TOLL LIKE RECEPTOR DEPENDENT CONTROL OF T HELPER 17 CELL DIFFERENTIATION AND INFLAMMASOME ACTIVATION

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DEDICATION

To my grandfather, for his everlasting love

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by

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Activation of pattern recognition receptors on dendritic cells (DCs) and macrophages leads to secretion of cytokines that control activation and differentiation of $CD4^+$ T cells. While IL-12 is critical for Th1 lineage development, IL-6 and TGF-β play an important role in differentiation of Th17 cells *in vitro*. IL-1 has also been implicated in regulating Th17 differentiation. In this dissertation, we examined the relative contribution of IL-6 and IL-1 for *in vivo* Th17 differentiation, and found that requirements for Th17 polarization depend entirely on the site of priming. While IL-6 plays a critical role in Th17 lineage priming in the skin and the mucosal tissue such as the gut and the lung, it is completely dispensable for Th17 priming in the spleen. IL-1R signaling in T cells is however necessary for Th17 priming in all tissues.

Moreover, the differential cytokine requirements for Th17 lineage commitment are guided by differential population of DCs resident in different tissues. These results reveal fundamental differences by which the systemic, mucosal, and cutaneous immune systems guide Th17 cell lineage commitment.

Pathogenic infections and tissue injuries trigger the assembly of inflammasomes, cytosolic protein complexes that activate caspase-1 leading to cleavage of pro-IL-1β/pro-IL-18, and pyroptosis, a pro-inflammatory cell death program. Although microbial recognition by Toll-like receptors (TLR) induces synthesis of pro-IL-1β and pro-IL-18, its role in inflammasome activation is insufficiently understood. In this dissertation, we have discovered that simultaneous activation of TLRs and NLRP3, which likely mimics natural infection by pathogenic microbes, triggers rapid caspase-1 cleavage, release of alarmins and pyroptosis. This acute caspase-1 activation is independent of new protein synthesis and depends on the TLR signaling molecule IRAK-1 and its kinase activity. Importantly, *Listeria monocytogenes* induces NLRP3-dependent rapid caspase-1 activation and pyroptosis, both of which depend on IRAK-1. These results demonstrate that simultaneous sensing of microbial ligands and virulence factors by TLRs and NLRP3 respectively leads to a rapid TLR- and IRAK-1-dependent assembly of the NLRP3 inflammasome, and such activation is important for release of alarmins, pyroptosis, and early IFN-γ production by memory T cells, all of which could be critical for early host defense.

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APC – Antigen presenting cell

ASC – Apoptosis-associated speck-like protein containing CARD

ATP – Adenosine triphosphate

BMDM – Bone marrow derived macrophage

CARD – Caspase recruitment domain

CFA – Complete Freund's adjuvant

CFU – Colony forming units

cGAS – cyclic GAMP synthase

CHX – Cycloheximide

DAPI – 4′,6-diamidino-2-phenylindole

DC – Dendritic cell

DKO – Double knockout

DLN – Draining lymph nodes

DMSO – Dimethyl sulfoxide

DPI – Diphenyleneiodonium Chloride

EAE – Experimental autoimmune encephalomyelitis

ELISA – Enzyme linked immunosorbent assay

FCS – Fetal calf serum

Foxp3 – Forkhead box P3

HBSS – Hank's Balanced Salt Solution

HMGB1 – High mobility group box 1

IFA – Incomplete Freund's adjuvant

IFN–Interferon

IκBα – NF-κB inhibitor α

IKK– IκB kinase

IL–Interleukin

IRAK– Interleukin-1 receptor associated kinase

IRF– Interferon regulatory factor

KD–Kinase Dead

KO – Knockout

LCMV– Lymphocytic choriomenigitis virus

LDH – Lactose dehydrogenase

LP – Lamina propria

LPL– Lamina propria lymphocytes

LPS–Lipopolysaccharide

MAPK – Mitogen-activated protein kinase

MAVS–Mitochondrial antiviral signaling protein

MDA5– Melanoma differentiation associated protein 5

MDP – Muramyl dipeptide

MHC–Major histocompatibility complex

mLN – Mesenteric lymph node

MSU– Monosodium urate

mtDNA – Mitochondria DNA

MyD88– Myeloid differentiation primary response 88

ND – Not detected

NFAT – Nuclear factor of activated T-cells

NF-κB – Nuclear factor of kappa light polypeptide gene enhancer in B cells

NIG – Nigericin

NK cell –Natural killer cell

NLRC– NOD-like receptor family, CARD domain containing

NLR–NOD-like receptor

NLRP– NOD-like receptor family, pyrin domain containing

NOD– Nucleotide-binding oligomerization domain

OVA–Ovalbumin

PAMP–Pathogen associated molecular pattern

PBS–Phosphