had a dynamic localization during HSV-1 infection that changes from diffused nuclear and nucleoli distribution in uninfected cells to discrete nuclear puncta early in infected cells. Mass spectrometry showed that IFIX associated with multiple transcriptional regulatory proteins mediated by its HIN domain, to suppress viral transcription (199).

Ifi204/p204

Though mice lack a clear homolog to IFI16, the mouse PYHIN p204 (also called Ifi204) is the closest in secondary structure to IFI16 and is considered its ortholog. Similar to IFI16, it contains a pyrin domain and two conserved HIN200 domains. Alongside IFI16, knockdown of p204 in mouse macrophages had partially inhibited IRF3, NF-κB and type-I IFN activation in response to DNA and HSV-1 infection (20). However, the p204 knockout mice showed no clear immune phenotype in response to DNA virus infection, possibly due to other IFN-activating pathways (e.g. cGAS) that protect the host in absence of p204.

Ifi203/p203

Using a murine leukemia virus (MLV) variant that induces a strong IFN-β response, and HIV, Ifi203 (along with DDX41 and cGAS) was identified as a sensor for the reverse transcribed DNA that required STING and TREX-1 to stimulate immune responses. In addition the group demostrated that the IFN-inducible cytidine deaminase, APOBEC3 (apolipoprotein B editing complex 3), which inhibits reverse transcription in the infectious retroviuses by introducing lethal mutations in the virus, restricting the viral load *in vivo*, serves as the primary defense. The cytosolic sensing is the secondary response and is critical for IFN-induction of anti-retroviral genes such as *Apobec3* (200).

Ifi205/p205

The mouse PYHIN protein, p205 (also called Ifi205) is a nuclear protein with the characteristic pyrin domain and a conserved HIN domain subtype A. Aside from

the potential role of p205 as a tumor suppressor, it has been reported that Ifi205 also acts as a senor for cytosolic DNA. Ifi205 sensed cytosolic, self-DNA derived from endogenous retroelements in macrophages and activated cGAS and cGAMP-independent, STING-dependent type-I IFN production. This pathway was blocked by AIM2 by sequestering cytosolic Ifi205 from STING (201). In this dissertation, I demonstrate that Ifi205/p205 acts as a nuclear co-transcriptional activator that controls the expression of immune genes such as ASC, and thereby regulates inflammasome activation in macrophages.

Several mechanisms have evolved within the host system that regulates the PYHIN proteins and the immune responses activated by them. As described previously in Section, POP3 and p202 limit the ability and activity of AIM2/Aim2 and/or IFI16 to form the inflammasomes through their Pyrin and HIN domains respectively. p202 has two HIN200 domains, the first of which sequesters cytosolic dsDNA available for initiating immune responses while the second HIN200 domain is responsible for binding and inhibiting AIM2 resulting in the prevention of AIM2-ASC clustering. Another example of regulation within the PYHIN family is regulation of AIM2 by IFI16, where IFI16 can bind AIM2 and inhibit AIM2 mediated caspase-1 activation (202), by reducing the levels of AIM2 available for associating with ASC. Similar to NLRP3, AIM2, in a ligand-free environment, is found in an auto-inhibited state, where the PYD is in association with the HIN domain. Only after binding of the HIN domain with dsDNA, the pyrin domain is freed and becomes available for binding with the ASC-pyrin domain (203).

1.6.2 PYHINs as Tumor Suppressors and in DNA Damage Response

The PYHIN proteins were initially characterized as a family of potential tumor suppressors. AIM2 or Absent in melanoma-2, as the name suggests was described as a protein that lost its expression in malignant, human melanoma cells (204). In addition to its function as a DNA sensor, AIM2 is also differentially expressed in multiple tumors, and is often down regulated in various cancers. AIM2 has been reported to inhibit fibroblast and breast cancer cell growth in vitro (205). *AIM2* gene mutations have been found to correlate with gastric and endometrial cancers (206), and *AIM2* gene expression can be silenced by DNA methylation in immortalized cells (207). Aim2 gene therapy in a mouse model of mammary tumor growth induced tumor cell death, and Aim2 expression greatly suppressed NF-κB transcriptional activity and was unresponsive to TNFα-mediated NF-κB activation (205).

The AIM2 gene contains a site of microsatellite instability that results in gene inactivation in almost 50% of colorectal tumors (208). AIM2 deficiency has been correlated to poor prognosis in patients with colorectal cancer. One study showed that AIM2 is required to mediate protection against colorectal cancer, in an inflammasome-independent mechanism. It suppressed tumor growth by blocking proliferation in enterocytes and expansion of the intestinal stem cell population. They also demonstrated that lack of Aim2 changed the composition of colon microbiota in mice, which favored tumor growth (209). Another study showed that in resting cells, AIM2 interacts with DNA-dependent protein kinase (DNA-PK), a phosphatidylinositol 3-kinase (PI3K)-related family member that promotes protein kinase B (Akt) phosphorylation. AIM2

reduces Akt activation and controls tumor burden in colorectal cancer models, while *Aim2*$^{-/-}$ mice had increased tumor load, which was reduced by the use of an Akt inhibitor (210). A recent study rescued AIM2 expression in a colorectal cancer cell line and demonstrated that AIM2 inhibited increased apoptosis and blocked cell growth by inhibiting cell cycle transition from G1 to S phase of the cancer cells. Further analysis showed that AIM2 promoted apoptosis by suppressing the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway (211). Interestingly, all the studies agree that the mechanism, by which AIM2 suppresses tumor growth in colon cancer, is inflammasome-independent.

DNA Damage Response (DDR)-induced signaling by DNA-damaging agents like ultraviolet light, ionizing radiations, or cancer chemotherapeutic agents, activates the ATM-p53 and ATM-IKKα/β-interferon (IFN)-β signaling pathways, leading to an induction of the p53 and IFN-inducible IFI16 gene. Further, upon DNA-damage, DNA accumulates in the cytoplasm, thereby inducing the IFI16 protein and STING-dependent IFN-β production as well as activation of the inflammasome. Increased expression of IFI16 in cells also promotes p53-mediated transcriptional activation of genes, inhibition of cell cycle, cell growth and proliferation while fostering cellular senescence. IFI16 constitutively binds to BRCA1 breast cancer tumor suppressor and is involved in p53-mediated regulation of cell growth and apoptosis. Analyses of breast cancer cell lines and specimens revealed that decrease in the levels of IFI16 is closely associated with tumor progression. IFI16 deficiency induces levels of NBS1 (nijmegen breakage syndrome protein 1), which activates of DNA-PK (DNA-dependent kinase), phosphorylation of p53 and accumulation of $p21^{WAF1}$. Hence, IFI16 negatively affects

p53-dependent transcription of p21 as well as p53 and p21 protein stability. Localization of IFI16 is determined by the status of BRCA1 protein under conditions of DNA damage, such as ionizing radiation. More recently, it has been shown that levels of IFI16 are increased by oxidative stress. Together, these results illustrate that IFI16 is involved in DNA damage signaling and responses in controlling tumor growth.

Initially, IFIX was discovered to function as a tumor suppressor. Expression of IFIX is diminished in breast tumor tissues and breast cancer cell lines like MCF-7 (195), and its overexpression suppressed the growth of breast cancer cell. IFIX negatively regulates HDM2 (a RING finger E3 ubiquitin ligase that degrades p53) by promoting its self-ubiquitination and degradation (196). IFIX exerts similar tumor suppressive activity by upregulating a metastasis suppressor called maspin (197). In both pathways involving HDM2 or maspin, IFIX is directly involved in ubiquitination reactions.

MNDA (myeloid nuclear differentiation antigen) is important for neutrophil apoptosis, where the nuclear MNDA is cleaved by caspases and relocated to the cytoplasm. A study reported that when healthy neutrophils are challenged with mediators of sepsis, apoptosis is induced, during which MNDA promotes proteasomal degradation of MCL-1 (myeloid cell leukemia-1), causing mitochondrial dysfunction that promotes the progression of apoptosis, and inflammation (212).

p205 localizes in the nucleus of hematopoietic and non-hematopoietic cells and acts as putative tumor suppressor by controlling p53-dependent expression of $p21^{CIP/WAF}$ in Saos2 cell line. p205 can block cell growth in a p53- and Rb- independent

manner, but it also induces the expression of Rb protein and can directly bind p53 and Rb.

These reports indicate that besides their roles in host defense and regulation of immune responses against invading pathogens, the PYHIN proteins play major role in tumor progression. The proteins may recognize the released or damaged self-DNA in the cytosol (due to damaged nuclear envelope) or nucleus, and co-ordinate multiple immune pathways to block tumor development and survival.

1.6.3 Roles of PYHINs in health, infection and autoimmunity

Multiple pathogens have co-evolved with their host to combat the cytokine induction and early detection by the PYHIN proteins and have mechanisms for bypassing host immune signaling. HSV-1 presented the first example of a virus targeting a PYHIN protein. The HSV-1 immediate early protein ICP0 is known to be capable of inhibiting IFN-α/β signaling, however its mechanism of inhibition remained had been unclear. One of the mechanisms suggest that ICP0 target IFI16 for proteasomal degradation to evade immune responses (213). Further, Johnson et al., showed that ICP0 co-localized with IFI16 after infection with HSV-1, and likely targeted IFI16 for degradation by its phosphorylation. Additionally, HSV-1 infection induced the NLRP3 inflammasome along with IFI16, but it also blocked NLRP3 induced activation, at later time points, by immobilizing caspase-1 in actin clusters. Another study argued that though ICP0 impacts IFI16-related responses but that IFI16 may not necessarily be an ICP0 substrate (214). However, it agrees that HSV-1 infection leads to IFI16 degradation, which is a very effective in limiting prolonged viral detection. Recently, HSV-1 has also been shown to

target the PYHIN protein IFIX that co-localizes with nuclear proteasome post- infection and its pyrin domain is rapidly degraded in a proteasome-dependent manner. Unlike other host proteins targeted by HSV-1, degradation of IFIX is independent of ICP0, but requires immediate-early viral gene expression, suggesting a distinct viral host suppression mechanism (199). Human MNDA may also be targeted by viral proteins, such as Latency-associated nuclear antigen (LANA) from KSHV that co-localizes with MNDA, but the physiological relevance of the interaction are yet unknown (215). It is becoming more apparent that innate host sensors frequently utilize aggregation dependent amplification mechanisms for signal activation. Indeed, The HCMV protein, pUL83 also blocks IFI16 activation by interacting with its pyrin domain and blocking its oligomerization (216). Li et al. demonstrated in a HCMV strain lacking pUL83, that IFI16 oligomerization is required for phosphorylation and nuclear translocation of IRF1 and IRF3. But there was no change in caspase 1 activation. Surprisingly, the amino acids in the IFI16, which are required for oligomerization are not well-conserved in AIM2 (217), raising the possibility that during inflammasome activation AIM2 (and, may be IFI16 as well) oligomerizes differently or only through ASC recruitment, though overexpression of the pyrin domain of AIM2 results in filament formation (216). pUL83 has also been reported to bind other nuclear PYHIN proteins, IFIX and MDNA, and can disrupt their ability to form nuclear oligomers (216).

The roles of PYHIN proteins in sensing DNA as well as regulating immune gene responses protect the host from from invading pathogens, but dysregulation of the PYHINs and their pathways can harm the host leading to diseases and autoimmune disorders. As describe before, differential expressions of the PYHIN proteins have been

reported in numerous diseases, from viral infections to cancer. However, it is not always clear whether the expression patterns are the cause or an effect of the disease in question, especially since the PYHINs inducible proteins. For example, PYHIN proteins have been reported to lie within a susceptibility locus for the autoimmune disease, systemic lupus erythematosus (SLE) (218) and the *PYHIN1* locus has been identified as a novel susceptibility locus for asthma, especially for people of African descent (219). Protective roles for AIM2 has been indicated in prostate diseases with increased levels of AIM2 mRNA and protein in benign prostate hyperplasia and lower levels of AIM2 expression in prostate cancer epithelial cells. Genetic and epigenetic inactivation of AIM2 is prevalent in patients with mismatch repair-deficiency that is a major mechanism for colorectal tumorigenesis and sporadic colorectal cancers (220). Contrastingly, levels of AIM2 and inflammasome activity are enhanced in psoriatic lesions, a chronic autoimmune disease (221), possibly via sensing of self-DNA as well as in chronic vascular inflammation in abdominal aortic aneurysm (222). Increased levels of AIM2 and IFI16 have also been correlated to occurrence of oral squamous cell carcinoma, which inactivates the p53 system (223). Altered PYHIN1 and MNDA levels in a number of conditions have also been associated with diseases such as asthma, breast cancer, Lymphoma Myelodysplastic syndrome (MDS). Chronic autoimmune diseases such as SLE, systemic sclerosis, rheumatoid arthritis and Sjögren's syndrome have been reported to have anti-IFI16 autoantibodies (224-227). IFI16 re-localizes to the cytosol following UV treatment leading to DNA damage or upon viral DNA detection. It is hypothesized that IFI16 can also be released into the extracellular matrix that can bind to endothelial cells, propagating the stress signal and causing further damage. IFI16 acts as an immune

sensor for HIV and HIV patients often have elevated IFI16 mRNA levels, possibly due to chronic immune activation of IFI16. However, more insights into IFI16 activation, its subcellular localization in HIV patients will determine the detrimental or beneficiary role of elevated IFI16 levels in HIV patients.

Inflammasomes are critical immune regulators that help in maintaining a normal abundance of gut microbiota, an intact epithelial barrier and a continuous turnover of intestinal epithelial cells to prevent intestinal diseases such as inflammatory bowel diseases (IBD) and colorectal cancer. Increased expression of AIM2 in epithelial cells and IFI16 in both lymphocytes and epithelial cells was associated with increased inflammation in the mucosa of IBD patients, and anti-TNF treatments decreased expression of AIM2 and IFI16 in the patients (228). Further, another group reported intestinal epithelial cells are a primary source of IFI16 that is increased in the inflamed gut mucosa of IBD patients. IFI16 localized to both intestinal epithelial cells and lamina propria leukocytes, and was inducible by TNF, IL-1β and IL-33. However, as mentioned before, a controlled AIM2 expression is protective against colorectal cancer, and is critical for maintaining the proper gut microbiota that detects and clears harmful pathogens such as *Listeria monocytogenes*, *Francisella tularensis* and *Aspergillus fumigatus* and blocks the epithelial translocation of *Salmonella typhimurium* in the gut.

Further mechanistic studies with the PYHIN proteins are required in the pathogenesis of diseases, cancer and autoimmunity, to understand the contribution of these proteins in protection as well as in dysregulated host immune responses such that they may be considered as therapeutic targets or disease bio-markers.

Dissertation Objectives

This dissertation has two major objectives:

(1) To characterize the functions of the nuclear, murine PYHIN protein, p205/Ifi205 in innate immune responses, and its mechanism of action in regulating the pathways in macrophages, using a series of loss-of-function and gain-of-function approaches.

(2) To determine the involvement of caspase 8-dependent inflammasome activation during *Paracoccidiodes brasiliensis* fungal infection *ex vivo* and *in vivo* using *Caspase8*$^{-/-}$ *Rip3*$^{-/-}$ mouse model.

Preface to Chapter II

Most of the content of this chapter has been submitted for the following manuscripts/ publications:

Sreya Ghosh, Christina Wallerath, Sergio Covarrubias, Veit Hornung, Susan Carpenter and Katherine A. Fitzgerald. *The PYHIN protein p205 regulates the inflammasome by controlling Asc expression.* Journal of Immunology, 2017 Sep 20; 199 (9): 3249–3260

- The project was conceptualized by K.A.F., S.C. and S.G.
- S.G. designed and performed the experiments.
- C.W. and V.H. made the CRISPR/Cas9 knockout in B16 melanoma cell lines
- S.G. and K.A.F. wrote the manuscript for publication.

Chapter 2

The PYHIN protein p205 controls innate immune responses by regulating the expression of the inflammasome adapter, Asc and other immune genes

Abstract

Members of the interferon-inducible PYHIN protein family such as Absent in Melanoma-2 and IFI16 bind double-stranded DNA (dsDNA) and form caspase-1 activating inflammasomes, important in immunity to cytosolic bacteria, DNA viruses or Human Immunodeficiency Virus. IFI16 has also been shown to regulate transcription of type I Interferons during Herpes Simplex Virus infection. The role of other members of the PYHIN protein family in the regulation of immune responses is much less clear. Here, we identified an immune regulatory function for a member of the murine PYHIN protein family, p205 (also called as Ifi205). Examination of immune gene expression in p205 knockdown lines in bone marrow derived macrophages revealed that inflammasome responses to dsDNA as well as ligands that engage the NLRP3 inflammasome were severely compromised in these cells. Further analysis revealed that p205 knockdown cells showed reduced expression of Asc at the RNA and protein level. p205 knockdown resulted in reduced binding of actively transcribing RNA Polymerase II to the endogenous *Asc* gene resulting in decreased transcription and processing of Asc pre-mRNA. Deletion of p205 in B16 melanoma cells using CRISPR/Cas9 showed similar loss of Asc expression. Ectopic expression of p205 induced expression of an *Asc* promoter-luciferase reporter gene and collaborated with other transcription factors such as c/EBPβ and p65/RelA to further enhance expression. Together these findings suggest that p205 controls expression of Asc mRNA to regulate inflammasome responses. These findings expand on our understanding of immune regulatory roles for the PYHIN protein family.

Introduction

Foreign nucleic acids play a critical role in initiating innate and adaptive immune responses. Nucleic acid (NA) sensors expressed in distinct cellular compartments survey the extracellular and intracellular environment for signs of infection and initiate immune defenses against bacterial, viral and eukaryotic pathogens (229). NA sensors include RNA Sensors such as TLR3, 7/8 and RIG-I/MDA5 (6, 11, 138) as well as DNA sensors such as TLR9, AIM2, cGAS and IFI16 (8, 18-20). In addition, recognition of foreign nucleic acids especially dsDNA leads to assembly of an inflammasome, a large caspase-1 activating multiprotein complex that controls the proteolytic processing and release of IL-1β. Inflammasome activation also results in a proinflammatory form of cell death called pyroptosis. While most inflammasomes are composed of members of the NLR family, the dsDNA-activated inflammasome is formed following dsDNA binding to a PYHIN protein, Absent in Melanoma-2 (AIM2) (18). Work from several labs including our own has defined AIM2 as a cytosolic DNA binding innate immune receptor (18, 230-232). AIM2 binds pathogen-derived dsDNA that accumulates in the cytosol during infection with DNA viruses or cytosolic bacterial pathogens (171, 185). In some instances, AIM2 can also recognize host dsDNA that gains access to the cytosol leading to autoinflammation (233). The related PYHIN protein IFI16 also forms an inflammasome during infection with Kaposi's Sarcoma Herpes Virus (KSHV) and Human Immunodeficiency 1 (HIV1) (21, 22).

The PYHIN proteins were first characterized as a family of interferon

inducible proteins that are predominantly nuclear localized (183). PYHINs are constitutively expressed in different hematopoietic cell types, although most members of this family are also inducible by type I Interferon in non-hematopoietic cells (183). Phylogenetic analysis of the mammalian PYHIN proteins also called the AIM2-like receptors or ALRs show strong evolutionary and functional diversity (172). The murine PYHIN locus has undergone extensive gene duplication with more than 13 members encoded in the murine genome in contrast to the human gene family with 4 genes (172, 173). PYHIN proteins have been implicated in a wide-range of cellular processes including transcription, tumor suppression, cell cycle, cell growth, differentiation and cell death(183). The majority of the PYHIN proteins share the same structural domains. They contain an N-terminal α-helical domain known as the Pyrin domain capable of homotypic protein-protein interactions and one or more HIN200 domains. Most PYHIN proteins contain a nuclear localization signal in their N-terminus that can be either monopartite, bipartite or both. Some family members also contain a nuclear export sequence that enables them to shuttle between the nucleus and the cytosol. Aim2 is the most conserved family member. Unlike the other PYHINs, AIM2 is localized in the cytosol. Phylogenetic analysis indicates that, with the exception of AIM2, there is a complete lack of orthology among mammalian ALRs.

While the role of AIM2 and IFI16 in dsDNA recognition and immune activation has been well established, the role of other members of the PYHIN protein family, especially those in the mouse remains unclear. Recently, genetic studies in mice lacking the entire PYHIN locus and analysis of type I IFN induction following dsDNA treatment

in cells from these animals demonstrated no clear link to dsDNA recognition and induction of type I IFN in murine myeloid cells (176). It remains to be defined therefore whether the PYHINs have other immune regulatory functions. In this study we examined the contribution of the murine PYHIN protein p205 (also called p205) in innate immunity. p205 is primarily expressed in macrophages and granulocytes (234). p205 shares the highest homology with p204 and the human PYHIN protein, MNDA (235). p205 has been implicated in cell growth, and differentiation playing roles in hematopoiesis, adipogenesis and osteogenesis (236-238). To evaluate a possible immune function for p205, we investigated the ability of p205 to control immune gene expression. Using a series of loss of function and gain of function approaches combined with functional studies we demonstrate that p205 regulates transcription of important immune genes. In particular, we found a critical role for p205 in controlling expression of the inflammasome adapter molecule Asc. This effect of p205 was not related to the prior work linking AIM2 and IFI16 to sensing of foreign DNA. Rather we find that p205 works in the nucleus to control Asc gene expression. These findings add to our understanding of PYHIN proteins in innate immunity, expanding their functions beyond dsDNA sensing to regulating innate immune responses through gene regulation.

Material and Methods

Reagents and Plasmids:

Lipopolysaccharide (LPS) and poly-dAdT (pdAdT) were obtained from Sigma-Aldrich and Immunostimulatory DNA oligonucleotides were synthesized as described (20). Nigericin and ATP were from Invivogen and Sigma respectively. Polyinosinic-polycytidylic acid (poly I:C) was obtained from Invivogen. Sendai virus (Cantrell strain) was purchased from Charles River Laboratories (Wilmington, MA). Lipofectamine 2000® Transfection Reagent was from Invitrogen. GeneJuice was from Novagen (Madison, WI). Universal type I IFN and IFN-γ were from PBL Interferon Source (Piscataway, NJ) and PeproTech (315-05), respectively. *S. typhimurium* (SL1344 lab strain) was from M. O'Riordan. The plasmids used were p65-pCMV4, c/EBPβ-pcDNA (Addgene), pGL3-enhancer luciferase reporter (Promega). Other plasmids such as Asc in pMSCVneo (Clontech), p205-HA in pRZ-retro, Aim2-FLAG, p204-HA, p205-HA in pEF-BOS or HA-tagged ΔHIN-p205, ΔPYD-p205 and ΔH/ΔP-p205 in pMSCV-PIG (Addgene) were made in the lab.

Cell culture, Stimulations, ELISA and Cell death assays:

Primary bone-marrow derived macrophages (BMDM) from C57BL/6J mice, cultured with L929 supernatant as a source for Macrophage Colony Stimulating Factor (MCSF), were transformed using CreJ2 virus to make immortalized BMDM (iBMDM). The cells were cultured in DMEM with 10% FCS and PenStrep. Antibiotics for selection were used as required. Cells were primed with repurified LPS at 100 ng/ml for 2-3h and stimulated

with *Salmonella* sp., Nigericin or ATP for 1h, pdAdT and Interferon Stimulatory DNA (ISD) for 6h, poly I:C and Sendai virus for overnight. Cells infected with *Salmonella typhimurium* and media containing gentamicin (100 µg/ml) was added to kill the extracellular bacteria. Supernatants from the stimulated cells were analyzed for the cytokines, IL-1β (eBiosciences) and IFN-β by ELISA. Cell death was measured by quantitating the amount of LDH, which is released into the supernatant upon cell lysis, using CytoTox96 Non Radio Cytotoxicity Assay (Promega) kit. 10% Triton-X was added to the cells as a representation of 100% cell death.

shRNA mediated silencing:

The shRNA sequences targeting p205 were cloned into a lentiviral pLKO.1 TRC cloning vector. Two separate shRNA sequences for p205 used either targeted the coding region (KD CDS) (Dharmacon TRCN0000095887 or TRCN0000095885) or the 3' untranslated region (KD 3'UTR) (Dharmacon TRCN0000095884). HEK 293T cells were transfected with 4 µg shRNA along with 3 µg pSPAX (gag/pol) and 1 µg pMD2 (VSV-G) plasmids for production of lentiviral particles. Viral particles were collected at 48 h, filtered and added to immortalized BMDM. As controls, BMDM were transduced with either an empty pLKO.1 vector (EV) or pLKO.1 containing an shRNA sequence targeting GFP (GFP shRNA). The cells were selected for effective transduction by selection with puromycin (5 µg/ml).

qRT-PCR and Nanostring:

RNA was extracted using Qiagen RNeasy Kit. cDNA was synthesized from 1 µg total RNA using either iScript cDNA synthesis kit (Bio-Rad) or QuantiTect Reverse Transcription Kit (Qiagen). Quantitative RT-PCR was performed using iQ SYBR Green supermix (Bio-Rad) or QuantiNova SYBR Green PCR Kit (Qiagen). Primers were constructed to respective genes (Table 1). Target genes expressions are relative to housekeeping genes expression and were normalized to respective controls. The expression of a subset of genes including p205, p204, Mnda, Mndal and Aim2 were measured using nCounter (Nanostring). Briefly, endogenous RNA transcript counting was performed on total RNA hybridized to a custom gene expression CodeSet and analyzed on an nCounter Digital Analyzer (29). The counts were normalized to internal spike-in and endogenous controls per Nanostring Technologies' specifications. The heatmaps were generated using the Morpheus software.

Table 1: qRT- PCR Primers for mRNA

Gene	Forward (5' → 3')	Reverse (5' → 3')
p205	GTATGAGTGAAGAAAAGACTGAC	GGATATTGGTGACTGGCATG
p205	AAGATCAAGGCATCTGGGAAAG	CCTCTGGGAATGTTCTGGTTC
p204	GACAACCAAGAGCAATACACCA	ATCAGTTTGCCCAATCAGAAT
Mnda	TGTGAAGAACCCACAGCCAT	TGATTTTTGGTTCTTAGCCGAAA
Gapdh	ATTGTCAGCAATGCATCCTG	ATGGACTGTGGTCATGAGCC
Hprt	TGAAGAGCTACTGTAATGATCAGTCAA	AGCAAGCTTGCAACCTTAACCA
Asc (3'UTR)	CCAAACATGCACAAATCAGTC	AAATGGGGAGCCAGGAATCA
Asc int2-ex3	CACCCTTGCACAGCCTATCT	CTCCGTCCACTTCTGTGACC
Asc ex2/3-ex3	CAGCCAGAACAGGACACTTT	CTCCGTCCACTTCTGTGACC
Asc ex1-ex1/2	CTGCGAGAAGGCTATGGG	CTCCAGACTCTTCTTTAGT
Asc ex1-in1	CTGCGAGAAGGCTATGGG	GAACAAGGGGACACACT
IL-1α mature	TCTCAGATTCACAACTGTTCGTG	AGAAAATGAGGTCGGTCTCACTA
IL1α int5-ex6	CACACACACACACACATCTGC	GGGCTGGTCTTCTCCTTGAG
IL1α int1	CGCTCTTCCCGTTTTGTAAG	GTGGCCATGTGTGTGTCACT
IL1α int2	TCCTCCTCCTCCTCCTTCTC	GAACCTGATGGCCTCTCTCA
IL-6 ex3-ex5	AACGATGATGCACTTGCAGA	GAGCATTGGAAATTGGGGTA
IL-6 ex2-ex2/3	GACTGATGCTGGTGACAACC	TTGCACAACTCTTTTCTCAT
IL-6 ex2-int2	AACGATGATGCACTTGCAGA	TTGAAAGTAAACGTGACAAG

Western Blot and Co-immunoprecipitation:

For detecting caspase-1 and IL-1β in the supernatants and lysates, 20% vol RIPA buffer and 30% vol SDS-loading dye were added directly to the wells containing cells and media. The samples were boiled at 100°C for 15'-30' and were run on 13% polyacrylamide gels. Cytosolic and nuclear fractions for protein detection were prepared by using Active Motif Nuclear Extract Kit. Co-immunoprecipitation assays were carried out with $5x10^6$ cells (treated or untreated) in ice-cold RIPA buffer (without SDS and DOC) using Protein A Dynabeads (Novex/Life Technologies, Cat#10001D) conjugated with specific antibodies, and immunoblotted for proteins of interest. Antibodies used were against Asc (Santa Cruz, Cat# sc-22514-R or Cell Signaling, Cat# 67824), IL-1β (R&D Systems, Cat# AF-401-NA), caspase-1 (Adipogen, Cat# AG-20B-0042), Aim2 (eBioscience, 14-6008), Nlrp3 (Enzo Life Sciences), Histone 3 (Abcam, Cat# ab1791), c/EBPβ (Santa Cruz, Cat# sc-150), Usf2 (Santa Cruz, Cat# sc-862), Gapdh (Sigma, Cat# G9295), β-actin (Sigma, Cat# A3854) HA-tag (Anti-HA-Peroxidase; Roche, Cat# 12 013 819 001), and FLAG-tag (Sigma, Cat# A8592). An affinity purified polyclonal antibody against p205 was generated using the following peptide: AGLDRLINFCERVPTL-amide) was generated (21^{st} Century Biochemicals).

Confocal Microscopy:

HEK 293T cells were transfected with p205 tagged with CFP and 24h post-transfection, the cells were washed with PBS and stained with Acridine Orange and/or Cholera toxin-B (CtxB) (Thermofisher) and were visualized by confocal microscopy (Leica 8000) for localization.

Luciferase assays:

The *Asc* gene promoter from -2000 +10 bp was cloned into pGL3-Enhancer reporter vector upstream of the firefly luciferase gene. The promoter-reporter construct was either transfected alone or co-transfected with plasmids expressing p205, p204, Aim2, p205 deletion mutants, p65/RelA or c/EBPβ or a combination thereof in HEK 293T cells. A plasmid expressing Renilla luciferase gene under thymidine kinase (pGL4-TK Renilla) promoter was included as transfection efficiency control. Data is represented as fold change over *Asc* reporter construct alone, and relative to transfection efficiency.

Chromatin Immunoprecipitation:

Cells were crosslinked with 1% formaldehyde for 10 min with gentle shaking. The crosslinking reaction was stopped by 125mM Glycine solution for 10 min on shaker. The crosslinked cells were lysed in cell lysis buffer containing 1% SDS and sonicated for 10 cycles (30s on, 30s off) using Bioruptor®300. The DNA was quantified, and 5 µg of total chromatin was immunoprecipitated with specific antibodies and Dynabeads Protein G (Novex/Life Technologies, Cat#10009D) overnight. The beads were washed with high and low salt buffers, and the crosslinked protein-DNA was eluted. The DNA was then reverse crosslinked, purified and quantitated by qPCR amplification with primers at *Asc*, *Gapdh* genes (Table 2). Antibodies used were against total RNA Polymerase II (RNAPII; Active Motif Cat# 39097), phospho Serine-2 RNA Polymerase II (Ser2 RNAPII; Abcam Cat# ab5095), phospho Serine-5 RNA Polymerase II (Ser5 RNAPII; Abcam Cat# ab5131) or IgG isotype (Abcam, Cat# AB37415 and Cell Signaling, 5415).

Table 2: Primers for Chromatin Immunoprecipitation

Gene	Forward (5' → 3')	Reverse (5' → 3')
Asc upstream	TCAGCCTAGCCAAAAAGCCA	GACTCCCCCACCCTCTTTTC
Asc TSS 1	CACGAGATGCCATCCTGGAC	CCCATAGCCTTCTCGCAGTT
Asc TSS 2	CTGCAGATGGACGCCATAGA	TGTGAGCTCCAAGCCATACG
Asc downstream	CACTCATTGCCAGGGTCACAG	CCTGGAGGAGGGAGTCCTTG
Gapdh TSS	TGAAGGTCGGTGTGAACGG	CAATCTCCACTTTGCCACTGC

CRISPR/Cas9-mediated gene knockout:

B16 mouse melanoma cells were cultured in DMEM containing 10% FBS, 0.5% Ciprofloxacin and 0.0075 % β-mercaptoethanol and transfected with 200 ng of plasmid containing mCherry-Cas9 and a U6 promoter-driven gRNA against p205 (Target Sequence: ATGAAGCCGAAGATGAGACCTGG) using Lipofectamine 2000® according to manufacturer's protocol (239). Two days after transfection cells were sorted for mCherry expression. Positive cells were plated at limiting dilution to obtain single cell clones. Genotyping of the B16 clones was conducted by deep sequencing (Illumina, MiSeq) as previously described (240) using the following primer sequences: 5'-ACACTCTTTCCCTACACGACGctcttccgatct**CGTGAAGAAGATCAAGGCATCTG**-3' and 5'-TGACTGGAGTTCAGACGTGTGctcttccgatct**AAATCTCAGGGAGAAGTGGGGGA**-3' (uppercase letters: 1st PCR adapter sequences, lowercase letters: linker sequences, uppercase letters: target site specific primer sequences). Cell clones harboring all-allelic frame shift mutations were then selected as p205 KO cell clones.

Statistics:

ELISA and luciferase assays are presented as mean ± SD from three independent biological replicates and are representative of at least three separate experiments. Data was analyzed using two-way ANOVA by Prism 6 Software (GraphPad, San Diego, CA). The *p* values < 0.05 were considered significant (*p < 0.05, **p < 0.001, ***p < 0.005, ****p < 0.0001) and n.s. = non-significant, unless otherwise indicated.

Results

p205 is highly inducible by LPS, type I and type II Interferons

The *p205* gene is encoded on chromosome 1q, the last of the 13 consecutive genes of the mouse PYHIN locus. *p205* is encoded on the reverse strand and flanked by *p202b* at the 5'-end and several olfactory receptor genes as well as the *Spta1* gene at its 3'-end (**Figure 2.1.A**). p205 is expressed in murine primary bone marrow derived macrophages and treatment of these cells with type-I IFN, IFN-γ and LPS treatments further upregulated its expression, similar to other PYHIN genes such as *Mnda* and *p204* (**Figure 2.1B, C and D**). The inducible expression of p205 was as robust as the well-characterized immune genes such as *Il6* or *Cxcl10* that are used as positive controls.

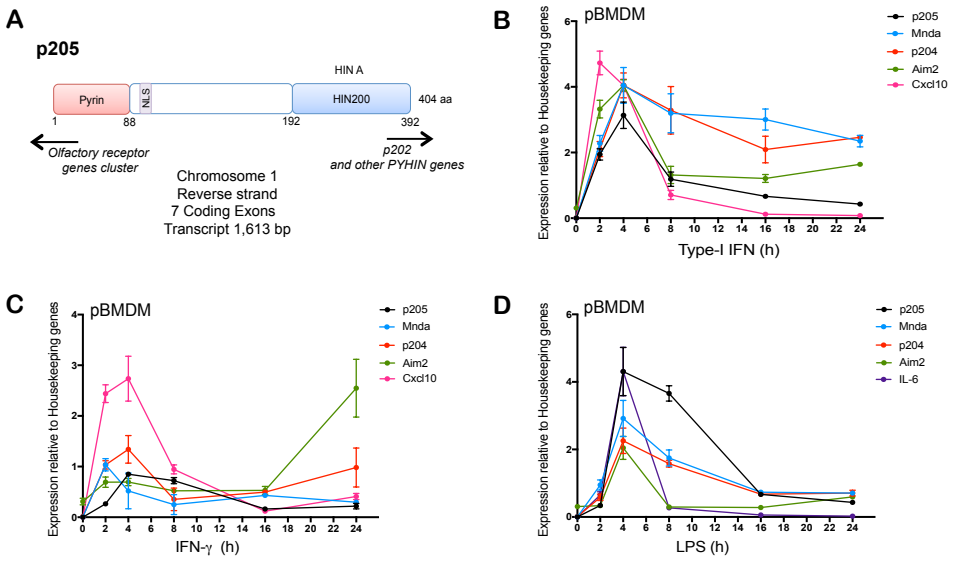

Figure 2.1: p205 is highly induced by Type I and Type II Interferons, and LPS. (A) Schematic of the p205 mouse gene. Primary BMDMs stimulated with (B) Type-I IFN (100 U/ml), (C) IFN-γ (20 ng/ml) or (D) LPS (200 ng/ml), at different time points (0, 2, 4, 8, 16, 24 h) were tested for p205 mRNA expression as well as other PYHIN genes, *Mnda*, *p204* and *Aim2*. Levels of IL-6 or Cxcl10 mRNA were included as positive controls. Gene expression is reported relative to a combination of three housekeeping genes- *Gapdh*, *Hprt*, *β-actin*.

Inducible and overexpressed p205 localizes primarily to the nucleus

The NLS Mapper prediction software projected p205 to have a monopartite signal, 'PTLKKRAEIL' (score >5) and two bipartite signals, 'TAQKRKGMSEEKTDVKKIKAS', 'FHLKRERGQPKLVCGDHSFVKVTKAGKKK' (score >6) that predict the protein is preferentially nuclear. However, as Brunette et al showed previously, overexpressed p205 may change localization as with change in stimuli, such as STING overexpression (173). Hence, we tested p205 localization when overexpressed as well as upon induction. We looked at the levels of induced p205 protein in macrophages and tested for its subcellular localization. Treatment of primary BMDM with LPS, type-I IFN and IFN-γ increased p205 protein expression and it was mainly expressed in the nucleus (**Figure 2.2A**). Further, we stimulated primary BMDM with type-I IFN at different time points and separated the cell lysates into nuclear and cytosolic fractions, which were immunoblotted for endogenous p205. Upon IFN-treatment, p205 expression was robustly increased over time in the nucleus (**Figure 2.2B**).

Furthermore, immortalized macrophages transduced with the hemagglutinin (HA) tagged p205 were fractionated into nuclear and cytosolic extracts. Analysis of these fractions by Western blot showed that overexpressed p205 localized to the nucleus in the immortalized macrophages as well (**Figure 2.2C**). Similarly, we observed the localization of ectopically expressed p205 in HEK 293T cells by imaging a CFP-tagged p205 construct using confocal microscopy and p205 appeared to be predominantly in the nucleus (**Figure 2.2D**).

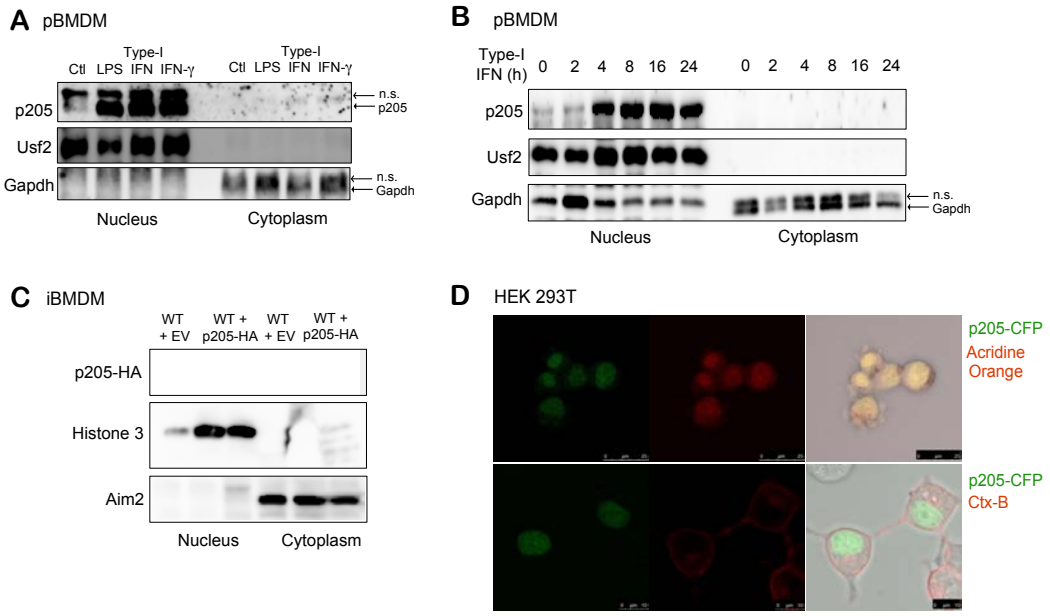

Figure 2.2: Inducible and overexpressed p205 primarily localizes to the nucleus. (A) Primary BMDM untreated (Ctl) or treated with LPS for 6h, with type-I IFN for 16h or with IFN-γ for 16h were separated into nuclear and cytosolic fractions and immunoblotted for endogenous p205 (n.s.- non-specific band). (B) Western blot analysis of endogenous p205 expression in the nuclear and cytosolic extracts of primary macrophages treated with type-I IFN as indicated. Usf2 and Gapdh were used as controls for nuclear and cytosolic fractions respectively (n.s.- non-specific band). (C) Immunoblot analysis of the nuclear and cytoplasmic fractions of wild-type BMDM transduced with empty vector (EV) and wild-type BMDM overexpressing HA-tagged p205 using anti-HA antibody. Histone 3 and Aim2 were used as controls for nuclear and cytosolic extracts respectively. (E) Confocal microscopy of CFP-tagged p205 (green) in HEK 293T cells stained for nucleus using Acridine orange in first panel and Cholera Toxin B (CtxB) staining plasma membrane in second panel. Data is representative of two independent experiments.

Generating shRNA-mediated p205 knockdown in immortalized bone marrow derived mouse macrophages

Bone marrow derived macrophages or BMDM from C57BL/6 wild-type mice were transformed and immortalized by CreJ2 virus. Resting macrophages express modest levels of p205 that can be robustly induced upon stimulation with Interferon or LPS. The immortalized macrophages were stably transduced with pLKO.1 lentiviral particles either containing short-hairpin RNA (shRNA) targeting p205 (p205 KD) or GFP (GFP shRNA) or, with no shRNA sequence (EV Ctl). The shRNA to p205 either targeted the 3' untranslated region (KD 3'UTR) (**Figure 2.3A**) and the coding region (KD CDS) (**Figure 2.3B**). After positive selection with puromycin for transduced cells, the levels of p205 were assessed by quantitative PCR (qPCR). The shRNAs targeting the coding region showed efficient knockdown of p205, with no effects on other closely related family members, p204 and Mnda. This effect was also evaluated using Nanostring where p205 mRNA was reduced with little impact on 4 other related PYHIN genes (*p204*, *Mnda*, *Mndal* and *Aim2*) (**Figure 2.3C**).

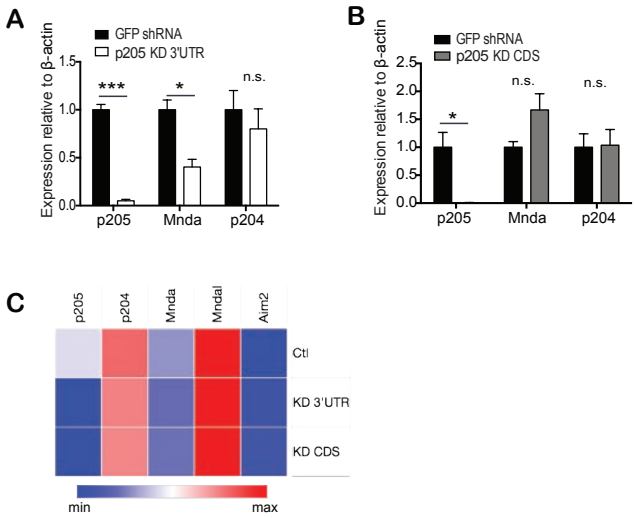

Figure 2.3: shRNA-mediated p205 knockdown in immortalized BMDM. Immortalized BMDM transduced with shRNA targeting either (A) 3'UTR or (B) CDS of p205 gene were inspected for expression of p205, Mnda and p204 mRNA relative to β-actin mRNA and normalized to expression in GFP shRNA BMDM. (C) Heatmap of PYHIN gene expression in p205 KD 3'UTR and KD CDS BMDM compared to control (Ctl) BMDM.

Knockdown of p205 in macrophages results in compromised inflammasome activation

In order to ascertain the potential immune functions of p205, the macrophages with reduced p205 expression were tested for their abilities to respond to various stimuli and launch effective cytokine expression. Following the involvement of other PYHIN proteins in the IFN-pathway, we first evaluated the induction of type-I IFN in these macrophages. The p205 knockdown and control BMDM were treated with LPS, poly dAdT (pdAdT), Interferon Stimulatory DNA (ISD), Sendai virus (SV) or poly I:C, all of which induces type I IFN, the levels of which were measured by ELISA. For most of these ligands the inducible expression of IFNβ was largely unaffected by the absence of p205 (**Figure 2.4A**). However, transfection with pdAdT, a dsDNA mimetic, showed a heightened IFNβ response in the macrophages lacking p205. Besides the IFN-pathway, PYHIN proteins such as AIM2 and IFI16 have also been implicated in the inflammasome pathway that produces IL-1β. Hence, the macrophages were treated with LPS (200 ng/ml) for two hours and stimulated with transfected pdAdT or ISD, or Nigericin that activate the Aim2 and Nlrp3 inflammasomes respectively. LPS behaves as Signal 1 that primes the cells via TLR signaling allowing transcriptional induction of several pro-inflammatory genes such as pro-IL1β, while stimulation with DNA or Nigericin (a bacterial toxin from *Streptomyces hygroscopius* that forms pores in membranes) activates the inflammasome, which processes pro-IL1β into active IL-1β. Macrophages lacking p205 showed a much-diminished response to pdAdT stimulation indicative of an impaired Aim2 inflammasome but surprisingly, the cells were also unresponsive to Nigericin stimulation that induces the Nlrp3 inflammasome (**Figure 2.4B**).

Pro-forms of caspase 1 and IL-1β are unaffected in macrophages lacking p205

To discern whether p205 knockdown was affecting the priming (Signal 1) or the processing of pro-IL1β (Signal 2), the lysates and the supernatants from the stimulated macrophages were tested for both the inactive and proteolytically processed forms of both caspase-1 and IL-1β by Western blot (**Figure 2.4C**). Cells with reduced p205 lacked the active IL-1β, as expected; however, there was no difference in the transcription of pro-IL-1β upon LPS signaling ruling out an effect of p205 on TLR signaling. Moreover, even though no change was observed in the levels of quiescent caspase 1 in the p205 knockdown macrophages with the control cells, no cleaved, active form of the caspase 1 was being made. Taken together these observations implied that reduction in p205 affected inflammasome dependent activation of caspase-1 leading to reduced processing of pro-IL1β.

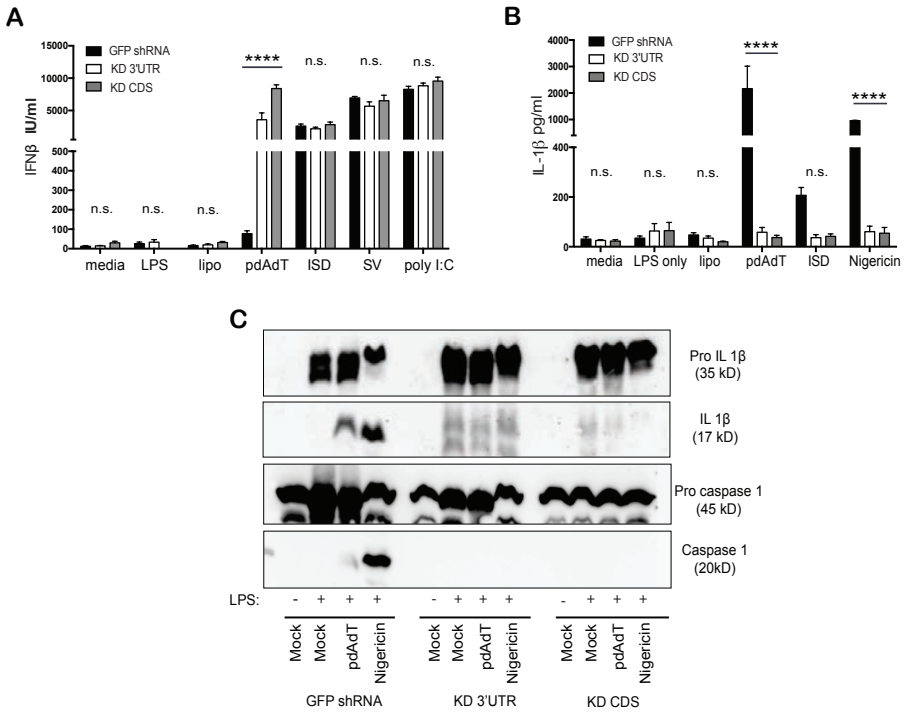

Figure 2.4: p205 knockdown in macrophages results in impaired inflammasome activation. The p205 knockdown BMDMs were primed with LPS (200ng/ml) for 3h and then stimulated with transfected pdAdT (1μg/ml for 6h), transfected ISD (3μM for 6h), Nigericin (10μM for 1h), ATP (5μM) or, stimulated alone with Sendai virus (SV; overnight) or poly I:C (overnight). Secreted (A) IFNβ and (B) IL-1β levels were assessed by ELISA. (C) GFP shRNA CTL, p205 KD 3'UTR and KD CDS were primed with LPS (200ng/ml) for 3h and then stimulated with pdAdT (1 μg/ml for 6h) or Nigericin (10 μM for 1h) and the supernatants and the lysates from the macrophages were immunoblotted for pro-IL1β (35 kD), cleaved form of IL-1β (p17), pro-caspase 1 (45kD) and the active subunit of caspase 1(p20).

Reduced expression of p205 impacts Asc expression

For further mechanistic analysis of the lack of inflammasome activation in absence of p205, we inspected the levels of the receptors Nlrp3 and Aim2 as well as the inflammasome adapter Asc in LPS-stimulated macrophages. There was no difference in the levels of Nlrp3 or Aim2, however Asc protein expression was completely abrogated in the macrophages with reduced p205 expression (**Figure 2.5A**). We established the loss of p205 in the LPS-stimulated macrophages using a newly generated antibody against p205, and the macrophages with reduced p205 showed diminished Asc expression (**Figure 2.5B**). Further analysis showed that the Asc mRNA levels were also impacted in the macrophages lacking p205 (**Figure 2.5C**). We inspected the shRNA sequences and they showed no sequence similarity with the Asc mRNA, indicating that the decrease in Asc transcript levels was not due to an off-target effect of the shRNA. Collectively, these results show that p205 affects Asc mRNA expression.

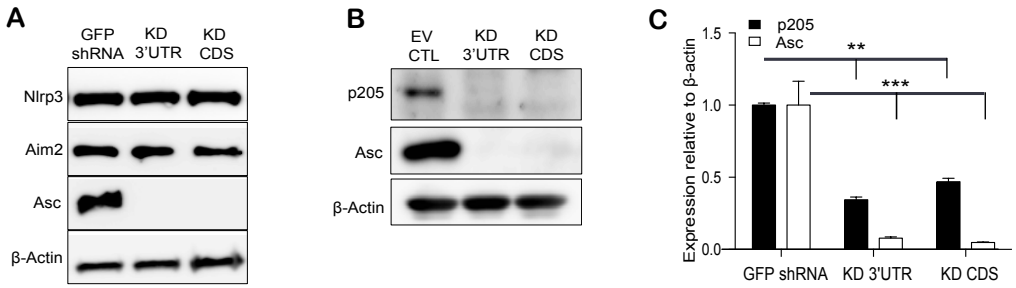

Figure 2.5: Loss of p205 leads to a defect in Asc expression.
(A) Levels of Nlrp3, Aim2, Asc and β-actin proteins were elucidated by Western blot in LPS stimulated (200 ng/ml) GFP shRNA CTL, p205 KD 3'UTR and KD CDS macrophages. (B) Immunoblot of p205, Asc and β-actin proteins in LPS-stimulated (200 ng/ml for 6h) p205 knockdown macrophages. (C) Levels of p205 and Asc mRNA (relative to β-actin and normalized to GFP shRNA BMDM) were detected by qPCR in the same cell lines.

p205 controls inflammasome activation via its adapter molecule, Asc

The Asc cDNA was cloned into a retroviral vector, pMSCV, and viral particles were generated to transduce macrophages lacking p205 to rescue Asc expression. The transduced macrophages were selected with neomycin, and the levels of restored Asc were inspected by Western blot. Asc proteins levels in the p205 knockdown macrophages were stably restored comparable to the control cell lines (**Figure 2.6A**). Inflammasome activation and IL-1β production with were fully restored in these macrophages with renewed Asc expression, following LPS priming and pdAdT transfection or Nigercin treatment (**Figure 2.6B**).

Salmonella typhimurium also activates the inflammasome. When *Salmonella* is in its log phase of growth i.e. the bacteria are actively dividing they are recognized by Nlrc4, while when in their non-dividing or stationary growth phase, *Salmonella* is primarily recognized by Nlrp3. Unlike Nlrp3 or Aim2, Nlrc4 itself contains a CARD domain, and can directly recruit and activate pro-caspase 1 as well as engage Asc to do so. Hence, Nlrc4 activates the inflammasome in a manner that is only partially dependent on Asc. The p205 knockdown cells were challenged with *Salmonella* sp. either in their stationary phase or in log phase after LPS stimulation for one hour. In the stationary phase (overnight culture), *Salmonella* sp., which primarily activates Nlrp3 to trigger inflammasomes showed minimal IL-1β production by ELISA. But when the same cells were infected with the actively dividing *Salmonella* (4-6hr fresh culture), the macrophages retained part of their inflammasome activating capability that was again rescued to its full potential upon reconstitution with Asc (**Figure 2.6C**). The IFN-β

responses were largely unaffected by Asc reconstitution except upon pdAdT transfection where p205 knockdown enhanced IFN-β production (**Figure 2.6D**).

Furthermore, stimulation with LPS and subsequent pdAdT transfection in the p205 knockdown macrophages reconstituted with Asc showed renewed processing of caspase 1 and IL-1β into their active, cleaved forms signifying a functional inflammasome (**Figure 2.6E and F**). Taken together, these findings attribute the loss of inflammasome responses in p205-knockdown cells directly to p205-dependent effects on Asc expression.

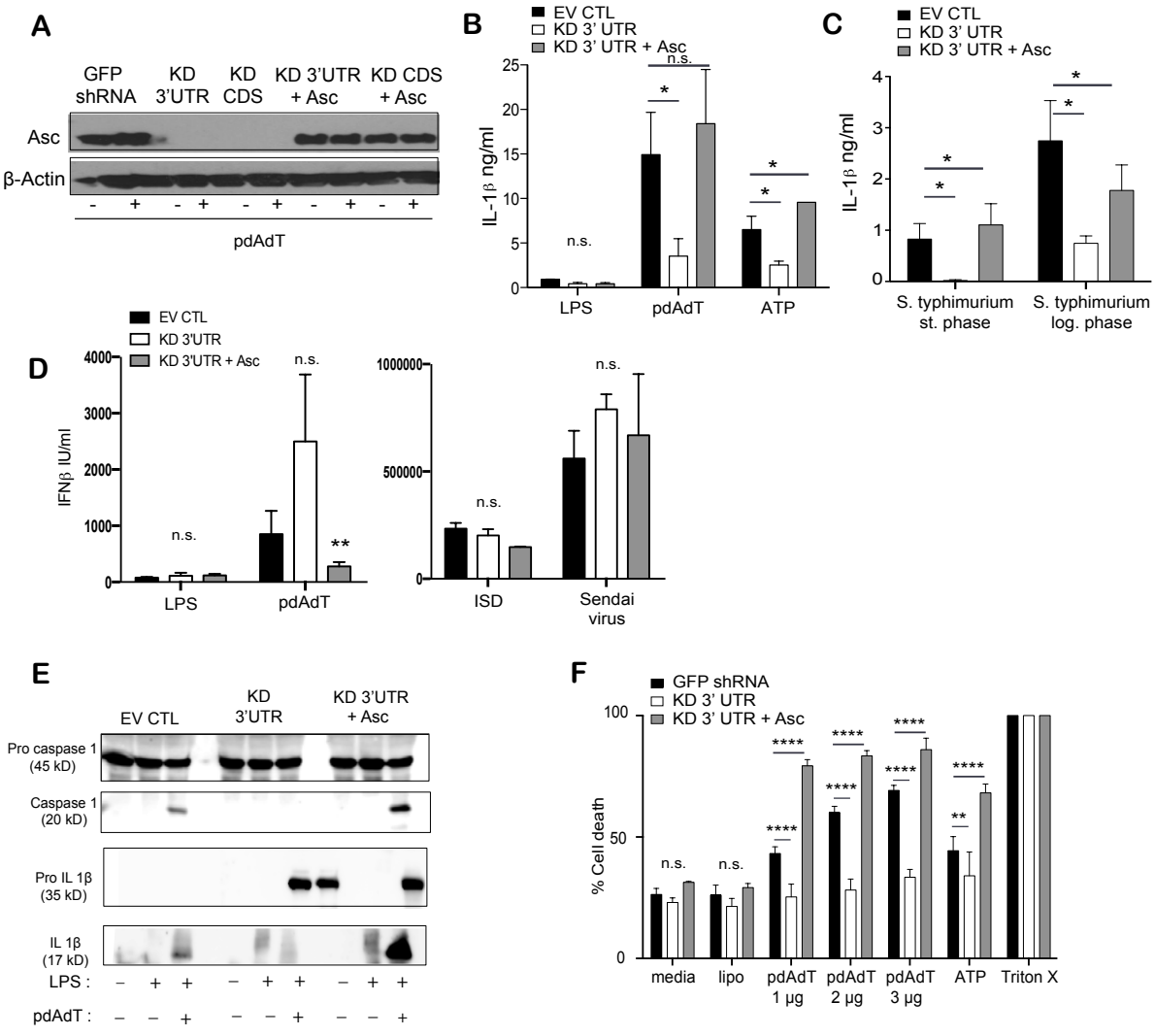

Figure 2.6: p205 controls inflammasome activation through its adapter molecule, Asc. (A) Western blot analysis of Asc overexpression in the p205 knockdown BMDM either left untreated or treated with LPS treated (200 ng/ml for 3h). Asc reconstituted cell lines tested for IL-1β production (B) on stimulation with LPS (200 ng/ml for 3h) and pdAdT (1 μg/ml for 6h) or ATP (5 μM for 1h) and (C) with overnight culture (stationary phase) or log phase culture of *Salmonella typhimurium* by ELISA (D) IFN-β levels were tested by ELISA in the Asc-reconstituted macrophages stimulated with LPS (200ng/ml for 3h), transfected pdAdT (1 μg/ml for 6h) or ISD (3μM for 6h), or Sendai virus (overnight) (E) Levels of caspase 1 and IL-1β processing in the Asc reconstituted cells were detected by Western Blot and (F) cell death was measured by amounts of LDH released with LPS (200 ng/ml for 3h) and pdAdT (1 μg, 2 μg, or 3 μg per ml for 6h) and/or ATP (5 μM for 1h) stimulation.

Restoring p205 expression rescues Asc expression and inflammasome activation

We next strived to restore the expression of p205 in the knockdown cells. p205 cDNA with a hemagglutinin (HA) tag at the C-terminal was cloned into pRZ retroviral vector. Viral particles were generated and stably transduced into p205 knockdown macrophages containing shRNA targeting the 3'UTR of p205 transcripts. The transduced cells were selected with zeocin (200 ng/ml). The p205 gene expression was first measured by qPCR (**Figure 2.7A**). Reconstitution of p205 restored Asc mRNA expression in these knockdown cell lines (**Figure 2.7B**). p205 protein expression was detected using an anti-HA antibody. Cell lysates from GFP shRNA, p205 KD 3'UTR cell lines along with the p205 KD 3'UTR cell line reconstituted with p205-HA were separated into nuclear and cytoplasmic fractions. The reconstituted HA-tagged p205 was found to be primarily nuclear. Level of Asc protein in both nucleus and cytoplasm was increased in cells expressing p205-HA (**Figure 2.7C**). Analysis of IL-1β release by ELISA in these macrophages expressing p205-HA further demonstrated that reconstitution of p205 restored inflammasome dependent responses (**Figure 2.7E and F**). Collectively these findings provide further evidence that p205 behaves as a regulator controlling Asc expression and inflammasome responses.

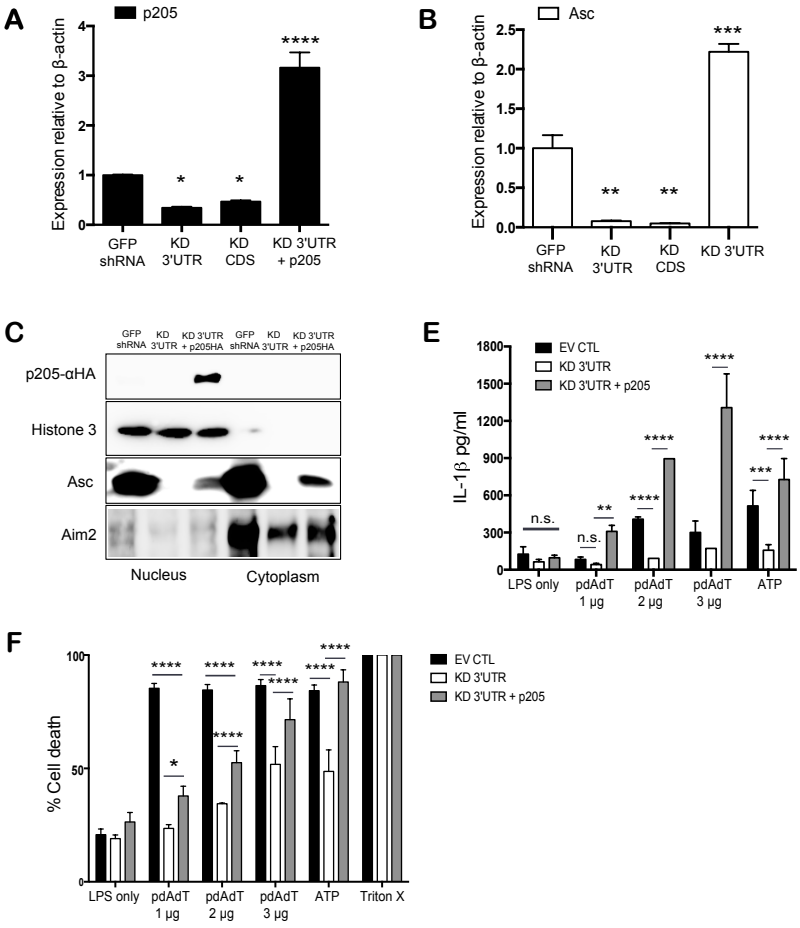

Figure 2.7: Reconstitution with p205 rescues Asc expression and inflammasome activation. (A) p205 and (B) Asc mRNA expression relative to β-actin and normalized to GFP shRNA BMDM were measured in BMDMs reconstituted with p205. (C) Western blot analysis of nuclear and cytoplasmic fractions of p205 reconstituted cell lines to detect p205-HA, Histone H3, Asc and Aim2. (E) IL1β production and (F) cell death with LPS (200ng/ml for 3h) and pdAdT (1 μg, 2 μg, or 3μg per ml for 6h) or ATP (5 μM for 1h) by ELISA and by measuring amount of LDH released respectively.

p205 overexpression enhances Asc expression in macrophages but the reverse is not true

The HA-tagged p205 was ectopically expressed in the wild type immortalized BMDM (**Figure 2.8A**) resulted in the increase of Asc mRNA levels compared to that found in the control cell lines (**Figure 2.8B**). However, the ectopic overexpression of Asc in the p205 knockdown macrophages had no obvious effect on the reduced p205 transcript levels. Furthermore, stable overexpression of p205-HA in wild type macrophages showed a concomitant increase of Asc mRNA (**Figure 2.8C**).

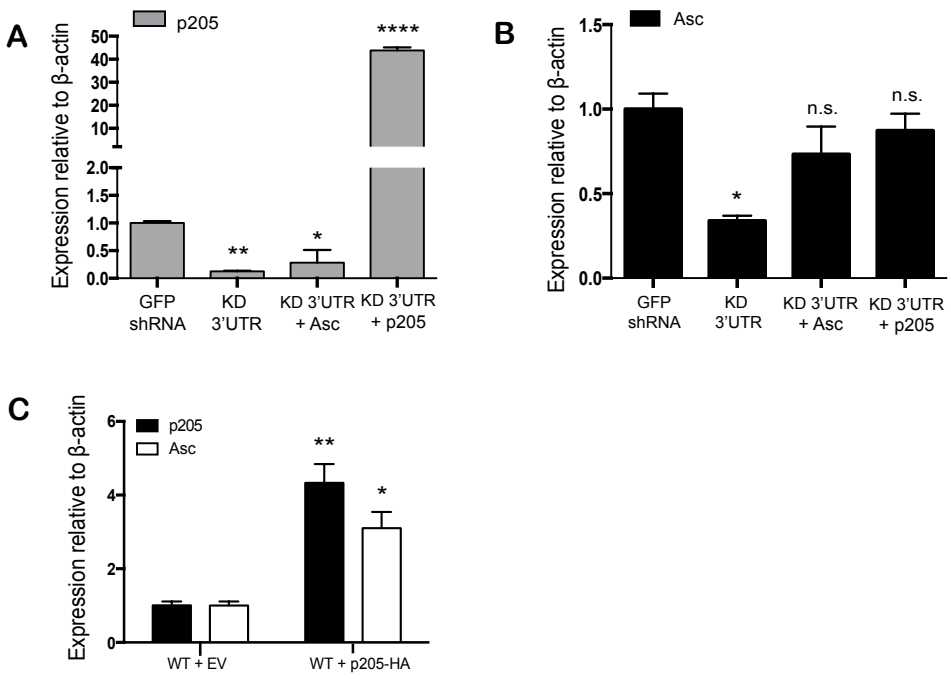

Figure 2.8: p205 overexpression enhances Asc expression but the reverse is not true. Expression of (A) p205 and (B) Asc mRNA in Asc- and p205-reconstituted BMDM relative to β-actin and normalized to GFP shRNA. (C) p205 and Asc mRNA levels by qPCR (relative to β-actin; normalized to WT + EV control) in p205-overexpressing BMDM.

Knockdown of p205 protects the macrophages from pyroptotic cell death

Activation of inflammasome also leads to an inflammatory cell death called pyroptosis that can be monitored by measuring the amount of lactate dehydrogenase enzyme (LDH) released. Transfection of pdAdT and stimulation with ATP in macrophages leads to Aim2 and Nlrp3 inflammasome dependent release of LDH respectively. Consistent with the other effects on inflammasome function, the release of LDH, hence, cell death was also significantly affected in macrophages lacking p205. Restoration of Asc in the p205 knockdown cell lines rendered these cells susceptible to more cell death compared to the levels seen in the control cell line (**Figure 2.6F**). Similarly, more cell death was observed upon reconstitution with p205 in the knockdown cells stimulated with LPS and transfected pdAdT or ATP (**Figure 2.7F**). Hence, loss of p205 diminishes the inflammatory responses and protects from inflammasome-dependent pyroptotic cell death.

Loss of p205 in macrophages results in reduced recruitment of actively transcribing RNA Polymerase II to the endogenous Asc gene

The reduced mRNA levels of Asc in cells lacking p205 could be due to either effects on *Asc* gene transcription or Asc mRNA stability. To further explore how p205 affected Asc mRNA levels we performed chromatin immunoprecipitation assays to explore the binding of RNA Polymerase II (RNA Pol II) to the endogenous *Asc* promoter, as a marker for active transcription, in both the GFP shRNA and p205 KD BMDM. **Figure 2.9A** shows a schematic of the *Asc* gene locus and the location of the primers used to evaluate RNA Pol II binding. Using antibodies to RNA Pol II and an isotype

control, IgG we evaluated binding of RNA Pol II to the endogenous *Asc* gene. RNA Pol II binding was considerably lower in the p205 KD cell lines using a series of primers as indicated (**Figure 2.9B**).

We further analyzed the functional status of RNA Pol II using antibodies to RNA Pol II phosphorylated either at position Serine 5 (Serine-5P RNA Pol II) or at Serine 2 (Serine-2P RNA Pol II) in its C-terminal domain. The RNA Pol II carboxy-terminal domain (called CTD or CT7n) is responsible for controlling the transcriptional activity of the polymerase. The CTD contains multiple repeats of a heptapeptide sequence, YSPTSPS (e.g. 26 in yeast, 44 in *Drosophila melanogaster* and 52 in mammals) rich in hydroxyl groups that are differentially phosphorylated and essential for its function. When preferentially phosphorylated at Serine 5 alone, there is only abortive transcription where the RNA Pol II can initiate, but not elongate further or process functional mRNA. The change from an initiating to an actively elongating or transcribing RNA Pol II occurs with the increased phosphorylation of the Serine 2 residue on the CTD repeats. Consistent with the findings with antibodies to total RNA Pol II, both Serine-5P RNA Pol II (**Figure 2.9C**) and Serine-2P RNA Pol II (**Figure 2.9D**) distributions on the *Asc* gene were considerably lower in the p205-shRNA cells compared to the GFP shRNA BMDM.

As controls, the recruitment of total RNA Pol II, Serine-5P RNA Pol II and Serine-2P RNA Pol II to the housekeeping gene, *Gapdh*, was measured and the occupancy levels were unchanged between the GFP shRNA and p205 KD macrophages (**Figure 2.9E**).

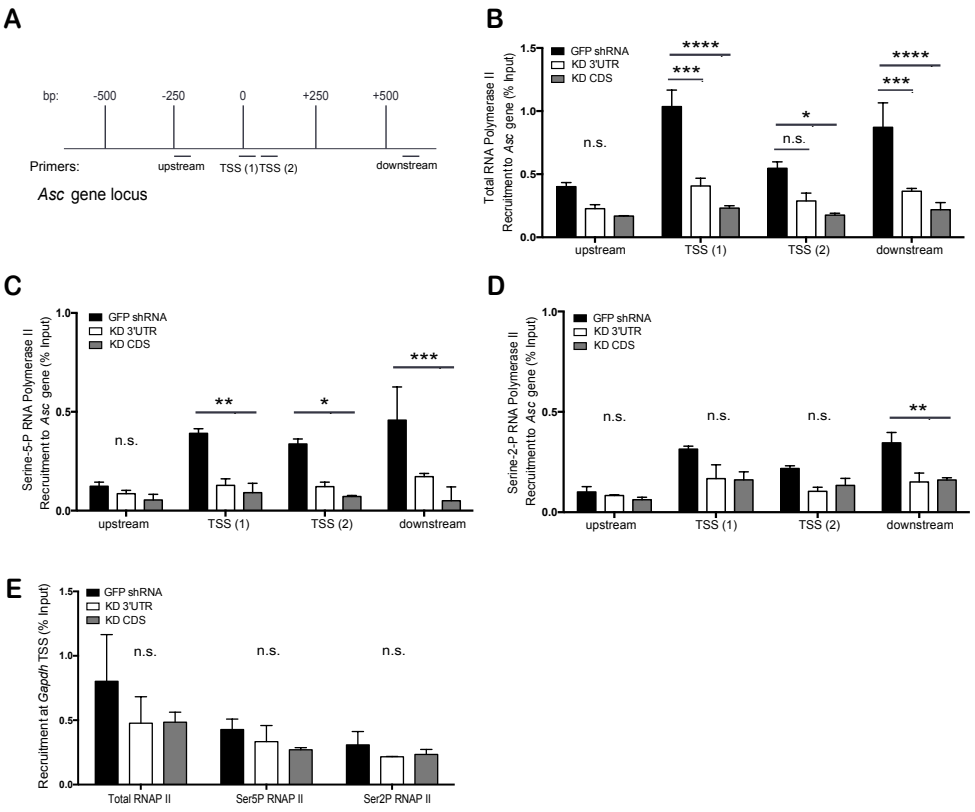

Figure 2.9: Loss of p205 affects transcription from the endogenous *Asc* gene in macrophages. (A) Schematic of the Asc gene locus and Chromatin-IP primers locations on the gene. (B) Recruitment of total RNA Pol II to endogenous Asc gene in GFP shRNA CTL, p205 KD 3'UTR or KD CDS macrophages. Recruitment of (C) Serine-5-P RNA Pol II and (D) Serine-2-P RNA Pol II on endogenous Asc gene. (E) Recruitment of total, Serine-5-P or Serine-2-P RNA Pol II to the Gapdh transcription start site. All values are represented as percent fraction of total input DNA. Data was calculated against the IgG isotype control and is representative of three independent experiments.

p205 knockdown impacts transcription as well as the mRNA processing of Asc mRNA

However, the decreased occupancy of RNA Pol II at the endogenous *Asc* locus in the p205 KD macrophages was less severely affected than what the Asc mRNA levels suggested. Compared to the near ablation of Asc mRNA expression observed in the absence of p205, there was still some RNA Pol II being recruited to the *Asc* gene. Recent studies have shown that RNA Pol II is also a major player in successful mRNA processing and splicing. Serine-2 phosphorylated RNA Pol II is responsible for mRNA elongation, recruitment of spliceosome and mRNA processing. Transcription and splicing are dependent on each other- a phenomenon termed co-transcriptional splicing. Hence, to understand if p205 was regulating transcription as well as mRNA processing of the *Asc* transcript, we measured the levels of Asc pre-mRNA as well as that of the processed and mature mRNA in macrophages with or without p205 (**Figure 2.10**). By designing primers that specifically measure either pre-mRNA or mature mRNA (Table), we observed that though there was some leaky transcription going on in the BMDM lacking p205, there was minimal processing of the Asc pre-mRNA into its mature form. We also tested for the GAPDH pre-mRNA and mature mRNA status and observed no difference between the knockdown and control macrophages. Thus, we inferred that p205 controls Asc expression both at the level of gene transcription as well as processing of mRNA.

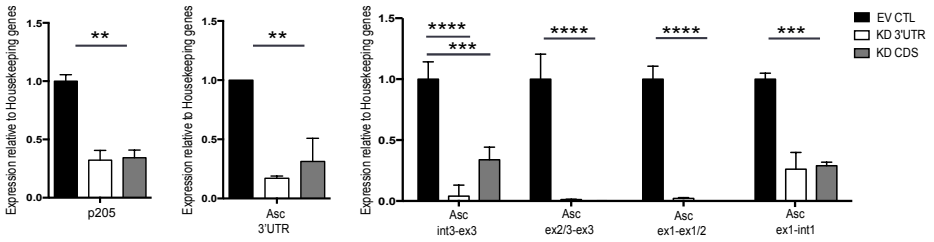

Figure 2.10: Knockdown of p205 affects transcription and processing of Asc mRNA. qRT-PCR analysis of p205, nascent or mature Asc mRNA expression (relative to the housekeeping genes, *Hprt* and *Gapdh*, and normalized to EV CTL BMDM) in p205 knockdown macrophages.

CRISPR/Cas9-mediated knockout of p205 affects Asc expression

In addition to shRNA–based loss-of-function approach, we strived to define the influence of p205 on *Asc* gene expression using an independent strategy and in a different cell-type that expresses p205. We carried out CRISPR/Cas9 mediated knockout of p205 in B16 melanoma cell line derived from C57BL/6 mice. Plasmids expressing Cas9 and the sgRNA targeting specific regions of p205 were transfected into B16 melanoma cells to generate p205 knockout (p205 KO) cells. A clone of these cells was produced and evaluated for p205 deficiency using sequencing, qPCR and Western blot, and similar clone without p205 knockout was used as a control (p205 WT). No induction of p205 mRNA was observed in p205 KO after overnight stimulation with IFN-γ compared to the WT (**Figure 2.11A**). Additionally, unlike the WT cells, there was no detectable p205 protein expression in the KO cells when stimulated with Sendai virus **(Figure 2.11B)**.

We tested these p205 KO cell lines for Asc expression and found that the B16 melanoma lines lacking p205 had a similar loss of mature Asc mRNA (**Figure 2.11C**) and protein (**Figure 2.11D**). These observations further support our shRNA-based studies and emphasize upon the importance of p205 as a regulator of *Asc* gene expression.

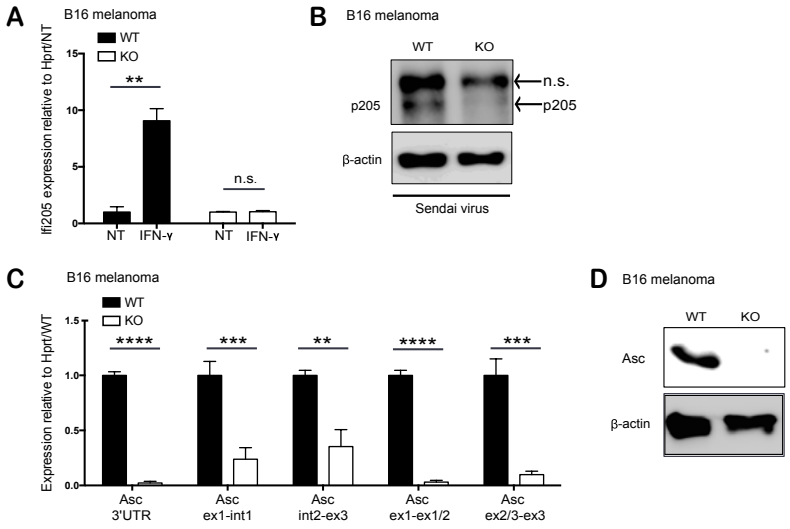

Figure 2.11: CRIPSR/Cas9-mediated knockout of p205 affects Asc expression. (A) Expression of p205 mRNA in IFNγ-stimulated wild type (WT) and p205 knockout (KO) B16 melanoma cell lines relative to Hprt and normalized to non-treated (NT). (B) p205 and β-actin protein expression in WT and p205 KO cell lines stimulated with Sendai virus (n.s.-non-specific band) (C) Mature mRNA and pre-mRNA profile of Asc expression (relative to Hprt and normalized to WT) and (D) Western blot analysis of Asc and β-actin in WT and p205 KO B16 melanoma cell lines.

p205 drives expression from an *Asc* gene-luciferase reporter in a dose-dependent manner

To understand the mechanisms involved in the transcription of *Asc,* we cloned the *Asc* promoter region (-2000 to +10 *Asc* gene) as a reporter gene upstream of a firefly luciferase gene. This *Asc* gene-reporter construct was transfected into HEK 293T cells together with increasing concentrations of p205. Ectopic expression of p205 led to a dose dependent increase in the *Asc*-luciferase reporter gene activity. This effect was specific to p205, as transfection of two related DNA-binding PYHIN proteins p204 and Aim2 had no effect on reporter expression (**Figure 2.12A**). Further to determine the roles of the specific domains of p205 on reporter gene expression, we generated deletion mutants of p205 that either lacked the pyrin domain (ΔPYD), the HIN domain (ΔHIN) or both (intermediate region only; ΔH/ΔP). We tested the deletion mutants for their ability to drive the *Asc* gene reporter but the mutants lacking either the HIN or pyrin domain failed to induce any significant expression from the reporter (**Figure 2.12B**) suggesting the importance of both the DNA-binding HIN domain and protein-binding pyrin domain of p205 in controlling transcription.

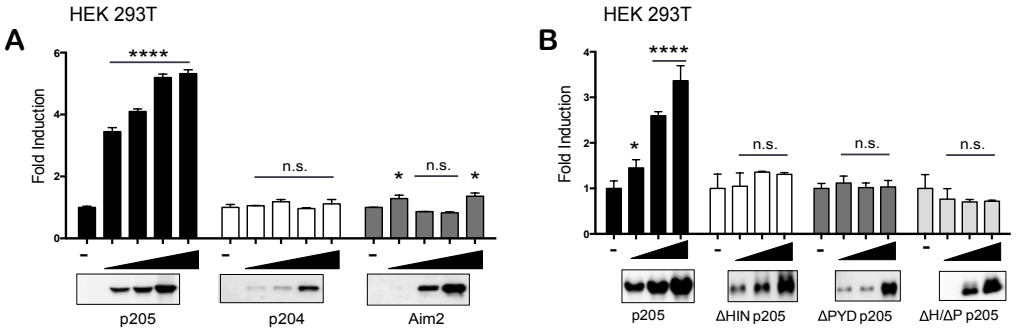

Figure 2.12: Full-length p205 protein drives expression from *Asc* gene-luciferase reporter. HEK 293T cells were transfected with a mixture containing 10 ng of TK-Renilla luciferase with 1 ng of *Asc* promoter firefly luciferase reporter and increasing amounts of (A) p205-HA, p204-HA or Aim2-FLAG, or (B) with full length p205 or p205 deletion mutants as indicated. Corresponding immunoblots show the expression of the respective constructs in the HEK 293T cells. All luciferase values were measured and normalized to Renilla values. Values are displayed as fold change over the *Asc* reporter construct alone. Data is representative of three independent experiments.

p205 synergizes with other transcription factors to enhance expression from the *Asc* gene reporter

Bioinformatics analysis of the *Asc* promoter region for response elements of known transcription factors revealed binding sites for c/EBPβ and NF-κB amongst others. Previously, Parsons et al has shown that TNFα treatment could enhance Asc expression through p65/RelA in MCF7 cell lines, though the luciferase reporter assay results in the study were not conclusive. Furthermore, p205 has been shown to co-localize and bind directly with the CCAAT/enhancer binding protein-β (c/EBPβ) in mouse adipose-derived stem cells. Also, c/EBPβ has also been implicated in the induction of several proinflammatory genes in macrophages. Therefore we tested the effect of ectopic expression of c/EBPβ or p65/RelA on *Asc* reporter gene expression. In both cases these inducible transcription factors showed modest increase in *Asc* luciferase reporter activity in a dose dependent manner (**Figure 2.13 left panel**). In addition, co-expressing p205 together with either c/EBPβ or p65/RelA further enhanced *Asc* reporter gene expression (**Figure 2.13 middle panel**). However, when minimal concentrations of c/EBPβ and p65/RelA were transfected together with increasing concentrations of p205, we observed a strong, synergistic increase of activity from the *Asc* gene-luciferase reporter (**Figure 2.13 right panel**).

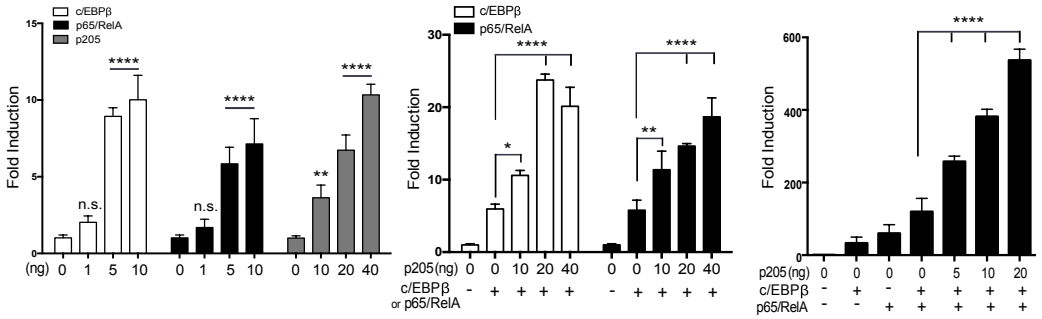

Figure 2.13: p205 synergizes with c/EBPβ and p65/RelA to drive *Asc* gene luciferase reporter activity. Transfection of increasing concentrations of c/EBPβ, p65/RelA and p205 alone or, co-transfection of increasing concentrations of p205-HA with either c/EBPβ or p65/RelA with *Asc* promoter-reporter or, transfection of the *Asc* promoter-reporter with increasing concentrations of p205 with both c/EBPβ and p65/RelA, as indicated.
All luciferase values were measured and normalized to Renilla values. Values are displayed as fold change over the Asc reporter construct alone. Data is representative of three independent experiments.

p205 interacts with c/EBPβ in HEK 293T and macrophages

In addition to c/EBPβ, p65/RelA and p205 synergistically activating expression from the *Asc* gene reporter in HEK 293T, we wanted to test whether proteins interact in a complex. We transiently transfected HA-tagged p205 and c/EBPβ in HEK 293T and observed that p205 specifically interacted with overexpressed c/EBPβ (**Figure 2.14A**). To test whether p205 and c/EBPβ interacted endogenously as well, we co-immunoprecipitated the proteins in resting and LPS-stimulated (200ng/ml for 3h or 6h) wild-type macrophages. p205 could successfully pull down c/EBPβ, and vice versa, in both unstimulated and LPS-induced macrophages, as was observed by co-immunoprecipitation assays (**Figure 2.14B**). Together these observations imply that p205 can increase *Asc* gene expression, and can collaborate with c/EBPβ or p65/RelA as well to do so.

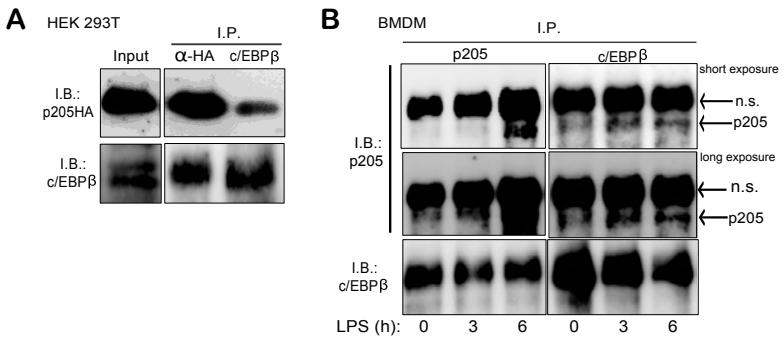

Figure 2.14: p205 interacts with c/EBPβ in HEK 293T and BMDM.
(A) Co-immunoprecipitation and immunoblot of overexpressed HA-tagged p205 and c/EBPβ in HEK 293T cells using anti-HA and anti-c/EBPβ antibodies.
(B) Co-immunoprecipitation and Western blot of endogenous p205 and c/EBPβ in LPS-stimulated BMDM using antibodies against p205 and c/EBPβ. Data is representative of two independent experiments (n.s.- non-specific band).

p205 and other transcription factors co-operate to drive Asc gene reporter activity

Bioinformatic analysis also displayed sites for transcription factors, c-Rel and p53 in the *Asc* gene promoter. Transfection of c-Rel and p53 alone showed modest increase from the *Asc* gene reporter (**Figure 2.15A**). Co-transfection of c-Rel and p53 with increasing concentrations of p205 with the reporter, compared to transfected p205 alone showed an additive effect (**Figure 2.15B**). Further, co-transfection of p205 with c/EBPβ and c-Rel did not show a synergy in the activation of the *Asc* gene reporter suggesting that c-Rel dependent activation occurs non-competitively and independently of p205 and c/EBPβ-mediated transcription of *Asc* (**Figure 2.15C**).

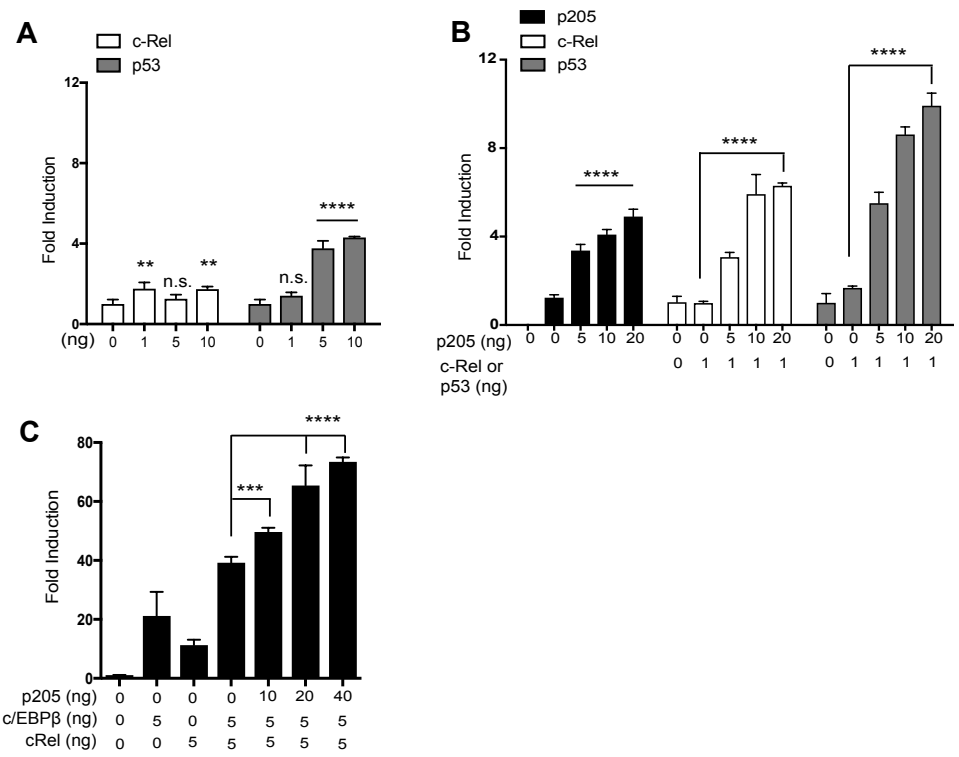

Figure 2.15: p205 co-operates with cRel and p53 to drive *Asc* gene reporter activity. (A) Transfection of increasing concentrations of cRel and p53 alone with *Asc* gene reporter (B) Transfection of p205 alone or co-transfection of increasing concentrations of p205 with either cRel and p53 with *Asc* gene-reporter, as indicated. (C) Transfection of increasing concentrations of p205 alone with fixed amount of c/EBPβ and c-Rel with *Asc* gene reporter. All luciferase values were measured and normalized to Renilla values. Values are displayed as fold change over the Asc reporter construct alone. Data is representative of two independent experiments.

Shorter mutants of *Asc* gene-luciferase reporter are not inducible by p205

We cloned shorter promoter-reporter constructs containing only 500 bp, 800 bp, 1000 bp or 1500 bp upstream of the transcription start site of the *Asc* gene and found that the p205-dependent increase in reporter gene expression was maximal in *Asc* gene construct that contained promoter sequence 2000 bp upstream, and reporter activity lost in constructs that were less than 1500 bp upstream of the transcription start site (TSS) (**Figure 2.16A**). We cloned the sequence between -2000 to -1500 *Asc* gene in the luciferase reporter to test whether that region was necessary and sufficient to drive Asc gene reporter activity, but p205 was unable to drive reporter activity from the construct (**Figure 2.16B**) indicating that the cis and distal regions of the *Asc* promoter region that is necessary for driving expression.

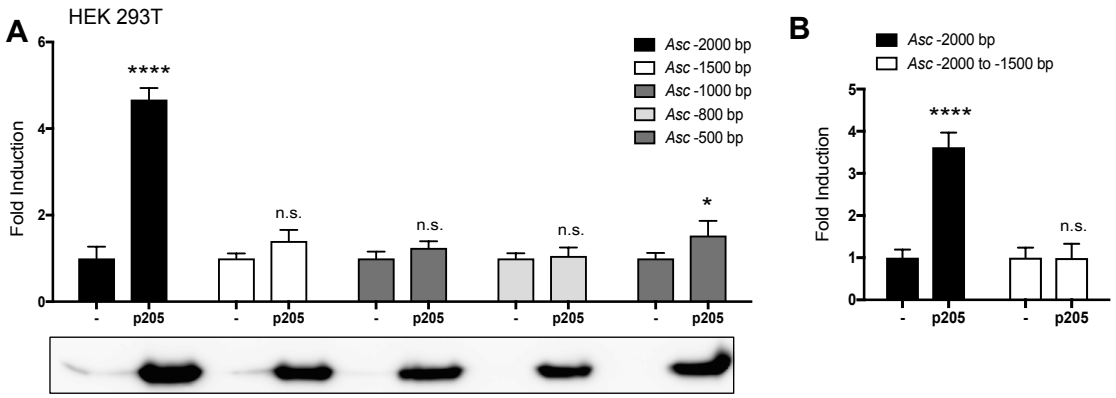

Figure 2.16: Shorter *Asc* gene-luciferase reporters are not inducible by p205. (A) HEK 293T cells were transfected with a mixture containing 10 ng of TK-Renilla luciferase with 1 ng of *Asc* promoter firefly luciferase reporter constructs (2000 bp, 1500 bp, 1000 bp, 800 bp and 500 bp upstream of the transcription start site) alone or with 25 ng of p205. (B) An *Asc* gene reporter construct with sequence -2000 to -1500 bp upstream was transfected similarly in HEK 293T cells with TK-Renilla and 25 ng p205. The immunoblot show p205 expression in the HEK 293T cells. All luciferase values were measured and normalized to Renilla values. Values are shown as fold change over the *Asc* reporter constructs alone. Data is representative of three independent experiments.

p205 drives expression from a NF-κB luciferase reporter as well

The effect of p205 was also studied on p125 IFNβ-luciferase reporter as well as a NF-κB luciferase reporter. p205 had no effect on IFN promoter construct but it showed substantial increased activity from the NF-κB reporter that consists of 5 tandem NF-κB sites as well as from an IL-8 reporter indicating that p205 could drive expression of other NF-κB genes in HEK 293T cells (**Figure 2.17A**). Induction of the NF- κB, IL-8, PRDIII and IFNβ-luciferase reporters were tested with transfected p65/RelA or mSTING as positive controls (**Figure 2.17B**). Following the specific effect of p205 on the NF-κB reporter, the loss of induction by p205 on shorter Asc reporter constructs and previous studies showing that 1200+ bp of *Asc* promoter was insufficient for induction, and that p65/RelA can interact with p205 and other pyrin-containing proteins, we concluded that p205 might be interacting with other factors on the NF-κB sites on the *Asc* promoter to affect gene expression.

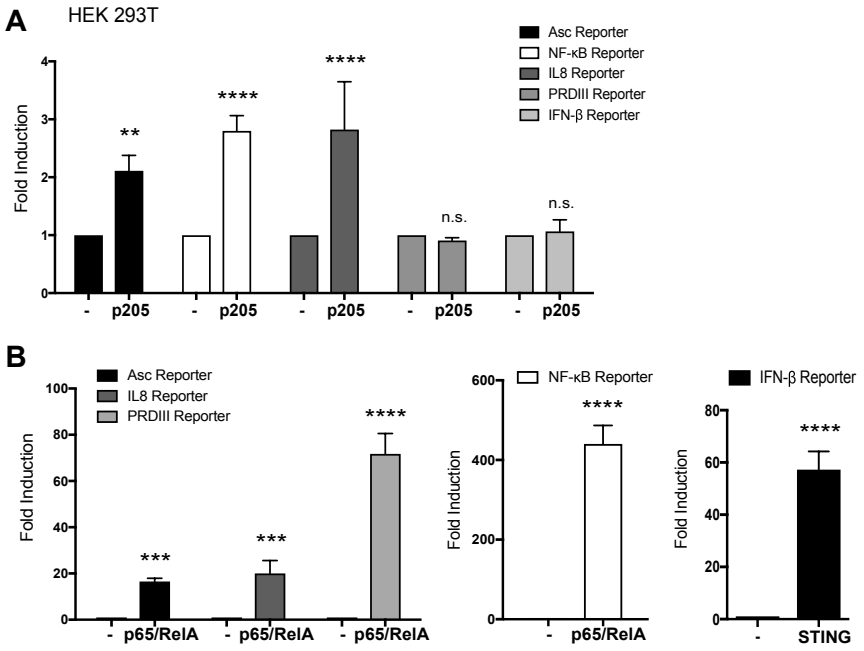

Figure 2.17: p205 drives expression from NF-κB luciferase reporter.
(A) HEK 293T cells were transfected with a mixture containing 10 ng of TK-Renilla luciferase and 25 ng of p205 with 1 ng of *Asc* gene reporter, 5 ng of NF-κB reporter, IL-8 reporter, PRDIII reporter and IFN-β reporter (B) The *Asc* gene reporter, NF-κB reporter, IL-8 reporter, PRDIII reporter and IFN-β reporter were transfected similarly in HEK 293T cells with TK-Renilla and 25 ng p62/RelA or STING. cells. All luciferase values were measured and normalized to Renilla values. Values are shown as fold change over the *Asc* reporter constructs alone. Data is representative of two independent experiments.

p205 regulates expression of other immune genes in LPS-stimulated macrophages

We wanted to determine the impact of p205 on the global expression of immune genes in resting and LPS-stimulated bone marrow derived macrophages. We collected RNA from either untreated or LPS-treated control and p205 knockdown BMDM for multiplex gene expression analysis (nCounter, NanoString) that included a selected panel of immune genes. Each immune gene in the NanoString panel is represented by fluorescently labeled probes that hybridize directly to the target mRNA, and gives a highly sensitive count of mRNA. We observed changes in multiples genes, especially in the LPS stimulated macrophages. Genes such as *MyD88*, *Aim2*, *Il18*, *Irf3*, *Irf5*, *Stat1*, *Stat3* showed no change between the control and the p205 knockdown lines. We selected a subset of genes that exhibited a considerable decrease in both the knockdown cell lines. As expected, we observed a decrease in the *Asc* gene expression in both the resting and LPS-stimulated macrophages. Other immune genes that showed a strong decrease in expression in the LPS-stimulated p205 knockdown macrophages were *Ccl4*, *Ccl5*, *Cd86*, *Cox2*, *Cxcl2*, *Il10*, *Il1α*, *Il12α*, *Il6 and Ifnα* as represented by the heatmap (**Figure 2.18**).

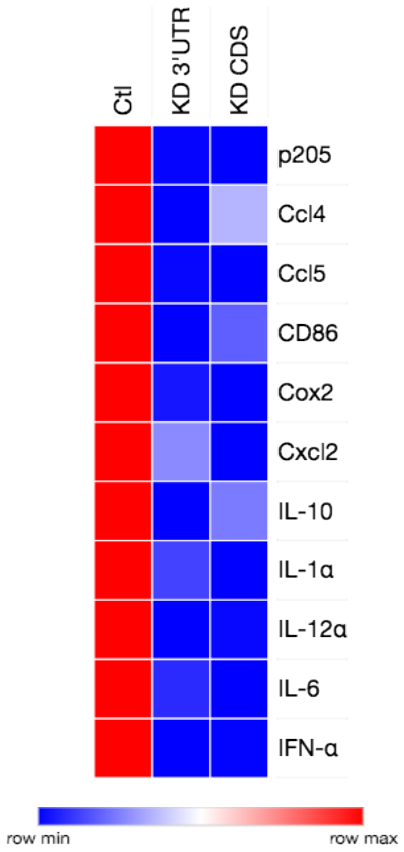

Figure 2.18: p205 affects expressions of other immune genes in LPS-stimulated macrophages. Heatmap of Nanostring analysis of immune genes expression in p205 knockdown macrophages stimulated with LPS (200 ng/ml for 3h).

We further inspected the levels of IL-1α and IL-6 mRNA in p205 knockdown macrophages. Since IL-1α, IL-6 are not expressed in resting state, we stimulated the macrophages with LPS for different times (0, 3, 6, 12 h) to observe if loss of p205 changed its expression over time. We observed that the induction of IL-1α and IL-6 mRNA was much lower in the knockdown cells with LPS stimulation, corresponding to the low p205 expression (**Figure 2.19A**). As expected, there was no Asc expression in LPS-stimulated p205 knockdown macrophages. Parallel to Asc mRNA expression in p205-deficient cells, we designed primers to distinguish between the levels of IL-1α and IL-6 pre-mRNA and mature mRNA in these cells (see Table 1). Both IL-6 and IL-1α pre-mRNA and mature mRNA levels were affected in p205 knockdown macrophages treated with LPS for 6 hours. Interestingly, the mRNA profile of IL-1α in LPS-stimulated macrophages mirrored that of Asc. The mature form of IL-1α mRNA was negligible whereas a low amount of its pre-mRNA was present in the macrophages lacking p205 (**Figure 2.19B**). Hence, p205 regulates immune responses by controlling transcriptional activation of other immune genes such as *Il1α*, *Il6*.

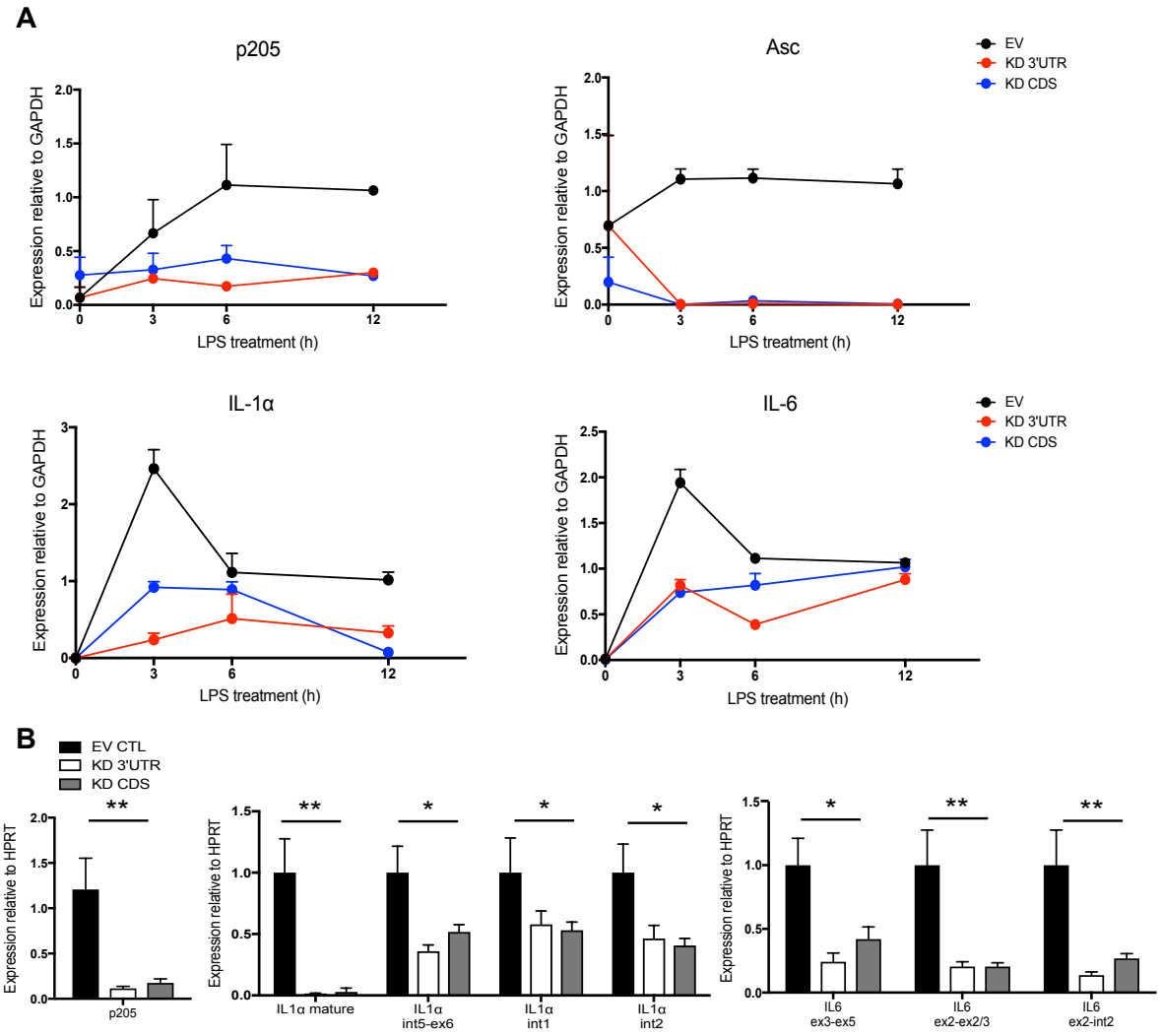

Figure 2.19: Knockdown of p205 affects expression of other inflamamtory genes. (A) qRT-PCR analysis of p205, Asc, IL-1α and IL-6 mRNA in p205 knockdown macrophages with LPS stimulation (200ng/ml) for 0, 3, 6, 12 hours. (B) Expression of p205 and nascent or mature L-1α and IL-6 mRNA in p205 knockdown macrophages (relative to the housekeeping genes, *Hprt* or *Gapdh*, and normalized to EV CTL BMDM)

NLRP3 and NLRC4 inflammasomes are partially compromised in *ALR*$^{-/-}$ mice

Stetson and lab generated an *ALR*$^{-/-}$ mouse that lacks the PYHIN/ALR locus on chromosome 1 containing the 13 PYHIN genes. Deletion of all the ALR/PYHIN genes indicates that it also lacks Aim2, and thus *ALR*$^{-/-}$ cells are unresponsive to triggers for the Aim2 inflammasomes. Since *ALR*$^{-/-}$ cells would also be lacking *p205* among other PYHIN genes, we wanted to test for activation of other inflammasomes such as NLRP3 and NLRC4 (**Figure 2.20A and B**). As expected, IL-1β production upon pdAdT transfection in both primary BMDM and BMDC was absent. Interestingly, NLRP3-dependent IL-1β secretion was partially impaired in *ALR*$^{-/-}$ BMDM and dendritic cells upon stimulation with Nigericin or ATP. Infection with *Salmonella typhimurium* in stationary phase (overnight culture) as well as log phase (4-6 h culture) also showed impaired IL-1β secretion. Hence, in these cells, there's a partial phenotype of p205 deficiency on IL-1β activation, but there's the possibility than the PYHIN proteins may cross regulate each other's functions and often, the PYHIN proteins have been observed to differentially regulate similar pathways.

Further, upon observation of the defect in IL-1β secretion in the *ALR*$^{-/-}$ primary BMDM and BMDC, we tested the expression of Asc in the cells. We generated *ALR*$^{-/-}$ immortalized macrophages that showed no p205 protein expression (**Figure 2.20C left panel**). Consistently, compared to wild-type immortalized macrophages, there was a marked reduction of Asc protein expression in these macrophages at basal level as well as after LPS stimulation (**Figure 2.20C right panel**). Hence, p205 is important for maintaining Asc expression in the cells, though some cross-regulation by the other PYHIN proteins (lacking as well in *ALR*$^{-/-}$ cells) may also be involved.

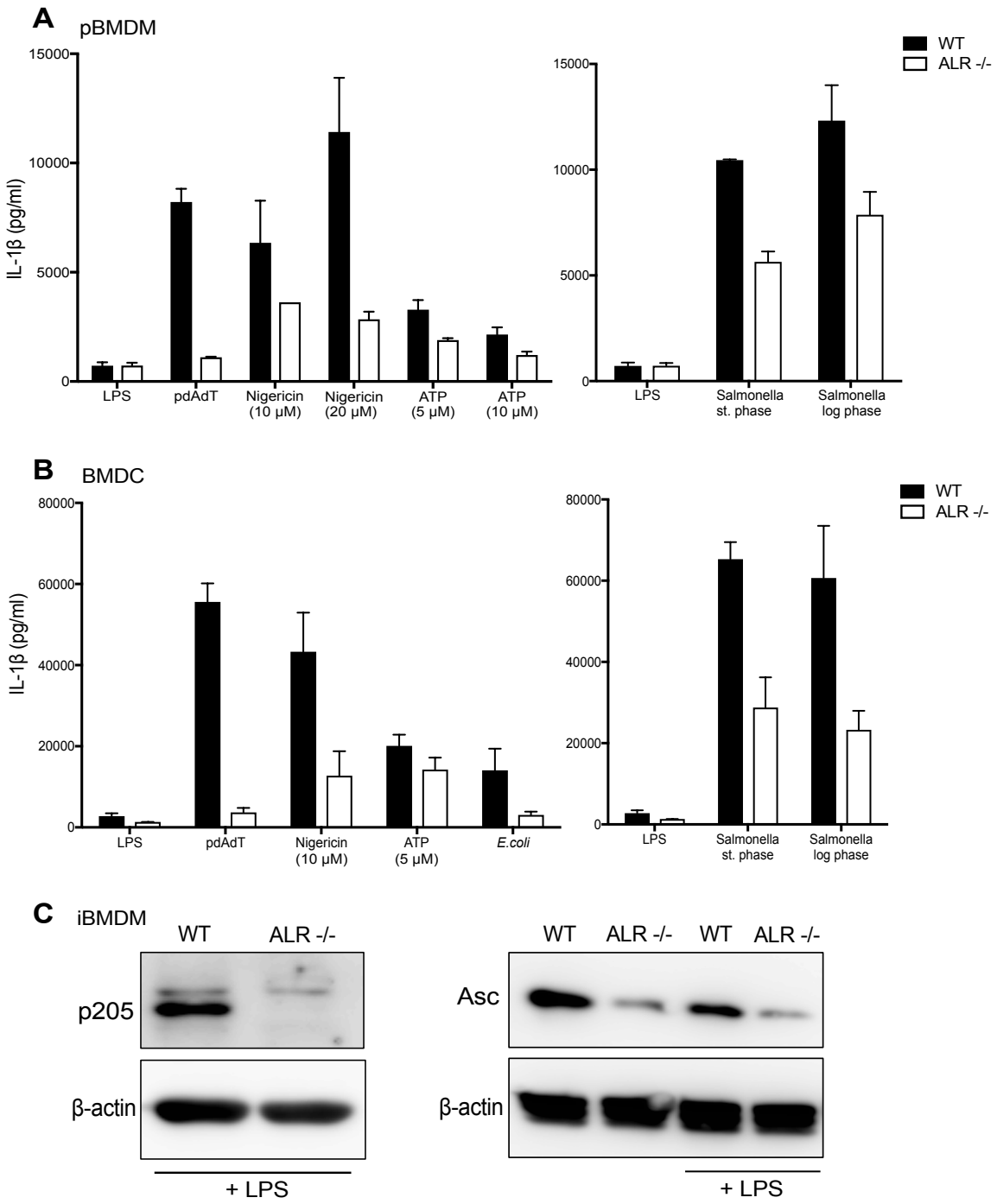

Figure 2.20: NLRP3 and NLRC4 inflammasomes are partially compromised in ALR-/- mice. IL-1β production in LPS-induced (200 ng/ml) ALR knockout (A) primary BMDM and (B) BMDC in response to pdAdT transfection (1 μg for 6h), stimulation with Nigercin (10 or 20 μM for 1h), ATP (5 or 10 μM for 1h) or infection with *E.coli* or *Salmonella typhimurium* (in stationary phase or log phase) by ELISA (C) p205, Asc and β-actin protein expression in immortalized wild-type (WT) and ALR knockout (ALR-/-) macrophages with or without LPS stimulation (200 ng/ml for 6h).

Discussion

Studies over the last decade have defined the importance of AIM2, a cytosolic DNA binding protein as a regulator of caspase-1 activity and proteolytic processing of IL-1β and IL18 (29). The role of the PYHINs in sensing dsDNA was further highlighted by the identification of IFI16 as a regulator of type I IFN gene transcription following HSV-1 infection (20).

Early studies had also linked members of the murine PYHIN family to type I IFN gene regulation. Knockdown of mouse Ifi203 and mouse p204 have been shown to dampen the IFN response to infection with multiple pathogens, including HSV-1, human immunodeficiency virus (HIV), murine leukemia virus (MLV), *Francisella tularensis*, and Mycobacterium tuberculosis (20, 241, 242). Despite these studies, a recent genetic study from Gray et al using mice lacking the entire PYHIN locus, lacking all 13 PYHIN genes, found that there was no change in the IFN signature when challenged with various immunostimulatory DNA ligands, DNA virus infection and lentivirus infection (176). This body of work indicates that the murine PYHIN proteins play limited roles in DNA ligand recognition at least in the myeloid cells and fibroblasts tested in these studies, raising the possibility that the PYHINs have alternative functions in these cells.

Prior to the discovery of AIM2 and IFI16 as sensors of microbial DNA, PYHIN proteins were shown to regulate cell growth, differentiation, tumor suppression and DNA damage responses (235). IFI16, IFIX, p202, and p204 regulate cell cycle transcription factors such as p53, p21, pRb, and E2F resulting in cell cycle arrest (178). p202 acts as a transcriptional repressor targeting NF-κB (179), AP-1 (180, 181), MYOD1 (182), and myogenin (181, 183). p204 also regulates gene expression during

monocyte/macrophage differentiation and osteoblast differentiation (243-245).

Here we expand upon these studies and define p205, a murine PYHIN protein as an additional regulator of gene expression in innate immunity. Using a series of loss of function approaches (both shRNA and CRISPR/Cas9 mediated gene editing), we report that p205 regulates expression of the inflammasome adapter protein Asc and in so doing controls inflammasome activation pathways broadly. Cells lacking p205 failed to activate caspase-1 and control inflammasome dependent processing of IL-1β in response to multiple ligands that engage the AIM2 inflammasome as well as the NLRP3 inflammasome. The abrogated inflammasome activation in the p205 KD macrophages upon pdAdT stimulation also likely explained the enhanced IFNβ levels detected in these cells, since prior work from our lab and others have shown that activation of the AIM2 inflammasome by intracellular DNA antagonizes the type I IFN pathway (171, 246). In cells with defects in inflammasome responses, there is a more robust dsDNA driven induction of type I IFNs.

By carefully measuring expression levels of key components of the Aim2 and Nlrp3 pathways, we found that cells lacking p205 had reduced expression of Asc. This effect was observed at both the protein and mRNA levels. The compromised inflammasome dependent responses observed in these cells could be fully rescued by ectopically expressing either p205 or Asc itself. Cells lacking p205 had reduced RNA Polymerase II binding to the *Asc* gene indicating that p205 functioned in part to control *Asc* gene transcription. In addition, by comparing the levels of the Asc pre-mRNA to those of the mature transcript, we could also observe additional effects on Asc mRNA processing. It is broadly accepted that the CTD of RNA Pol II is involved in efficient

transcription as well as mRNA processing (247, 248), and the Serine-2 phosphorylated polymerase determines the rate of mRNA elongation, spliceosome assembly and splicing efficiency (249). Thus we deduced from our ChIP experiments and qPCR analysis of Asc pre-mRNA and mature transcripts, that the absence of p205 not only affects *Asc* gene transcription but also affected the processing of the immature Asc mRNA.

Further, we show that in regulating gene expression, p205 cooperates with both c/EBPβ and p65/RelA to drive Asc expression. The transcription factor c/EBPβ is important in controlling macrophage differentiation. c/EBPβ remains fully active in resting macrophages and stays positioned on target genes, ready to stimulate transcription with other inducible transcription factors (250). p205 has previously been shown to interact with c/EBPβ in adipocytes. p205 interacts with c/EBPβ in unstimulated macrophages as well, and maintains basal expression of Asc, while under stimulated conditions, LPS-inducible p205 may interact with both c/EBPβ and activated NF-κB transcription factors to further enhance expression.

Previous studies have shown that Asc, originally identified as TMS1 (Target of methylation induced silencing-1) is influenced by DNA methylation. The *Asc* gene contains a 600-bp long CpG island located near the transcription start site and the methylation status of this CpG island correlates with the expression level of Asc/TMS1. The methylation status of the CpG island, as well as promoter proximal pausing of RNA Polymerase II in multiple cancer cell lines and tissues result in reduced expression of Asc (251-259). Our studies indicate that Asc levels are influenced by p205 in murine macrophages adding additional understanding to the regulation of this important immune gene. Our findings expand on our understanding of transcriptional regulatory roles for the

PYHIN proteins. p205 has previously been shown to control $p21^{CIP/WAF}$ gene expression via p53 in Saos2 cell lines and to impact gene expression in adipogenesis and osteogenesis via transcriptional mechanisms. p205 acts as a transactivator synergizing with p53 to induce expression of the cell cycle inhibitor p21 to inhibit cell growth. In adipocytes, p205 interacts with c/EBPβ and c/EBPα to further activate the transcriptional activity of c/EBPα and PPARγ. All of these studies help establish evidence for PYHIN proteins as regulators of gene expression. The discovery of p205 as a regulator of inflammasomes via transcriptional regulation of Asc further supports a role for the PYHIN proteins as regulators of gene expression and in addition emphasizes the importance of PYHIN family members in innate immunity.

Preface to Chapter III:

Most of the components of this chapter have appeared in the following manuscripts/ publications:

Natália Ketelut-Carneiro, Sreya Ghosh, Stuart M. Levitz, Katherine A. Fitzgerald[*] and João Santana da Silva[*]. *A Dectin-1-caspase-8 pathway licenses canonical caspase-1 inflammasome activation and IL-1β release in response to a pathogenic fungus.* Journal of Infectious Diseases, 2017 Nov 1; doi: 10.1093/infdis/jix568 (published ahead of print)

[*] These authors contributed equally to this work

- The project was conceptualized by N.K-C. and J.S.S., with intellectual contributions by S.G., K.A.F. and S.L.
- N.K-C. and S.G. designed and performed the experiments
- N.K-C., S.G., K.A.F. and J.S.S. wrote the manuscript

Chapter 3

Dectin-1/Syk signaling through caspase-8 promotes caspase-1/11-independent inflammasome activation upon *Paracoccidioides brasiliensis* infection

Abstract

Paracoccidioides brasiliensis is equipped with an arsenal of virulence factors that are crucial for causing infection. NLRP3 inflammasome has been defined as a mediator of *P. brasiliensis*-induced cell damage, and also in promoting an effective T_h1 immune response. However, the loss of caspase-1 only partially reduced IL-1β levels. Here we identify an additional pathway for IL-1β production in response to *P. brasiliensis* infection. By engaging dectin-1 and Syk signaling, *P. brasiliensis* initiated caspase-8-mediated IL-1β production, an event that was necessary and sufficient for transcriptional priming and posttranslational cleavage of pro-IL-1β. Caspase-8 subsequently synergizes with the canonical inflammasome pathway to control caspase-1 processing and caspase-1/11-independent IL-1β maturation, providing a regulatory role in host resistance to in vivo *P. brasiliensis* infection. Together these findings revealed a novel function for dectin-1 in innate immune response of host cells to *P. brasiliensis* infection, demonstrating a connected network between non-canonical caspase-8 and canonical caspase-1 inflammasomes to coordinate IL-1β production upon *P. brasiliensis* challenge.

Introduction

The systemic granulomatous disease, paracoccidioidomycosis (PCM), is initiated when airborne particles produced by the mycelial stage of *Paracoccidioides brasiliensis* are inhaled and converted into pathogenic yeast in the lungs due to an increase in temperature (260, 261). This morphological transition, crucial for the establishment of the infection, is accompanied by extensive structural changes in cell wall architecture and composition. Both forms have comparable contents of glucans (36‑47%), but differ in the type of glucan predominantly expressed (262). The polysaccharides shift (263, 264) has been proposed as a virulence factor during *P. brasiliensis* infection (265), as it presumably enhances the fungal survival in the mammalian host by modulating the β-glucan-triggered inflammatory response (266, 267).

β-1,3-glucans are recognized by dectin-1 (Clec7A), a C-type lectin receptor (CLR) that drives CARD9/Bcl-10/MALT1 scaffold assembly via Src/Syk- dependent signaling to activate the transcription factor NF-κB (268, 269). It was suggested that *P. brasiliensis*-dependent Syk kinase phosphorylation (270) culminates in pro-IL-1β production (102), and relies on the dectin-1 pathway (271). The expression, maturation and secretion of IL-1β are tightly controlled processes that require at least two signals: synthesis of the precursor pro-IL-1β through pattern-recognition receptor- mediated NF-κB activation (signal 1) and processing of the immature pro-IL-1β by caspase-1 or 11-dependent inflammasome complexes to generate mature, bioactive IL-1β (signal 2) (71, 272). However, besides dectin-1-induced IL-1β transcription, this CLR also functions as an extracellular sensor that directly activates caspase-8, which then cleaves pro-IL-1β

during *Candida albicans* infection (80, 273), emphasizing that diverse and complex molecular platforms can form inflammasomes. Caspase-8 cleaves the inactive form of IL-1β at the same site as recombinant caspase-1 producing similar mature IL-1β fragments (79) and similar to caspase-1, forms Asc (apoptosis-associated speck-like protein) puncta after *Salmonella typhimurium* and *Aspergillus fumigatus* infections (111, 274). Since there exists a caspase-1 and 11-independent inflammasome pathway regulating IL-1β maturation after fungal invasion, we were interested in understanding the mechanisms underlying the non-canonical inflammasome activation. Particularly, we focused on defining the contribution of caspase-8 in the modulation of inflammasome-mediated immune response during *P. brasiliensis* infection, as the absence of caspase-1 did not completely abrogate IL-1β levels in the infected macrophages (39). In this study, we show that caspase-8 activated by *P. brasiliensis* interacting with the adapter molecule Asc after engagement of dectin-1/Syk signaling orchestrates the caspase-1/11-independent IL-1β release and impairs fungal growth. The appearance of the non-canonical caspase-8 inflammasome as a partner for IL-1β secretion during *P. brasiliensis* infection demonstrates the versatility of this platform to recruit distinct effector proteins to tailor the immune response and sheds new light on the complexity of this host-pathogen interaction.

Material and Methods

Mice:

Mouse strains were maintained in specific pathogen-free conditions and bred at the University of Massachusetts Medical School. C57BL/6 mice were bred in-house or obtained from Jackson Labs (Bar Harbor, ME). *Casp1/11*$^{-/-}$ mice were provided by Dr. V. Dixit (Genentech, South San Francisco, CA) , and *Clec7a*$^{-/-}$ mice were from Dr. G. D. Brown (University of Aberdeen, King's College, Aberdeen, U.K.). *Asc*$^{-/-}$ were from Millennium Pharmaceuticals. *Rip3*$^{-/-}$*Casp8*$^{-/-}$ were obtained from Dr. W.J. Kaiser and Dr. E.S. Mocarski (Emory University School of Medicine, Atlanta, GA). *Rip3*$^{-/-}$ mice were provided by Dr. D. Green (St. Jude Children's Research Hospital, Memphis, TN) and in some cases by Dr. F. K.-A. Chan (University of Massachusetts Medical School, Worcester, MA). All mice were on a C57BL/6 background.

Fungal infection:

The yeast cells of *P. brasiliensis* (Pb) 60855 strain (ATCC 60855), obtained from Dr. B. S. Klein (University of Wisconsin, Madison, WI), were cultured for 7 days at 37°C in brain heart infusion agar medium (Sigma Aldrich) supplemented with 5% FBS (Life Technologies). To prepare the inocula, the yeast cells were harvested and maintained overnight under agitation in F12 Coon's Modification medium (Sigma Aldrich) at 37ºC. The yeast phase suspension was transferred to sterile tubes and centrifuged (400 x g) for 10 min at 4°C. The pellets were resuspended in PBS (pH 7.2–7.4), diluted and counted using a hemocytometer and a light microscope. The viability was determined by the

fluorescein diacetate (5mg/mL)-ethidium bromide (1mg/mL) method. Only fungal suspensions containing more than 90% of viable yeast cells were used. The cells were infected with viable fungi at a multiplicity of infection (MOI) of 1 or 5 whereas each animal was inoculated intravenously with $1x10^6$ yeast cells.

Recovery of CFUs (colony-forming units) and Cytokine Analysis:

The numbers of viable yeast cells in the organs (lungs, liver, and spleen) of the Pb60855-infected mice were determined at 30 days post-infection (dpi) by counting the CFUs. Fragments of the lung, liver, and spleen were aseptically collected, weighed, and homogenized using a sterile tissue grinder. The resulting macerate was diluted 10-fold in sterile PBS and plated onto Petri dishes containing brain heart infusion agar enriched with 5% FBS. The plates were incubated at 37°C for 7 days, and the number of CFUs per gram of tissue was calculated. For cytokine analysis of the lungs samples of the animals, the tissue in lysis buffer was tested at 10-fold dilution, and amount of cytokine released was calculated as per gram of tissue.

Histopathological analysis:

Animals selected at random from each group were sacrificed at 30 dpi. The lungs were excised, fixed with 10% formalin for 48 h, and embedded in paraffin. Tissue sections (5 mm) were stained with H&E for analysis of the lesions in the lung tissue using standard protocols.

Bone marrow-derived dendritic cell (BMDC) differentiation and stimulation:

Bone marrow progenitor cells were obtained from femurs and grown in RPMI 1640 medium with 10% heat-inactivated serum, 1% penicillin-streptomycin, 1% sodium pyruvate, 1% NEAA, 20 ng/mL of recombinant GM-CSF, 50 mM 2-ME for 7–10 days to differentiate into dendritic cells. On day 7, BMDCs were seeded in cell culture plates and the next day were primed or not with Pam3CSK4 (200ng/mL) for 3 hours and then were infected with *P. brasiliensis* at an MOI of 1 or 5 for 24 hours. Where indicated, Pam3CSK4-primed BMDCs were treated either with caspase-1 (z-YVAD-fmk), caspase-8 inhibitors (z-IETD-fmk) (both Santa Cruz; 50uM) for 2 hours or with 40uM of Syk inhibitor (piceatannol; InvivoGen) for 1 hour before adding the fungus. As a control, cells were incubated overnight with a canonical inflammasome activator such as pdAdT (poly(deoxyadenylic-deoxythymidylic) acid). For pdAdT transfection, each reaction consisted of 1ug of poly(dA:dT) per one million cells (Sigma-Aldrich) mixed with (1:2)(v/v) of Lipofectamine® 2000 Transfection Reagent (Invitrogen). After 15 min, DNA complexes were added to BMDCs in Opti-MEM (Gibco) and incubated for 16 hours.

Cytokine analysis:

Cytokines in culture supernatants were measured by ELISA (eBioscience or R&D Systems), according to the manufacturers' instructions.

Western blot:

Cell lysates and culture supernatants either combined or separated were denatured in loading buffer containing SDS and DTT, boiled and subjected to 13% SDS-PAGE gel. SDS-PAGE–separated proteins were transferred to nitrocellulose membranes, blocked with 1% nonfat dry milk and immunoblotted with primary antibodies against caspase-1

(Adipogen; clone casper-1), pro–caspase-8 (Enzo Life Sciences; clone 1G12 or Cell Signaling Technology; #4927), cleaved caspase-8 (Cell Signaling Technology; clone D5B2), IL-1β (R&D Systems), Asc (Millipore; clone 2EI-7 or Santa Cruz Biotechnology; sc-22514-R) and β-actin (Sigma Aldrich; clone AC15), followed by secondary anti-rabbit, anti-rat, anti-mouse or anti-goat HRP antibodies (BioRad Laboratories). In some cases, proteins from the cell culture supernatants were precipitated by the methanol-chloroform extraction method. The bands were quantified densitometrically using the ImageTool 2.0 software (University of Texas), and the results were expressed as arbitrary units.

Caspase-8 activity and cell death assay:

Caspase-8 activity in cell lysates was assayed using Caspase-Glo 8 Assay kit (Promega). Cell death was assessed detecting the release of lactate dehydrogenase (LDH) by CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega), according to the manufacturer's instructions. The percentage of cytotoxicity was calculated as (LDH infected - LDH uninfected)/(LDH total lysis - LDH uninfected) x 100. LDH total lysis was determined by lysing the cultures with Triton X-100.

Asc oligomerization assay:

Asc oligomerization assay was performed as described (275) with minor modifications (276). In brief, BMDCs were primed either with LPS or Pam3CSK4 (50ng/mL) overnight and incubated with pan-caspase inhibitor z-VAD (25uM) 30min before the 24-hour stimulation with *P. brasiliensis* or pdAdT. Cytosolic lysates from the cells were enriched

for inflammasome fractions by low-speed centrifugation and subjected to cross-linking with disuccinimidyl suberate (DSS) (2 mM). The crosslinked samples were analyzed for Asc oligomerization by immunoblotting.

Confocal immunofluorescence:

Following infection, BMDCs were washed twice with PBS and fixed in 4% paraformaldehyde for 30 min, followed by blocking with 5% normal goat serum (Dako) in Perm/Wash buffer (BD Biosciences) for 1 hr. Cells were incubated with a mouse anti-Asc antibody (1:500 dilution, clone 2EI-7; Millipore) overnight followed by incubation with a rabbit anti-caspase-8 (1:500 dilution, 8592; CST) for an additional 1 hr. The secondary antibodies used were Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 633 anti-mouse IgG. Cells were counterstained in DAPI mounting medium (1:1000 dilution; Vector Laboratories). Cells and inflammasomes were visualized and imaged using a Leica TCS SP8 confocal microscope at the Research Core Facility at University of Massachusetts Medical School.

Statistical analysis:

GraphPad Prism 5.0 software was used for statistical analysis. The data were represented as mean ± standard errors of the means (SEM) and the differences observed among the experimental groups after infection were examined by applying one-way ANOVA followed by the parametric Tukey's test for comparing multiple groups. $p < 0.05$ was considered statistically significant.

Results

P. brasiliensis-induced IL-1β secretion is dependent on both caspase-1 and caspase-8

Regulation of IL-1β production in macrophages and dendritic cells has focused on caspase-1, the predominant interleukin-1-converting enzyme (ICE) downstream of the NLRP3 (nucleotide-binding oligomerization domain–like receptor family pyrin domain–containing 3) inflammasome signaling complex, which is the best characterized enzyme for the proteolytic maturation of IL-1β (29). However, some studies have also implicated caspase-8 either as a direct executioner caspase for generating the mature 17-kDa IL-1β or as an initiator caspase for the activation of caspase-1 in response to infection by diverse microbial pathogens (76, 79). However, the 'if and how' of caspase-8 activation during *P. brasiliensis* infection is still unknown. Caspase-8 is synthesized as a single chain zymogen, procaspase-8 (p55), which consists of two death effector domains and two active domains (p18 and p10) (277). To monitor the activation status of caspase-8 after *P. brasiliensis* infection, we infected BMDCs, and 24 hours p.i., immunoblotted for the presence of the caspase-8 p18 subunit, which is yielded upon procaspase-8 proteolysis (278). The procaspase-8 processing into the small catalytic (p18) subunit as well as the intermediate 43kD form, that avoids apoptosis (279), was markedly augmented in Pam3CSK4-primed dendritic cells infected with *P. brasiliensis*, which explains the reduced procaspase-8 level in WT cells after incubation with the yeast form (**Figure 3.1A**). Additionally, the infection of BMDCs with *P. brasiliensis* induced caspase-8 activity in the two fungal concentrations tested (**Figure 3.1B**), denoting that *P. brasiliensis* recognition positively regulates caspase-8 activation.

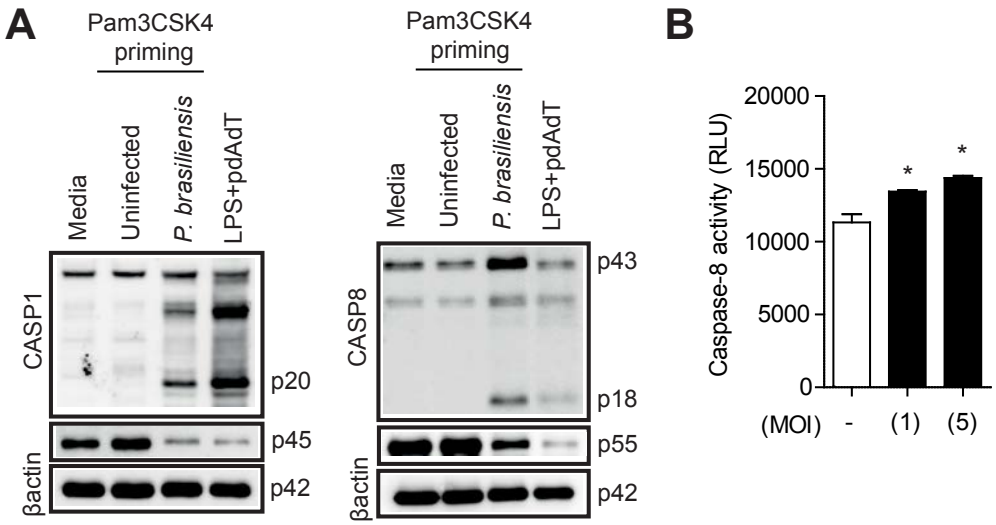

Figure 3.1: Caspase 8 is activated during *P. brasiliensis* infection.
(A) WT BMDCs were primed with 200 ng/mL of Pam3CSK4 for 3 hours and infected with *P. brasiliensis* (MOI 5) for 24 hours before lysates and supernatants were collected and immunoblotted for caspase-8 and caspase-1, respectively. Supernatant proteins were precipitated with methanol and chloroform. Control cells were stimulated with canonical inflammasome activator pdAdT after LPS priming. (B) Caspase-8 activity induced by P. brasiliensis in Pam3CSK4-primed WT BMDCs after 24 hours of stimulation with indicated MOI of fungus. The results are presented as relative light units.

As caspase-8 has been linked to pro-IL1β maturation in response to stimulation via TLR4 (79) as well as after fungal recognition by dectin-1 (80, 273), next we investigated whether caspase-8 was involved in *P. brasiliensis*-induced IL-1β production. Blocking caspase-8 activity with a chemical inhibitor (z-IETD-fmk) that binds irreversibly to active caspase-8 significantly attenuated the release of active IL-1β (**Figure 3.2A**), but did not affect TNF-α production after stimulation with *P. brasiliensis* (**Figure 3.2B**). Consistent with that, we also found suppressed cleavage of pro-IL-1β in z-IETD-treated cells compared to the untreated cells (**Figure 3.2C**), demonstrating the importance of caspase-8 for pro-IL-1β processing. Accordingly we noted that caspase-8 inhibition prevented cell cytotoxicity, resulting in lower LDH release, indicating decreased cell death (**Figure 3.2D**). Altogether these data suggest that infection with *P. brasiliensis* leads to processing of pro-IL-1β after fungal recognition not only through the activation of caspase-1-dependent inflammasome responses but also through caspase-8.

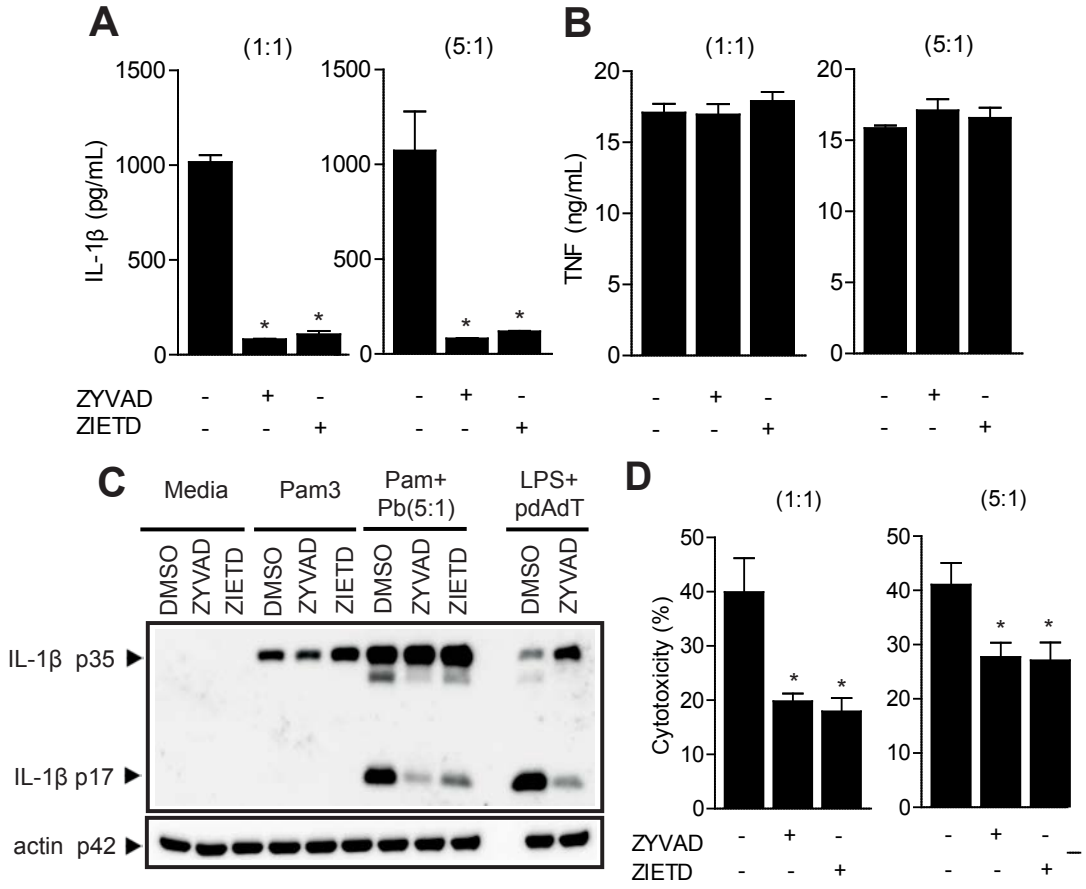

Figure 3.2: Caspase-8 activates *P. brasiliensis* dependent IL-1β processing and release. (C) Culture supernatants harvested from WT BMDCs activated for 3 hours with Pam3CSK4, treated the last 2 hours with caspase-1 (z-YVAD-fmk) or caspase-8 (z-IETD-fmk) inhibitors and subsequently challenged for 24 hours with *P. brasiliensis* were analyzed by ELISA to measure the levels of secreted IL-1β and (D) TNFα. (E) z-YVAD-fmk or z-IETD-fmk were added to Pam3CSK4 or LPS-primed BMDCs 2 hours before either infection with 5 yeast of *P. brasiliensis* per cell or incubation with pdAdT. After 24 and 16 hours, respectively, the samples prepared by combining cell lysates with culture supernatants were immunoblotted for IL-1β. (F) Pam3CSK4-primed BMDCs untreated or treated with z-YVAD-fmk or z-IETD-fmk were cultured with *P. brasiliensis* prior to the assessment of extracellular LDH. Values correspond to the percentage of LDH release compared with cells lysed with Triton X-100. Data show the averages ± SD from triplicate wells of at least three independent experiments.

Caspase-1/11 deficiency exacerbates *P. brasiliensis*-induced caspase-8 maturation

Though mouse BMDM lacking caspase-1 failed to produce appreciable levels of IL-1β (39, 280), the deficiency of caspase-1 only partially reduced IL-1β production. Therefore, it is possible that caspase-8 may also be playing a role in this process. To explore the relative contribution of caspase-8 for the caspase-1/11-independent IL-1β secretion, we analyzed caspase-8 activation and its enzymatic activity in the absence of caspase-1 and 11. Surprisingly, *P. brasiliensis*-induced caspase-8 processing and activity were greatly enhanced (41.5%) in caspase-1/11–deficient BMDCs (**Figure 3.3A and B**), These data correlated with the key role of caspase-8 in mediating IL-1β secretion in *Casp1/11*$^{-/-}$ cells, since the administration of caspase-8 inhibitor completely abolished the residual IL-1β production from the untreated dendritic cells that have no caspase-1 or 11 (**Figure 3.4A**). On the other hand, under the same conditions, the z-IETD treatment of *P. brasiliensis*-stimulated *Casp1/11*$^{-/-}$ BMDCs did not interfere with TNF-α production (**Figure 3.4B**). In agreement with the hypothesis that caspase-8 is essential for IL-1β release when caspase-1 and 11 are not present, the expression of the processed mature form of IL-1β, p17 was severely impaired in BMDCs from *Casp1/11*$^{-/-}$ mice, and it completely disappeared under caspase-8 blockade (**Figure 3.4C**). We conclude that *P. brasiliensis* activates the non-canonical caspase-8 inflammasome pathway, which is required for IL-1β release in the absence of caspase-1 and 11. Establishing that dendritic cells require caspase-8 activation when caspase-1 is absent and that this non-canonical caspase-8 inflammasome is suppressed by caspase-1 in WT cells, we set out to address whether Asc, a key component for caspase-1 activation after *P. brasiliensis* infection (39), was also involved in this process. Even though *Asc*$^{-/-}$ cells are

hampered in IL-1β secretion (**Figure 3.5A and B)**, Asc deletion boosted *P. brasiliensis*-induced caspase-8 proteolysis (**Figure 3.5C**) and activity (**Figure 3.5D**). Therefore, canonical caspase-1 inflammasome pathway inhibits the potent non-canonical caspase-8 inflammasome activation, but in the context of caspase-1/11 deficiency, IL-1β secretion is mechanistically dependent on the robust contribution of caspase-8 activity.

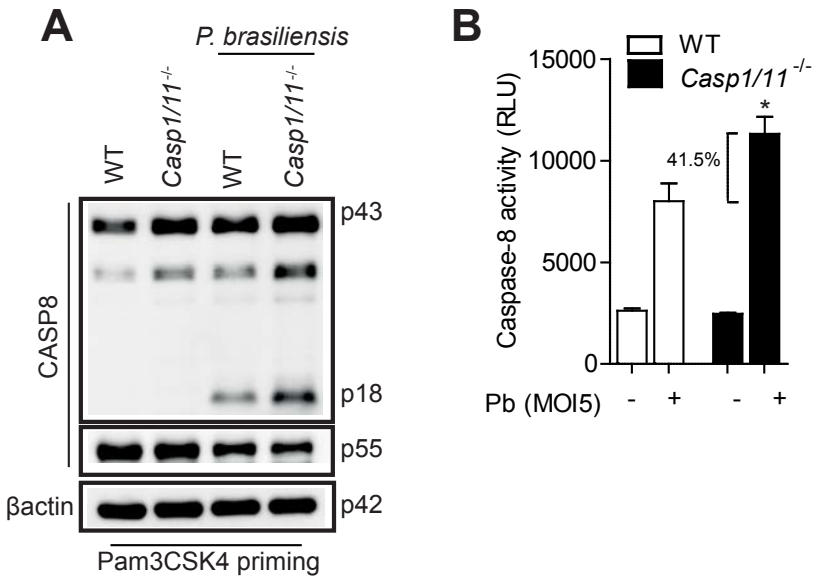

Figure 3.3: *P. brasiliensis* triggers caspase 1-independent caspase 8 activation. (A) BMDCs from indicated mice were primed with Pam3CSK4, infected with P. brasiliensis at (5:1) for 24 hours. The cell lysates were subjected to caspase-8 staining by Western blotting. (B) Caspase-8 activity in Pam3CSK4-primed WT or Casp1/11-/- BMDCs 24 hours after stimulation with Pb60855 was determined by the generation of luminescent signal as result of caspase-8 substrate cleavage (Caspase-Glo 8 Assay kit). The results are presented as relative light units (RLU)

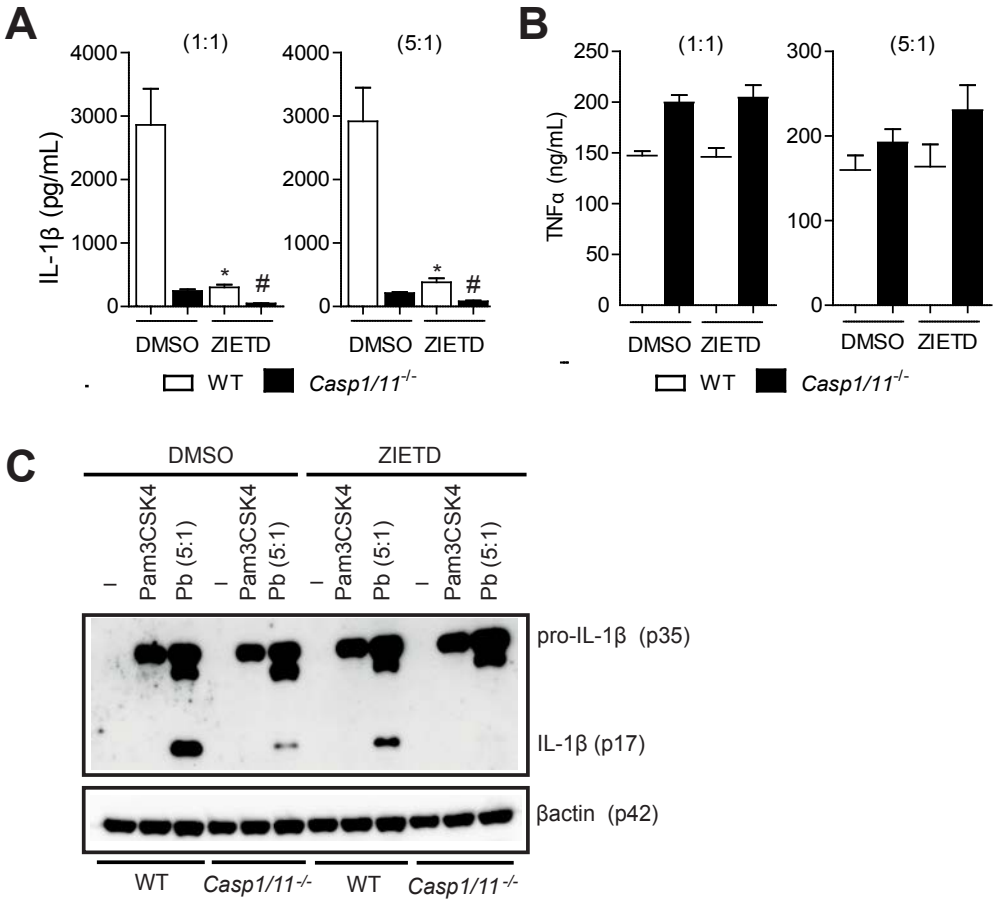

Figure 3.4: Caspase-8 is responsible for the caspase-1-independent IL-1β secretion triggered by *P. brasiliensis*. (A) BMDCs obtained from WT or Casp1/11-/- mice were primed with Pam3CSK4 and under caspase-8 activity blockade were incubated for 24 hours with *P. brasiliensis* at a MOI of 5:1. The amounts of IL-1β and (B) TNFα were quantified by an ELISA assay. (C) IL-1β processing on whole cell extracts was detected after incubation of Pam3CSK4-primed WT and Casp1/11-/- BMDCs with DMSO or z-IETD-fmk and infection with MOI-5 for 24 hours. Data are mean ± SD from one of three independent experiments.

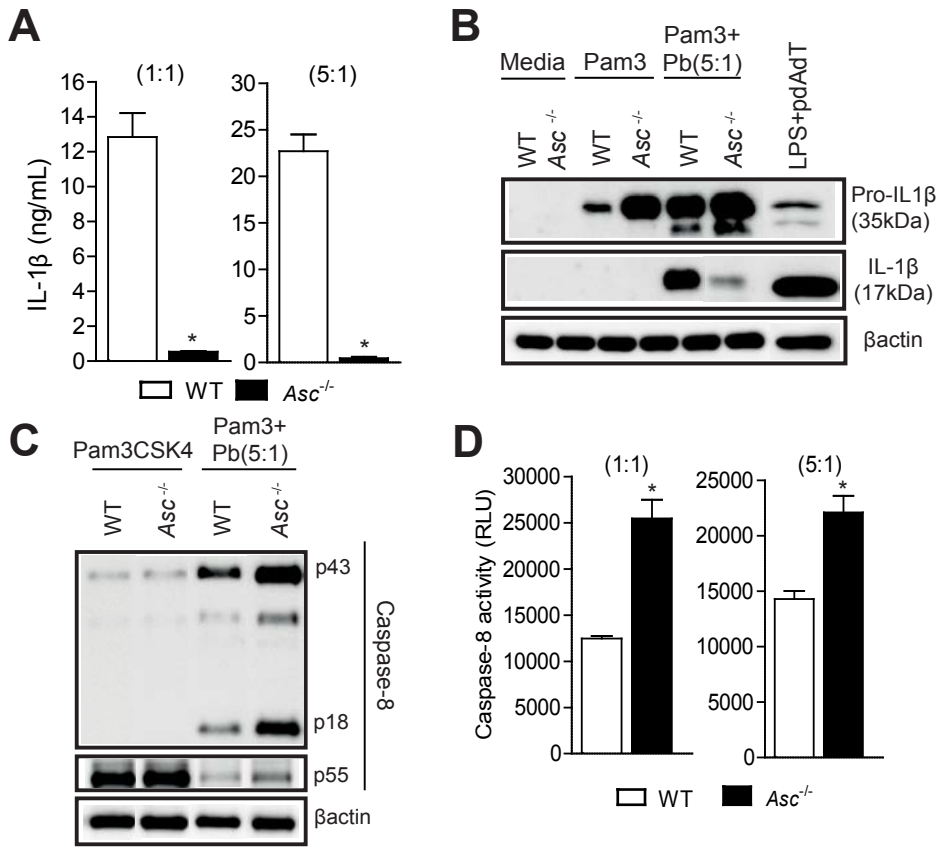

Figure 3.5: ASC deficiency activates caspase 8 after *P. brasiliensis* infection.
(A) IL-1β secretion by WT and Asc-/- BMDCs stimulated with Pam3CSK4 and P. brasiliensis for 3 and 24 hours, respectively, was defined by ELISA. (B) Pam3CSK4-primed WT and Asc-/- dendritic cells were incubated with P. brasiliensis. 24 hours later, pro-IL-1β conversion into biologically active IL-1β was analyzed by western blotting of total cell lysates. In parallel, WT cells were treated with LPS and pdAdT as control. (C) Caspase-8 activation and (D) activity were examined in BMDCs from WT and Asc-/- mice that were stimulated for 3 hours with Pam3CSK4 and for an additional 24 hours with P. brasiliensis.

Caspase-8 recruits to Asc speck assembly upon *P. brasiliensis* infection promoting IL-1β release through caspase-1 activation

Since caspase-8 plays a role in the regulation of inflammasome-generated IL-1β, we sought genetic evidence of the potential role of caspase-8 in the canonical inflammasome activation response. The genetic deletion of caspase-8 in mice results in embryonic lethality (281), which can be rescued by a simultaneous ablation of receptor interacting protein kinase-3 (RIP3) (113, 114). We differentiated BMDCs from caspase-8–deficient mice in a RIP3-deficient background and examined IL-1β release and caspase-1 activation following *P. brasiliensis* infection. Interestingly, *P. brasiliensis*-induced IL-1β production was diminished in *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ BMDCs (**Figure 3.6A**), but the amount of TNF-α produced by those cells was comparable to WT (**Figure 3.6B**). Similarly, the amount of mature IL-1β in the supernatants of stimulated BMDCs was also decreased in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ compared to WT and *Rip3*$^{-/-}$ cells (**Figure 3.6C**). Nevertheless, considering the known function of caspase-8 as a positive modulator of NF-κB transcriptional signaling (77, 78, 116, 118), the reduction in the quantity of mature IL-1β from the *P. brasiliensis*-stimulated *Rip3*$^{-/-}$*Casp8*$^{-/-}$ cells could reflect both attenuation of inflammasome assembly as well as less pro-IL-1β substrate available for activation. As shown in **Figure 3.6C**, the levels of pro-IL-1β after Pam3CSK4 priming were intact in WT, whereas dendritic cells lacking either RIP3 alone or caspase-8 and RIP3 both had less of the 35kD precursor form. However, this decrease did not happen when the cells were stimulated with *P. brasiliensis*, which confirms the effect of caspase-8 on signal 2 (inflammasome mediated pro–IL-1β processing) instead of signal 1 (NF-κB–mediated pro–IL-1β induction). Consistently, *Rip3*$^{-/-}$*Casp8*$^{-/-}$ BMDCs infected with *P.*

brasiliensis expressed normal levels of pro-caspase-1, but presented a significant defect in inducing caspase-1 activation compared to WT and *Rip3*$^{-/-}$ cells (**Fig 3.7A**), which correlated with the lower caspase-1 maturation observed on TLR2 agonist-primed WT cells treated with z-IETD-fmk, prior to *P. brasiliensis* stimulation (**Figure 3.7B**). To extend our findings, we evaluated whether even with the faint caspase-1 activation in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ BMDCs, caspase-1 still contributes to IL-1β maturation. Caspase-1 inhibition clearly limited the ability of Pam3CSK4-primed *Rip3*$^{-/-}$*Casp8*$^{-/-}$ BMDCs to produce mature IL-1β in response to *P. brasiliensis* (**Figure 3.8A and B**). Thus, these data indicate that caspase-8 licenses caspase-1 activation in the *P. brasiliensis*-triggered inflammasome, mediating efficient canonical inflammasome activation.

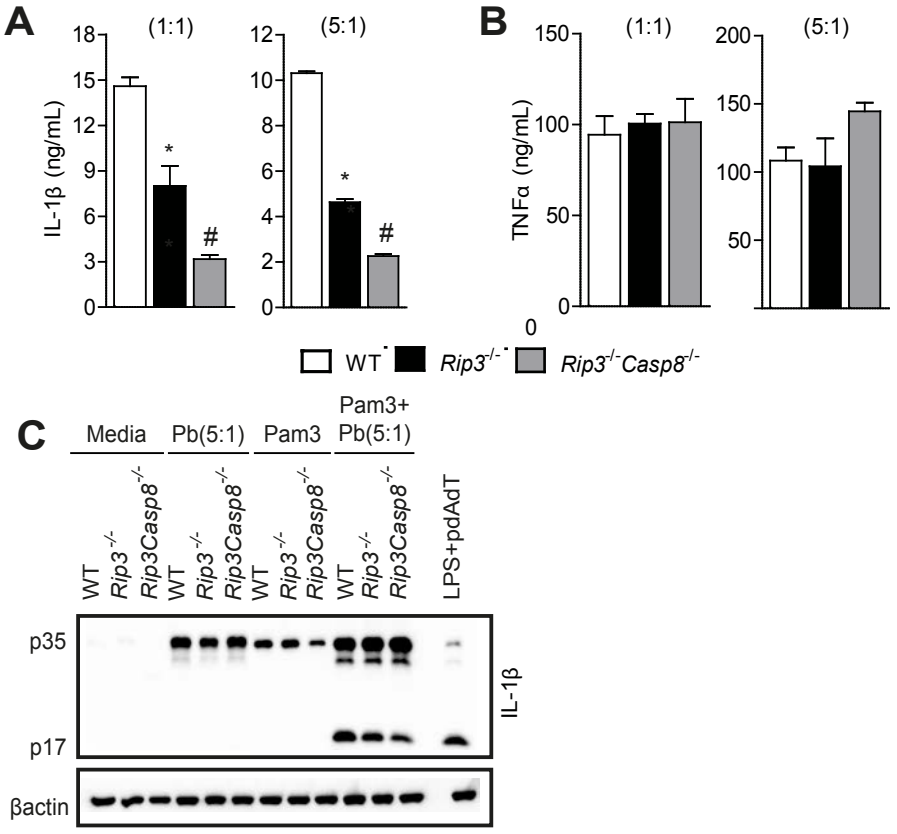

Figure 3.6: Caspase 8 is required for casapse 1 dependent IL-β production.
(A) Quantification of IL-1β and (B) TNF-α in the culture supernatant of Pam3CSK4-primed WT, Rip3-/- and Rip3-/-Casp8-/- dendritic cells 24 h postinfection with P. brasiliensis. (C) Unprimed or Pam3CSK4-primed BMDCs were left uninfected or stimulated with P. brasiliensis for 24 hours. The supernatant together with the cell lysate were harvested and immunoblotted to detect IL-1β cleavage.

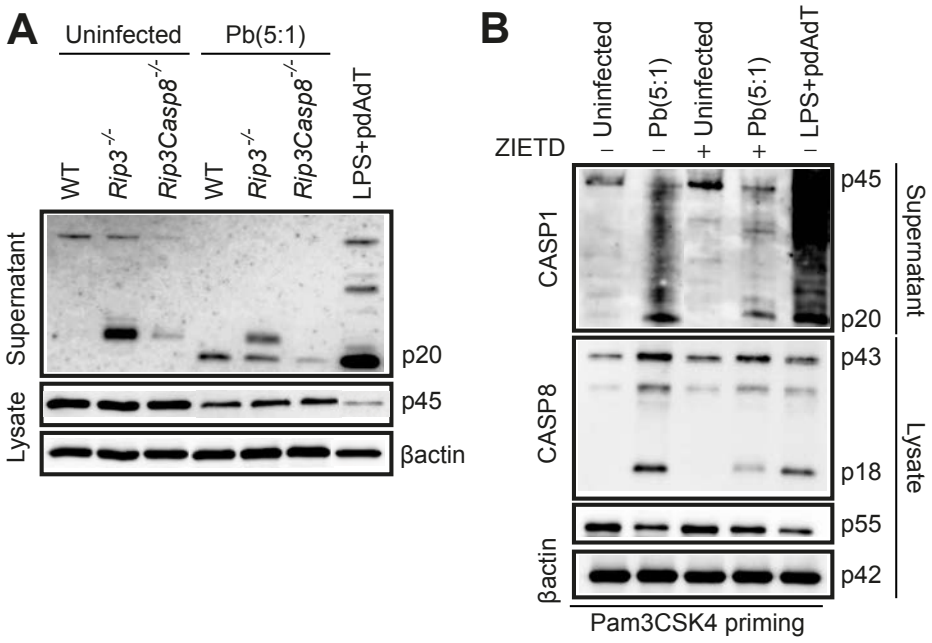

Figure 3.7: Caspase 8 is required for casapse 1 activation.
(A) p20 and p45 caspase-1 subunits were determined in supernatants and lysate, respectively, collected from P. brasiliensis-infected WT, Rip3-/- and Rip3-/-Casp8-/- BMDCs, previously primed with PamCSK4. (B) WT BMDCs were stimulated with 200 ng/mL of Pam3CSK4 for 3 hours, with the last 2 hours preceding the addition of *P. brasiliensis* in the presence of 50uM z-IETD. Precipitated supernatants and lysates were immunoblotted for the indicated proteins.

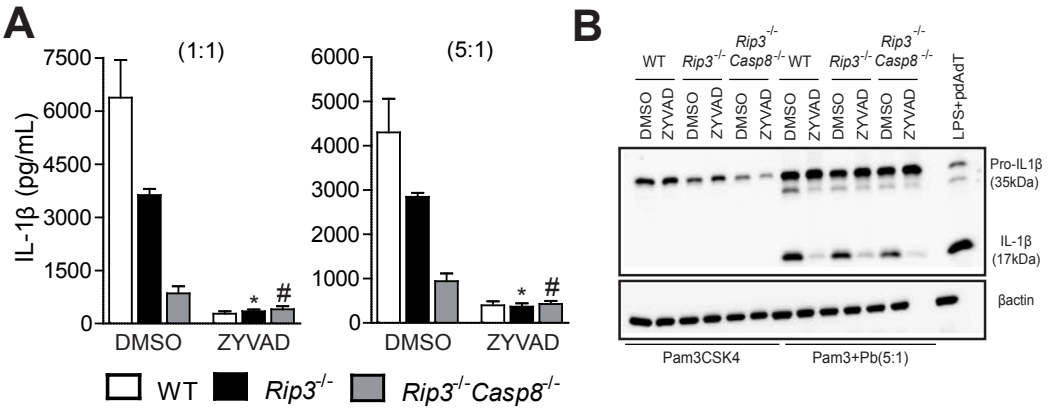

Figure 3.8: Caspase-1 controls IL-1β release in the absence of RIP3 and caspase-8. BMDCs from wild-type, Rip3-/- and Rip3-/-Casp8-/- mice were stimulated with Pam3CSK4. After 1 hour we added DMSO or z-YVAD-fmk (50uM) in the cell cultures and kept it for 2 hours until proceed to the 24-hour P. brasiliensis infection. The culture supernatants or the whole cell extract from the infected BMDCs were collected (A) to measure the concentrations of secreted IL-1β by ELISA and (B) to analyze the processed form of IL-1β by western blotting, respectively.

A key question that arises from these results is how does caspase-8 impact caspase-1 inflammasome activation. Inflammasome activation results in Asc redistribution in the host cytosol to arrange a single cytoplasmic focus, called an Asc speck, which can be visualized using microscopy techniques (282). Hypothesizing that caspase-8 may be recruited to the *P. brasiliensis*-induced Asc inflammasome platform, we stained for caspase-8 and Asc to determine whether caspase-8 forms a distinct speck-like protein and/or co-localizes to the same Asc puncta. Comparing the distribution of the fluorescently labeled proteins, our immunofluorescence analysis showed a significant co-localization of caspase-8 and Asc in dendritic cells stimulated with Pam3CSK4 and *P. brasiliensis* (**Figure 3.9A**). Hence, our observation places caspase-8 as a component of the Asc inflammasome complex during *P. brasiliensis* infection.

Currently it is known that Asc forms a single supramolecular platform composed of oligomerized Asc dimers that allows the recruitment and subsequent auto-activation of caspase-1 (275). In that case, one possibility is that caspase-8 facilitates Asc oligomerization. In order to find out the oligomeric state of Asc in *P. brasiliensis*-stimulated WT, *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ cells, we pelleted Asc aggregates with relatively low speed centrifugation and subjected it to chemical crosslinking. Asc was predominantly found as monomers and dimers in the pellets from WT, *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$ *Casp8*$^{-/-}$ BMDCs, and isolated in equal amounts between WT and *Rip3*$^{-/-}$ cells. In contrast, the oligomerizarion of Asc in *Rip3*$^{-/-}$*Casp8*$^{-/-}$- infected dendritic cells was strikingly weaker (**Figure 3.9B**). Thus, these results show that caspase-8 is an upstream mediator of the canonical caspase-1-dependent inflammasome, demonstrating a connection between these two signaling pathways during *P. brasiliensis* infection.

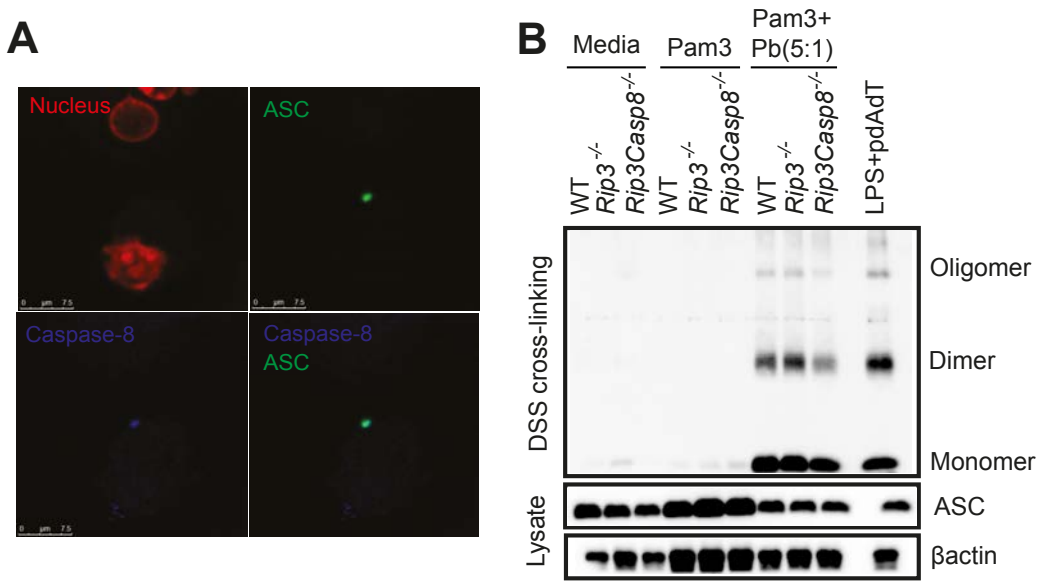

Figure 3.9: Caspase 8 promotes ASC oligomerization and speck formation.
(A) ASC oligomerization in inflammasome-enriched and cross-linked lysates of Pam3CSK4-primed WT, Rip3-/- and Rip3-/-Casp8-/- BMDCs that were put in contact with *P. brasiliensis* for 24 hours. All cultures contained the pan-caspase inhibitor z-VAD-fmk (25uM). Monomers, dimers, and oligomers of ASC are indicated accordingly. Where indicated wild-type BMDCs stimulated for 3 hours with LPS (200 ng/ml) and treated with pdAdT overnight were used as the positive control. (B) Caspase-8 (blue) and ASC (green) confocal micrographs taken from Pam3CSK4-primed BMDCs seeded on coverslips and stimulated with *P. brasiliensis* during 24 hours. DAPI was used for nuclear staining. Scale bars, 7,5μm.

***P. brasiliensis* recognition by dectin-1 engages IL-1β processing through a noncanonical caspase-8 inflammasome pathway**

IL-1β cleavage downstream of the C-type lectin receptor, dectin-1 in dendritic cells can proceed through a noncanonical pathway involving the activation of caspase-8 (80). Hence, we sought to understand how *P. brasiliensis* activates the non-canonical caspase-8 inflammasome, by checking whether dectin-1 was involved in caspase-8-induced pro-IL-1β processing. To ensure that the IL-1β cleavage elicited by *P. brasiliensis* occurs through β-glucan receptor we verified the processing of IL-1β in WT and *Clec7a*$^{-/-}$ cells after fungal challenge. To exclude the effects of dectin-1 on pro-IL-1β accumulation, we primed *Clec7a*$^{-/-}$ cells with Pam3CSK4 to guarantee dectin-1 independent pro-IL-1β production. Even though dectin-1 could play a role in priming, acting upstream of inflammasome signaling, this C-type lectin receptor was also important for pro-IL-1β / IL-1β conversion after *P. brasiliensis* infection, as the reduction in IL-1β released was prominent (**Figure 3.10A and B**). As we hypothesized, this defect was not caused by less pro-IL-1β production, but because Pam3CSK4-primed *Clec7a*$^{-/-}$ dendritic cells infected with *P. brasiliensis* did not provoke caspase-8 activation (**Figure 3.10C and D**).

Upon ligand binding to the extracellular carbohydrate-recognition domain of dectin-1, the immunoreceptor tyrosine-based activation motif (ITAM)-coupled cytoplasmic tail is phosphorylated by Src tyrosine kinases causing the subsequent recruitment and activation of Syk (268, 283, 284). To ascertain the participation of Syk kinase as a potential transducer for dectin-1-mediated IL-1β secretion, we treated Pam3CSK4-primed WT BMDCs with piceatannol, a Syk kinase inhibitor, prior to *P.*

brasiliensis infection. Unlike DMSO-treated control cultures, TLR2 agonist-primed WT cells that received piceatannol before *P. brasiliensis* infection had compromised release of IL-1β (**Figure 3.11A and B**), supporting the idea that *P. brasiliensis* recognition incites IL-1β production via Syk kinase. The next question we asked was, how is Syk kinase mediating IL-1β cleavage. The treatment of BMDCs with piceatannol accompanied by *P. brasiliensis* stimulation not only dampened caspase-8 activity (**Figure 3.11C**) and activation (**Figure 3.11D**), but also interfered with caspase-1 processing (**Figure 3.11E**), which may be a consequence of activation of multiple receptors that converge on Syk. Collectively, Syk kinase signaling is fundamental for IL-1β production upon *P. brasiliensis* recognition as it is necessary for both non-canonical and canonical inflammasome activation.

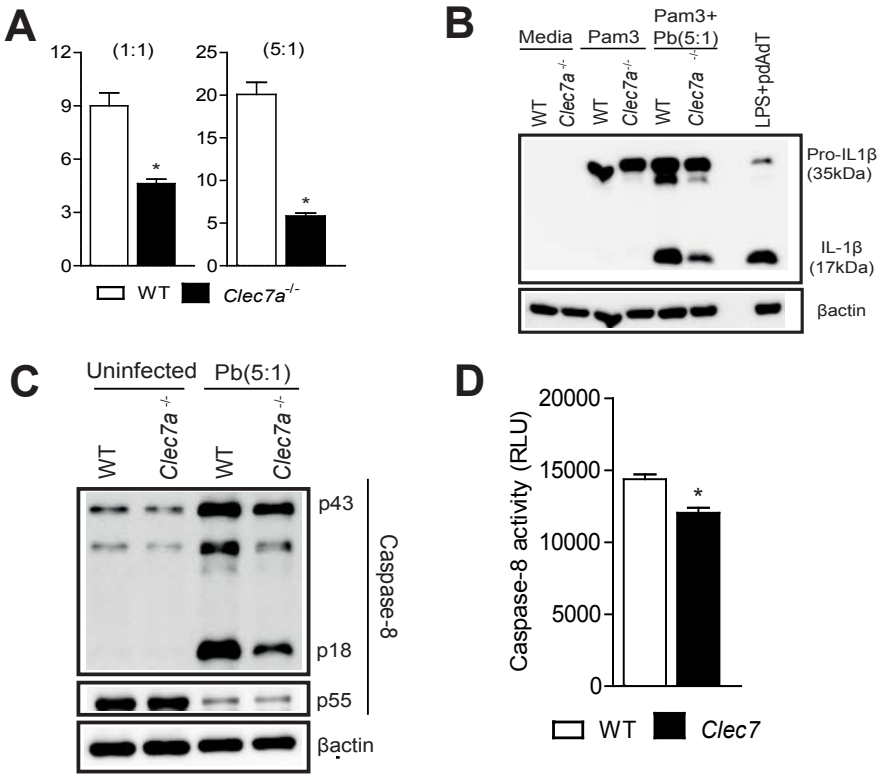

Figure 3.10: *P. brasiliensis* sensing by dectin-1 induces caspase-8 inflammasome -mediated IL-1β maturation. (A) IL-1β concentration in BMDCs from WT or Clec7a-/- mice stimulated with Pam3CSK4 plus *P. brasiliensis* at an MOI of 5 for 24 hours. (B) Western blotting of the processed IL-1β p17 subunit in whole cell extracts from Pam3CSK4-primed WT and Clec7a-/- dendritic cells infected with *P. brasiliensis*. LPS-primed WT BMDCs cultured with pdAdT overnight were used as the control for IL-1β production. (C) The lysates from Pam3CSK4-treated WT and Clec7a-/- BMDCs, which were infected or not with *P. brasiliensis* for 24 hours, were fractionated by SDS-PAGE and incubated with caspase-8 antibody. (D) Caspase-8 activity measured after 24 hours of incubation with *P. brasiliensis* in Pam3CSK4-treated wild-type BMDCs or in BMDCs lacking dectin-1.

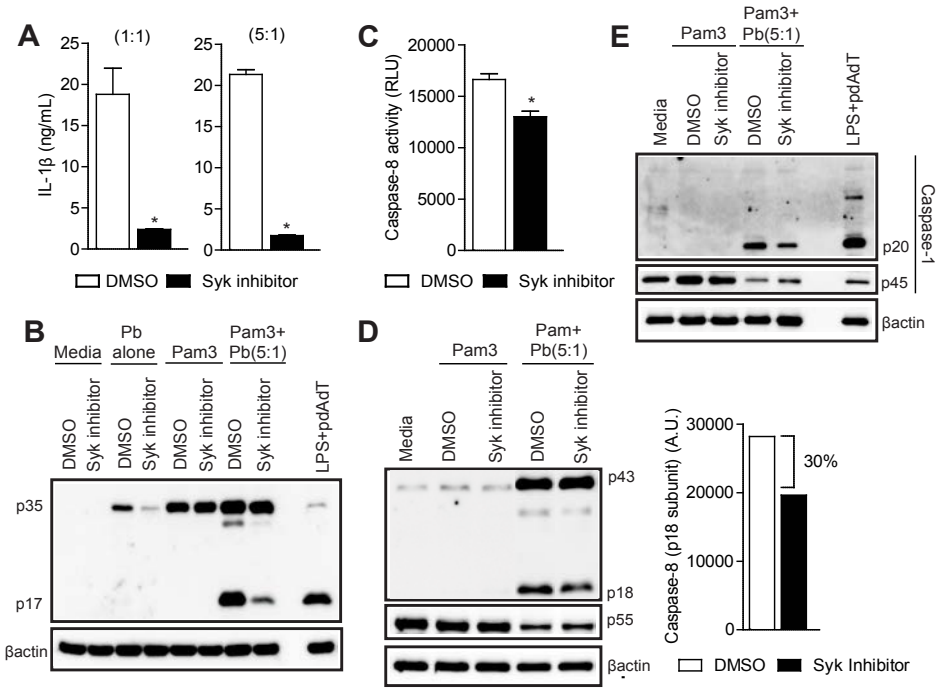

Figure 3.11: Syk kinase signaling activates both caspase 8 and caspase 1 inflammasome-dependent processing of pro-IL-1β. (A) Pam3CSK4-stimulated BMDCs from WT mice with or without Syk kinase inhibitor (piceatannol 40uM) treatment were infected with *P. brasiliensis* for 24 hours and then the supernatants were assayed for IL-1β. (B) BMDCs from WT mice were infected with *P. brasiliensis* (MOI 5) for 24 h in the absence or presence of 40μM Syk inhibitor. The combination of supernatant and cell lysate was analyzed by immunoblotting to determine the levels of IL-1β released. (C) Assay of caspase-8 activity in wild-type BMDCs stimulated with *P. brasiliensis* and treated or not with Syk inhibitor. (D) Pam3CSK4-primed WT BMDCs were pretreated with Syk inhibitor for 1 hour followed by 24-hour P. brasiliensis infection. Cell lysate samples were used to probe caspase-8 and (E) pro-caspase-1, while the supernatant was precipitated by methanol and chloroform before carrying out the caspase-1 western blotting. On the panel D, the intensities of immunoreactive caspase-8 p 18 bands in the western blotting were quantified through densitometric analysis.

Caspase-8 augments host resistance to *P. brasiliensis* infection

Subsequently, our effort was to look for *in vivo* evidence that would connect the non-canonical caspase-8 inflammasome to the immune responses against *P. brasiliensis*. To ascertain whether non-canonical caspase-8 inflammasome response protects against challenge with *P. brasiliensis*, we infected WT, *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice with 1x10^{6} viable yeasts of *P. brasiliensis* to analyze the fungal colonization in tissues. *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice had large numbers of fungi in their lungs compared to *Rip3*$^{-/-}$ mice, which behaved similarly as wild-type control mice, while no significant changes were observed in the liver and spleen (**Figure 3.12A**). The reduced ability in attenuating fungal growth in *P-brasiliensis*-infected *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice was also confirmed in histological sections by the presence of extensive lesions containing fungi colonies (**Figure 3.12B**). When the H&E staining was used to visualize the pulmonary fungal distribution, we found that *P. brasiliensis* was localized within granulomatous foci in WT and *Rip3*$^{-/-}$ mice, but was more widespread in the *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice, which failed to confine the pathogen inside granulomas (**Figure 3.12B upper panel**). Additionally, at 30 days post-infection, while the immune cells surrounded the granulomatous areas in the lung tissues from WT and *Rip3*$^{-/-}$ mice, the intense inflammatory reaction of *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice was diffuse and infiltrated the alveolar spaces (**Figure 3.12B lower panel**). Together these findings show that caspase-8 plays a critical role in mediating protective immune defense *in vivo* and that a deficiency in non-canonical caspase-8 inflammasome, leads to a dysregulated inflammatory response, which is unable to control *P. brasiliensis* replication.

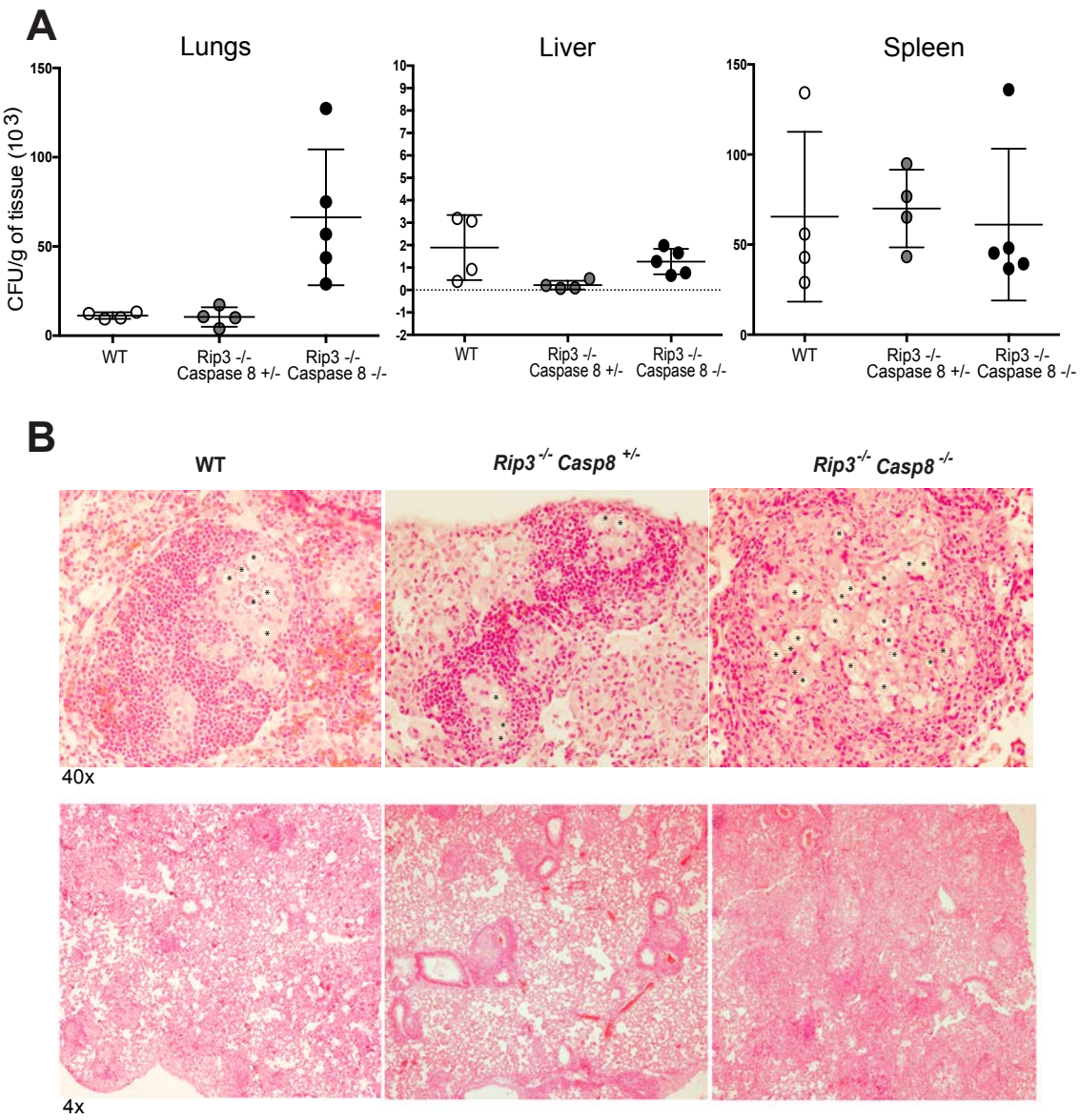

Figure 3.12: Caspase-8, but not RIP3, deficiency confers susceptibility to *P. brasiliensis* infection *in vivo*. (A) The fungal burden in the lungs, livers, and spleens from wild-type, *Rip3-/-* and *Rip3-/-Casp8-/-* mice after 30 days post infection with 1x106 yeast cells of *P. brasiliensis* 60855 strain. (B) Photomicrographs (original magnification, upper panel: 40x and lower panel: 4x) of granuloma lesions from the WT, *Rip3-/-*, and *Rip3-/-Casp8-/-* mice at 30 dpi. The lung sections were fixed in formalin, paraffin embedded, stained with H&E, and analyzed by light microscopy. The asterisks indicate the fungi in the tissue. Each dot represents a male mouse.

Discussion

A major signaling pathway involved in the release of IL-1β is the activation of the classical or canonical inflammasome pathway, a multi-protein platform that activates caspase-1 (285). Beside caspase-1, additional caspases such as caspase-8 and caspase-11 have also been implicated in the inflammasome-dependent control of IL-1β processing and maturation (286). As the inflammasome is a dynamic complex that recruits different components to a single molecular platform depending on the contextual cue, the emergence of non-canonical inflammasomes for pro-IL-1β processing emphasizes the diversity of the innate immune system to combat pathogens, indicating that several effectors can form inflammasomes during infection *in vivo*.

Caspase-8, first described in 1996, is classically known for its function as a mediator of the death receptor–induced extrinsic pathway of apoptosis, that is triggered by several death receptors such as TNF-receptor type 1 (TNFRI) and CD95, both requiring the FAS-associated death domain (FADD) protein (287, 288). However, unexpectedly, caspase-8 is not always involved in apoptotic signaling (289). Substantial evidence has built up regarding its non-apoptotic functions, including activation of the transcription factor NF-κB (116, 118, 290) and production of mature IL-1β (79). Although IL-1β secretion coordinated by caspase-8 activation is not restricted to fungal pathogens (76, 78, 111, 291), a body of literature reinforces the influence of this caspase in IL-1β production during *C. albicans*, *A. fumigatus* and, *Cryptococcus neoformans* infections (80, 273, 274, 292). Previosuly, we demonstrated that the production of IL-1β during *P. brasiliensis* infection is not exclusively due to caspase-1 signaling (39). In order to reveal an alternative route for IL-1β secretion, we expanded our views to clarify

the interplay between the non-canonical caspase-8 and the canonical caspase-1 inflammasome pathways after *P. brasiliensis* infection.

Here, we showed that the moderate level of caspase-8 activation observed in Pam3CSK4-primed WT dendritic cells incubated with *P. brasiliensis* was controlled by caspase-1/11, because in caspase-1 and -11-sufficient cells the expression and activity of these two proteases bypasses vigorous caspase-8 proteolysis and activity. However, caspase-8 served as an accessory to induce IL-1β production in *P. brasiliensis*-infected BMDCs under conditions in which canonical caspase-1 inflammasome activation is prevented. Consistent with these results, *C. neoformans*-infected dendritic cells also turn to caspase-8 activation when caspase-1 is absent, since caspase-1 suppresses the non-canonical caspase-8 inflammasome activation (292). Beyond that, the delayed export of mature IL-1β in conjunction with increased level of the 18-kDa caspase-8 subunit after sustained nigericin treatment also proceeds in a caspase-1 and -11-independent way (293). An explanation for the improved caspase-8 activation in *Casp1/11*$^{-/-}$ cells is that caspase-8 and caspase-1/11 compete with each other. Consequently, caspase-1 and -11 ablation assures that more Asc would available to bind to 55-kDa procaspase-8.

Like other caspases, caspase-8 is translated as a monomeric zymogen comprised of a pro domain followed by a large and small catalytic subunit. The pro domain of caspase-8 monomers, consists of two death effector domains (DEDs) that interact with the N-terminal PYRIN domain of Asc (275, 294) and mediate colocalization with the AIM2 inflammasome in *Francisella tularensis*-infected cells (295). Through confocal experiments, we found that caspase-8 interacts and colocalizes together with the adapter protein Asc in a single cytoplasmic speck during *P. brasiliensis* infection, similar

to earlier published observations (111, 274, 292, 294, 295). It will be interesting to investigate the spatial orientation of caspase-1 and *Nod-like* receptor proteins in the Asc-caspase-8 inflammasome in future studies to understand whether the components of the canonical inflammasome pathway also reside in the same Asc complex.

Even when caspase-1 and 11 are present, we demonstrate that caspase-8 mediates IL-1β maturation upon *P. brasiliensis* infection. The reduced IL-1β processing assigned to the pharmacological inhibition of caspase-8 was corroborated using the *Rip3*$^{-/-}$*Casp8*$^{-/-}$ double knockout mice. Caspase-8 deficiency, more than RIP3 deficiency, disturbs *P. brasiliensis*-induced IL-1β secretion in Pam3CSK4-primed BMDCs, disrupting caspase-1 activation and Asc oligomerization. The requirement of caspase-8 for caspase-1 activation suggests that caspase-8 might be upstream of caspase-1, but future studies will dissect the mode of action of caspase-8 to elucidate whether the protease acts directly or is a parallel pathway that converges with the canonical inflammasome to facilitate IL-1β production. Caspase-8 could regulate caspase-1 activating cellular inhibitors of apoptosis proteins (cIAPs), which have been shown to interact with caspase-1, through their respective N-termini, in a complex containing TRAF2 (TNF receptor-associated factor 2) to direct the non-degrading K63-linked polyubiquitination of caspase-1 that could favor either the assembly of inflammasome or coordinate its activity (81). Several groups have demonstrated the involvement of caspase-8 in the cleavage of the caspase-1 pro form over the last few years (78, 274, 291). The exception is the *Salmonella typhimurium*-sensing pathway, in which caspase-1 processing in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ macrophages is preserved (111). Nevertheless, how caspase-

1 and caspase-8 operate, whether synergistically or independently, as well as the nature of the activators that determine this relationship remain to be fully elucidated.

Mechanistic studies also described that apart from IL-1β cleavage, caspase-8 regulates the synthesis of pro-IL-1β mRNA (111). At the transcriptional level, caspase-8 is involved in NF-κB-dependent upregulation of pro-IL-1β in LPS-primed BMDCs (77). However, another observation revealed that the transcriptional role of caspase-8 is not confined to TLR4-induced signaling alone, but extends to NF-κB and MAPK activation by the TLR2 ligand Pam3CSK4 (78), which is in agreement with our data that showed a weaker protein band for the p35kDa precursor form of IL-1β in *Rip3*$^{-/-}$ *Casp8*$^{-/-}$ dendritic cells compared to *Rip3*$^{-/-}$ and WT cells. It has been established that the catalytic functions of caspase-8 as well as caspase-8-mediated IL-1β production in response to heat killed *C. albicans* are regulated by cFLIP (cellular FLICE-inhibitory protein) (296). Furthermore, caspase-8 forms a heterodimer with cFLIP (297) and the cFLIP cleavage fragments generated by association with caspase-8 are involved in NF-κB activation (298). Combined with our results, it raises the possibility that cFLIP proteolysis, a prerequisite for induction of NF-κB activation, does not occur in dendritic cells deficient for both caspase-8 and RIPK3, which could explain why the levels of pro-IL-1β are low after TLR2 engagement. Although the *Il1b* gene upregulation induced by caspase-8 is not applicable to the experimental model of *P. brasiliensis* infection, additional inquiries are needed to better comprehend the precise mechanism by which caspase-8 contributes to efficient NF-κB-mediated gene transcription.

In addition, we demonstrate that dectin-1-mediated IL-1β production after *P. brasiliensis* challenge requires the non-canonical caspase-8 inflammasome, permitting

us to uncover a previously unknown pathway for dectin-1 in *P. brasiliensis*-infected dendritic cells. In fact, in our fungal model, dectin-1 is connected to the posttranslational cleavage of pro-IL-1β, but alternatively could regulate IL-1β transcription on priming. Similarly, dectin-1-ligand β-glucan participates in both NLRP3 inflammasome activation and NF-κB-mediated priming (95, 299). Moreover, it has been shown that dectin-1 signals alone can activate NF-κB in BMDCs (268, 300), but not in BMDM due to the differential use of CARD9 (300). For IL-1β secretion, we saw that inflammasome activation was amplified in dendritic cells rather than macrophages (data not shown). Regardless of the undesirable IL-1β secretion in certain myeloid cells populations, quiescent dendritic cells have increased amounts of NLRP3 transcripts (301, 302), higher NLRP3 promoter activity (303) compared to macrophages, and GM-CSF treatment maximizes the pro-IL-1β production by NF-κB activating agonists (304), prompting them to achieve a satisfactory activation threshold for the NLRP3 inflammasome.

Several studies firmly underline the effectors and regulatory mechanisms of pattern recognition receptors (PRRs) in innate and adaptive immunity against this fungal pathogen (305, 306). However, they did not study their relevance for IL-1β induction and secretion, probably because the compensatory pathways seem to mask the immunological differences caused by PRR deficiencies. Based on our results, at least one additional pathway is responsible for pro-IL-1β induction in dendritic cells since a remnant IL-1β production was consistently observed in *Clec7a*$^{-/-}$ BMDCs. As dectin-1 manipulates the expression of *Toll-like* receptors (TLRs) during PCM (271), and *P. brasiliensis* is simultaneously sensed by TLR2 and TLR4 (307, 308) further investigations are necessary to unveil the roles of these TLRs for IL-1β production.

Interestingly, Pam3CSK4 priming greatly boosted this response compared to LPS (data not shown), suggesting a more prominent expression of the pro-IL-1β precursor form after engagement of TLR2 signaling in *P- brasiliensis*-infected BMDCs. In accordance, Netea et al., 2002 (309) verified that TLR2 neutralization in mononuclear cells, using a specific anti-TLR2 antibody, leads to a significant reduction of *C. albicans*-induced TNF-α and IL-1β production, whereas anti-TLR4 treatment did not influence the secretion of these proinflammatory cytokines. In contrast, the IL-1β production triggered by *Aspergillus fumigatus* hyphae on telomerase-immortalized human corneal epithelial cells is mediated through both signaling pathways, TLR2 and TLR4 (310). However, the sensing pathway engaged by *P. brasiliensis* to trigger pro-IL-1β synthesis varies with cell type. As the transcript levels of genes that encode TLRs and MyD88 are not elevated in *P. brasiliensis*-infected BMDCs (311), the induction of pro-IL-1β in BMDCs is only dependent on Syk kinase (102).

Even though Syk kinase operates on pro-IL-1β expression during *P. brasiliensis* infection, in our present work we also identified that it plays a pivotal function in controlling both non-canonical caspase-8 and canonical caspase-1 inflammasome activation. In contrast, Gringhuis et al. (80) demonstrated Syk signaling induced by non-phagocytosed *C. albicans* was restricted to caspase-8 activity in human dendritic cells. Considering that the requirement of Syk for dectin-1 function is confined to cell type (96, 269), this discrepancy may result from variations in the experimental approaches or reflect species-based differences that come from the use of either human or murine-derived dendritic cells.

Other studies also have linked the NLRP3 inflammasome to Syk-induced IL-1β processing in mouse dendritic cells and macrophages infected with fungi (42, 95, 299). The proposed mechanism is that Syk augments canonical inflammasome activation upon dectin-1 signaling by generating reactive oxygen species (ROS) (42, 95, 96, 299), chemical compounds that are relevant to NLRP3/caspase-1 inflammasome activation in *P. brasiliensis*-stimulated murine dendritic cells (102). Another important aspect is that Syk represents a common point in the signaling pathways of CLEC6A (also known as dectin-2) and CLEC4E (also known as Mincle). Unlike dectin-1, dectin-2 and Mincle have no known intracellular signaling motifs (312, 313). Instead, they associate with ITAM-containing adaptor protein Fc receptor γ chain (FcRγ) to propagate the signal (312, 314, 315). Future investigations are likely to dissect the necessity of these receptors during PCM.

Finally, we demonstrated a greater fungal load in *P. brasiliensis*-infected *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice denoting a critical role for caspase-8 in host protection. Likewise, the same phenotype was described in response to *Citrobacter rodentium* (78), *A. fumigatus* (274) and *Yersinia pestis* (291, 316). As mice lacking caspase-8 on a Rip3-deficient background (*Rip3*$^{-/-}$*Casp8*$^{-/-}$) or dendritic cells with deleted caspase-8 (Casp8$^{fl/fl}$Itgax^{cre}) have defects in cell death that cause lymphocytes accumulation (113, 122), and the capsular polysaccharide component galactoxylomannan from *C. neoformans* induces apoptosis of human T-cells, through caspase-8 activation (317), we believe that caspase-8 mediated cell death might prevent unwanted or excessive innate immune responses. Nonetheless, whether cell death is directly responsible for the inability of caspase-8-deficient animals to clear *P. brasiliensis* or for the mobilization of bystander cells that

rapidly produce cytokines and phagocytose pathogen-associated cell debris remain to be studied. The new insights gained from deciphering how pathogen recognition shapes the development of appropriate innate immune responses during *P. brasiliensis* infection allow a better understanding of the underlying cause of inflammation after host-pathogen interactions.

Chapter 4: Discussion, perspectives and implications

Innate immunity navigates a very fine line between launching an effective onslaught against an invading pathogen or self-derived dangers, and controlling these responses in such a way so that harm to the host is minimal. Hence, innate immune responses are regulated by multiple factors upon which the balance between pathogenesis and autoimmunity is hinged. Too less a response, the infection wins or damage persists, whereas prolonged or too intense a response, and the healthy host cells and tissues are damaged. Multiple layers of regulation have evolved that control immune responses, some of which have been described earlier in this dissertation. For example, stimulation of inflammatory pathways upon the detection of microbial agents or nucleic acids leads to the production of pro-inflammatory as well as anti-inflammatory cytokines and chemokines, that counteract each other. Temporal regulation, feedback inhibition of gene expression and degradation of effector components are a few critical factors that fine-tune a necessary and sufficient immune response to pathogens or danger molecules, and are involved in switching off the responses over time.

p205 as a Regulator of Immune Genes and Inflammation

In this dissertation, I outline a novel function of a PYHIN protein in regulating inflammation. Previous studies indicate that Asc is upregulated in response to cytokines such as IL-1β, IFN- γ, TNF- α and LPS in immune cells (318-320), but the mechanism of this regulation has not been clearly elucidated. p205 is a nuclear protein

that is highly inducible upon stimulation with the microbial PAMP, LPS as well as by the cytokines IFN-α/β and IFN-γ. Deficiency of p205 impaired the Aim2 and Nlrp3 inflammasomes and had a partial effect on Nlrc4 inflammasomes. In resting macrophages, loss of p205 abrogated the expression of the inflammasome adapter protein, Asc. Here, I show that p205 is required in maintaining Asc expression in resting macrophages as well as B16 melanoma cells, and induction of p205 (e.g. TLR4-dependent signaling through LPS or the interferons) during infection or cellular stress stimulates Asc expression further, in a feed-forward mechanism, which is recruited to form a caspase-1 dependent inflammasome required for maturation and activation of the inflammatory cytokine, IL-1β. This study illustrates that besides acting as receptors for DNA damage and invading pathogens, the DNA-binding PYHIN proteins also control the immune pathways by regulating immune gene expressions (**Figure 4.1**).

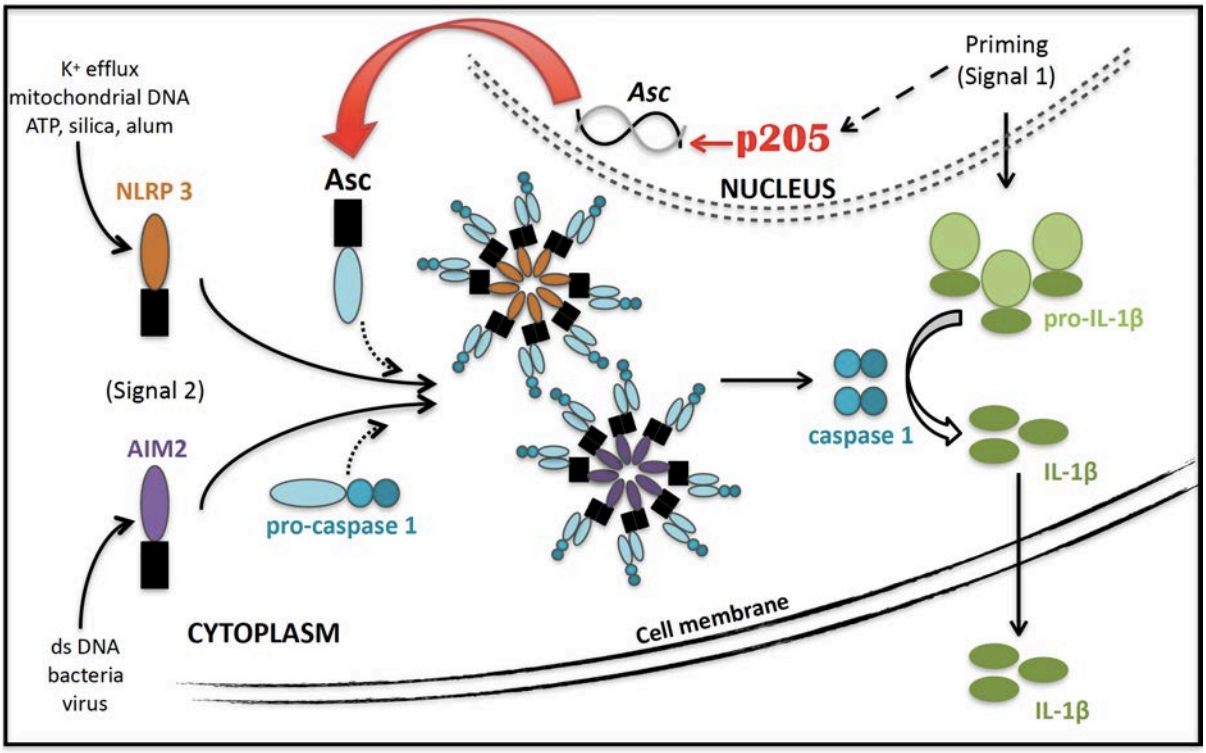

Figure 4.1: p205 and Inflammasome Activation. p205 expressed in the cell nucleus controls *Asc* gene expression in resting cells. Upon priming of macrophages by TLR agonists such as LPS (Signal 1) that induces pro-IL1β expression, p205 expression is also enhanced. In these primed cells, induction of the receptors like NLRP3 or AIM2 (Signal 2) by their respective ligands leads to the recruitment of the adapter Asc, and subsequently pro-caspase 1 that forms the inflammasome. The activated caspase 1 then cleaves the pro-IL1β into its active, secreted form that stimulates a downstream inflammatory response.

p205 deficiency diminished inflammasome activation due to decreased Asc protein and mRNA expression. Loss of p205 affected the RNA Polymerase II transcription machinery at the endogenous *Asc* gene. It reduced the recruitment of total RNA Pol II as well as the differentially phosphorylated RNA Pol II that are responsible for transcriptional initiation and elongation as well as mRNA processing and maturation. p205 also interacted with other known transcription factors such as c/EBPβ and p65/RelA to synergistically enhance *Asc* gene expression. ASC/TMS1 gene activity has been known to be regulated by DNA methylation, which blocks RNA Pol II recruitment, and by promoter proximal pausing, which recruits an RNA Pol II that can initiate transcription but does not allow transcriptional elongation. In the context of p205, I suggest that p205 is an integral part of the larger transcriptional complex containing RNA Pol II with or without other transcription factors that is required for effective *Asc* gene expression. Loss of p205 from this transcriptional complex allows for some leaky transcription at the endogenous *Asc* gene but blocks efficient transcription and mRNA maturation. Further, the reduced levels of the initiating as well as elongating RNA Pol II in the absence of p205 alludes to the inability of RNA Pol II to successfully initiate and/or elongate gene transcription even though it is recruited to the gene promoter. Hence, other immune genes that are regulated by p205 may not exhibit a similar phenotype of leaky pre-mRNA production, but may simply be inhibited by p205 at the transcription level - as RNA Pol II pausing and aborted initiation is characteristic of the gene promoter, and not the transcription factor.

The interaction of p205 with c/EBPβ opens up possibilities about the genes that may be regulated by p205, as well as how p205 is doing so. The transcription factor, c/EBPβ remains poised on multiple gene promoters in resting macrophages while upon induction by LPS, it initiates gene expression (250). c/EBPβ acts by binding to DNA, and recruiting other factors or co-activators to open up the chromatin structure to facilitate transcription. c/EBPβ can interact with NF-κB and other proteins for trans-activating gene expression. Interestingly, in our study we observed that p205 interacted with c/EBPβ in LPS-stimulated macrophages as well as in the resting cells. Hence, c/EBPβ and p205 cooperate in resting cells to sustain *Asc* gene expression that is expressed constitutively, whereas in context of inducible genes such as IL-1α or IL-6, c/EBPβ might collaborate with p205 to effectually initiate gene expression during stimulation, as indicated by the decreased mRNA levels of these genes in LPS-induced macrophages lacking p205. Similarly, NF-κB signaling via TLR4-LPS can induce p65/RelA collaboration with p205 that would enhance a LPS-mediated induction of Asc expression and other immune genes. p65/RelA induces gene expression by altering the chromatin signature towards successful transcription. It facilitates the recruitment of co-activator complexes (e.g. p300/CBP histone acetyltransferase complex), removal of inhibitory modifications, and recruits general transcriptional machinery to the gene. Hence, upon stimulation, p205, along with c/EBPβ, can recruit p65/RelA to generate a more accessible chromatin conformation that further augments inducible gene transcription (**Figure 4.2**).

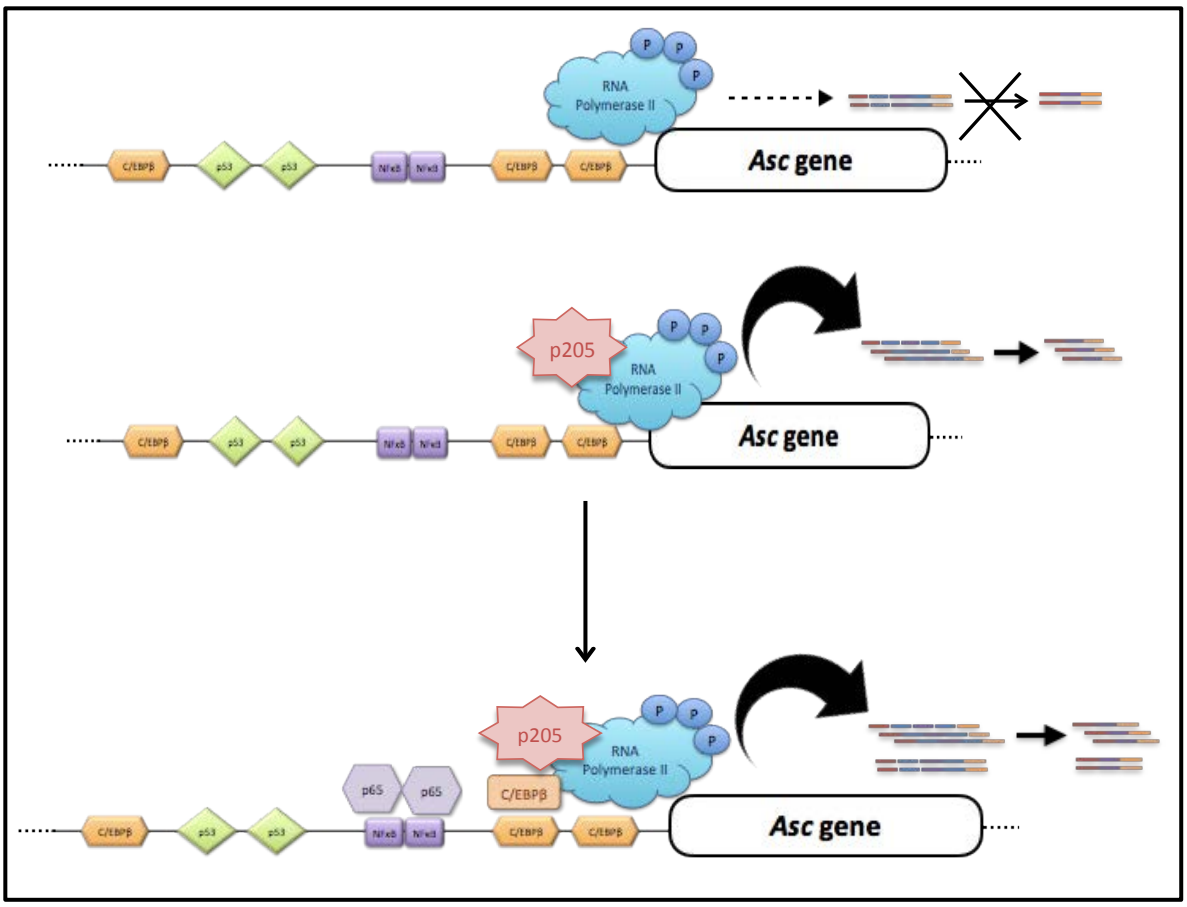

Figure 4.2: Schematic model describing the mechanism of action by which p205 regulates *Asc* gene expression by modulating transcription as well as mRNA processing.

In our study, we were unsuccessful in generating *p205*$^{-/-}$ knockout mice with ES-cell microinjection due to very low chimera percentage in the pups. Instead we used an *ALR*$^{-/-}$ mouse model that has the complete PYHIN/ALR locus deleted and hence, lacks the 13 PYHIN genes including *p205* (176). On testing primary bone marrow-derived macrophages and dendritic cells from these animals we observed a partial defect in the Nlrp3 and Nlrc4 inflammasomes as well. However, at first, we did not observe a strong effect on Asc protein expression in the primary macrophages and dendritic cells. Primary macrophages and dendritic cells are cultured in conditional media that contain Macrophage Colony Stimulating Factor (MCSF) and Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) that are responsible for differentiating hematopoietic stem cells, and thus stimulate multiple signaling pathways. Any such pathway, or a component of it (e.g. c/EBPβ), upon differentiation, can regulate signaling cascades that are otherwise controlled by p205 in the resting or LPS- and IFN-induced differentiated cells. However, in immortalized *ALR* $^{-/-}$ macrophages, Asc protein expression was markedly reduced, confirming our previous observations that p205 indeed regulates Asc expression. This can be due to multiple reasons including that immortalization of the cells augments the effect of p205 on Asc expression, which is not unlikely as both the proteins have been previously associated with tumor progression. Additionally, PYHIN proteins can interact with each other, and quite possibly be regulating themselves as well as similar pathways. Deletion of all 13 PYHIN genes could be overshadowing the singular effect attributable to p205. A point to be noted here, several PYHIN proteins, including p204, p205 have been reported to be induced and necessary during myeloid cell differentiation; hence, deletion of the complete PYHIN locus can change the make-up of

the primary macrophages and dendritic cells. However, the decrease in IL-1β production in the differentiating primary macrophages and dendritic cells was consistent across separate *ALR* $^{-/-}$ mice, which is in agreement with our observations that there is defect in the machinery responsible for IL-1β maturation upon Nlrp3 and Nlrc4 activation.

A previous study on ASC/TMS1 induction in breast cancer epithelial cells predicted the involvement of p65/RelA upon TNF-α stimulation but lacked experimental evidence. Using an *ASC* promoter reporter construct, 1254 bp upstream of the translation start site, they did not observe sufficient induction with TNF-α stimulation or p65/RelA transfection (321). They predicted that promoter regions distal to the translation start site would be involved. These observations agree with our study, where p205 could drive expression only from the Asc promoter construct containing up to 2000 bp upstream of the translation start site. Bioinformatics of the Asc promoter predicted transcription sites for c/EBPβ and NF-κB factors scattered within the gene sequence. Hence, it is likely that more than one DNA response element and a specific, folded secondary structure is essential to initiate effective *Asc* gene transcription.

So, which motifs of p205 are involved in mediating gene transcription? As mentioned earlier, the PYHIN proteins consist of at least one conserved 200 amino acid domain that is capable of binding DNA. Interestingly, HIN-A domain, as found in p205, is considered to bind single stranded DNA with higher affinity, compared to HIN-C domain of AIM2 and the combination of HIN-A and HIN-B domains of IFI16 that preferentially bind double stranded DNA (183). Thus, it is quite likely that the HIN

domain of p205 is responsible for binding to the transcriptional elements on the gene promoters. However, so far reports indicate that the binding of DNA by the HIN domains is sequence-independent, which is contradictory to how transcription factors act. Additionally, from our reporter studies we observed, that both pyrin and HIN domains of p205 were required to drive expression from the *Asc* gene luciferase reporter. This suggests that while p205 may bind the gene promoter DNA mediated by its HIN domain, the specificity of binding may be promoted by protein-protein interactions between the p205-pyrin domain with other factors. Hence, p205 binds to another transactivator or transcription factor and recruited to the gene promoter where it clamps on to the exposed single-stranded DNA in a sequence- independent manner.

For example, Pyrin protein, encoded by the gene *MEFV,* that forms inflammasomes and is the genetic basis for familial Mediterranean fever (FMF), is cleaved by caspase 1 and interacts with p65/RelA and other NF-κB factors to enhance NF-κB-mediated signaling (322). Intriguingly, a protease cleavage site prediction tool (ExPASy) predicts that p205 also has a single caspase 1 cleavage site within its pyrin domain, at Asp59 that would generate a N-terminal fragment, and it might interact with p65/RelA under stimulated conditions to induce gene transcription.

Future directions

Outstanding questions about the functions of p205 include determining the DNA sequence that is bound by p205 (by ChIP sequencing), the array of genes that are

regulated by p205, and whether it cell-type specific (using DNA microarray) and deciphering all the interacting partners of p205 (by Mass spectrometry) that form the transcriptional complex involved in gene regulation. Another model that can prove useful in studying the functions of p205 (independent of other PYHIN proteins) is the *ALR*$^{-/-}$ mouse; however, it has to be kept in mind, that PYHIN proteins may regulate each other and often, have been reported to be working in tandem to regulate different pathways (e.g. cell growth and proliferation, cell differentiation). Further, defining the functional homolog of p205 in humans that regulates Asc and other inflammatory genes would make the candidate an exciting therapeutic target in autoimmune and inflammatory disorders.

The innate immune system has evolved to detect microbial threats as well as self-derived danger molecules in the cells. In context of immune responses, Asc is expressed in resting cells and had been considered to be largely unaffected by microbial stimulations. However, expression of Asc is highly variable, and is often silenced, in context of cancers and tumors. Previously, p205 has been shown to regulate $p21^{CIP/WAF}$ expression in a p53-dependent manner (323). The tumor suppressor function of p205 may also be implemented through the action of Asc, which has been implicated in p53-mediated Bax-dependent mitochondrial apoptosis (324). This is supported by the observation that CRISPR/Cas9 mediated knockout of p205 in melanoma cell line also affected Asc expression potently. Thus, it is possible that Asc deficiency in murine tumors can be correlated to a lack of p205 expression. Also, p205 is inducible by type II interferon that is involved in viral infection as well as in DNA damage response. Hence,

it would be interesting to study the role of p205 in tumorigenesis, cell proliferation and/or cell death.

Humans have 4 PYHIN proteins including AIM2, IFI16, PYHIN1/IFIX and MNDA compared to 13 in mouse. The functional human ortholog of p205 is unknown. However, the convergence of genes in higher mammals signifies that their functions have converged as well. p205 shares high sequence homology with both PYHIN1/IFIX and human MNDA. Both PYHIN1/IFIX and MNDA contain a HIN200 subtype A motif. However, the functions of these proteins have remained unsolved. One study has reported that during viral infection, PYHIN1/IFIX can associate with other regulatory transcription factors to inhibit viral replication. On the other hand, multiple reports have observed differential expression of MNDA in tumor and cancer cell lines similar to gene silencing of ASC in tumors, and cancer patients. It is also interesting to note that phylogenetic analysis shows that p204, p211, p205 and p207 form a clade with human IFI16 and PYHIN1/IFIX in the pyrin domain tree, rather than other mouse proteins (172). Hence, depending on the cell-type and immune responses, the functions of p205 is likely carried out by one or more PYHINs in humans. The straightforward approach would be to determine the expression of these PYHIN proteins in cancer cell lines known to down-regulate ASC expression.

Caspase 8 as a Regulator of Inflammasomes Activation in Fungal Infections

Regulation of inflammation and inflammasomes by caspase 8 is context-dependent, and often contradictory. While caspase 8 itself can activate a non-canonical inflammasome, it is also involved in regulation of the canonical inflammasomes, inhibitory or activating. In BMDC infected with *Paracoccidioides brasileinsis*, NLRP3-dependent caspase 1/11 activation of inflammasomes is the major pathway but in our study we show that there is a parallel pathway regulated by activated caspase 8 that is also responsible for IL-1β release (**Figure 4.3**). Future studies will delineate the subtle interplay that occurs between caspase 1 and caspase 8 in regulating infection. In the absence of caspase 8 activity, there is higher caspase 1 being produced whereas caspase 8 induces higher IL-1β cleavage and secretion when caspase 1 is blocked. However, caspase 1 is activated primarily through the NLRP3 pathway and caspase 8 inflammasome is dependent on the dectin-1/Syk pathway. It will be interesting to study the crosstalk between the two pathways and whether they cooperate or compete with each other. Using a Syk inhibitor showed an effect on IL-1β and caspase 8 processing as well as a partial effect on caspase 1 proteolysis during *P. brasiliensis* infection. However, in dectin-1 deficient mice (*Clec7a*$^{-/-}$), *P. brasiliensis* infection caused reduction in IL-1β processing and release, but showed some amount of caspase 8 cleavage. Hence, further studies will be required to outline any other receptors that might activate the inflammasome as well as caspase 8 in fungal infection. The *in vivo* infection model of *P. brasiliensis* in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice clarified the critical involvement of caspase 8, but not Rip3, in controlling the fungal load in the lungs of animals that led to higher

inflammation in the lungs as well. At 30 days post-infection, *P. brasiliensis* infected *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice were also in worse conditions than *Rip3*$^{-/-}$ or WT mice, with the DKO mice showing symptoms of shivering and acute weight loss. Hence, caspase 8 is crucial in controlling fungal infection, and may be regulating inflammation and immune responses through more than one signaling pathway.

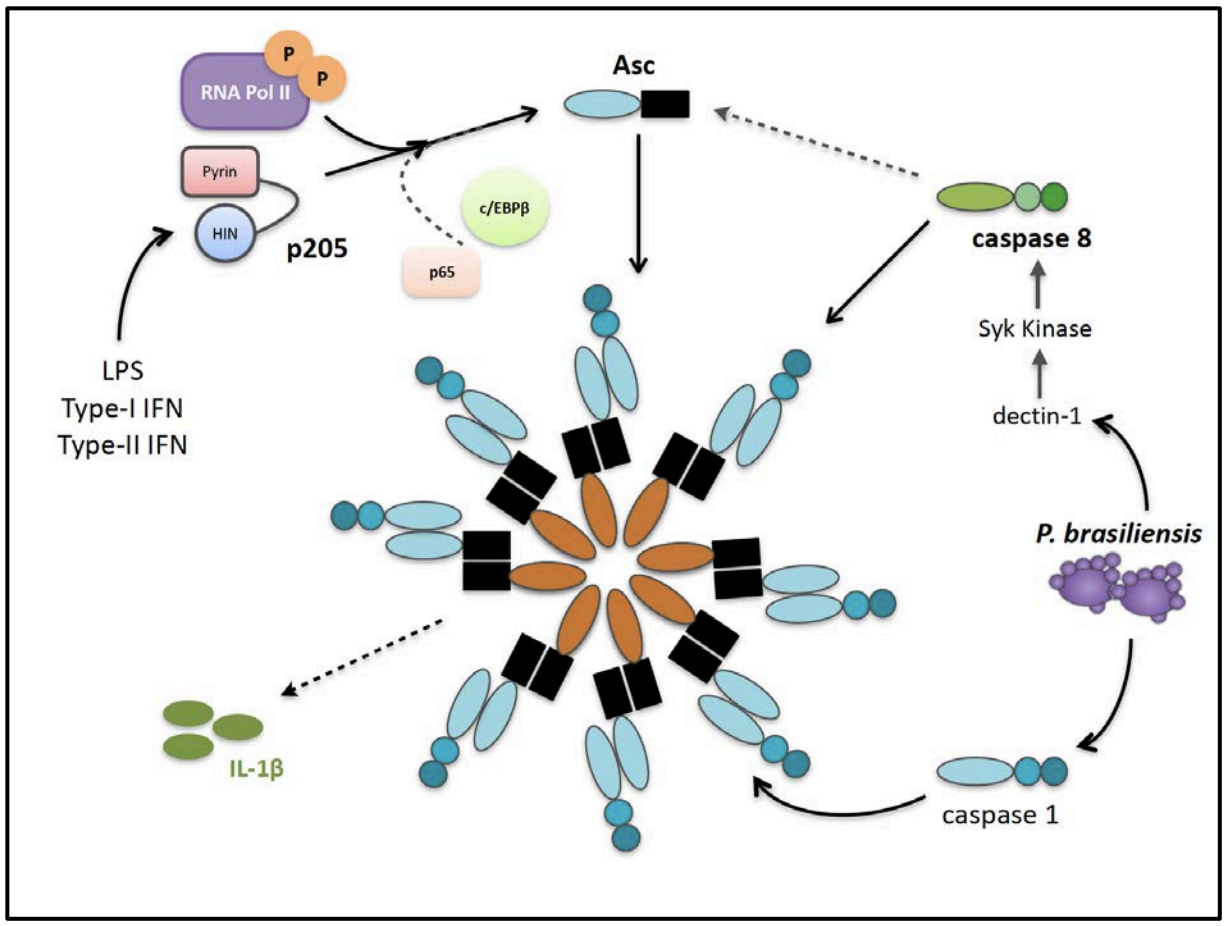

Figure 4.3: Regulation and Activation of the Inflammasome by p205 and Caspase 8. p205 maintains the expression of the inflammasome adapter protein, Asc in a RNA Polymerase II-dependent manner. Upon stimulation with LPS or Interferons, p205 can collaborate with other transcription factors like c/EBPβ and p65 to enhance Asc expression. Asc is required for activation of the inflammasome (e.g. NLRP3, AIM2) to induce IL-1β secretion, cell death and other inflammatory responses. Infection with the fungal pathogen, *Paracoccidioides brasiliensis* stimulates caspase 1- as well as caspase 8- dependent inflammasomes to activate IL-1β secretion. The caspase 8-dependent inflammasome requires dectin-1, Syk kinase and Asc. Caspase 8 also controls inflammasome activation by regulating the activity of mature caspase 1 (NLRP3 inflammasome).

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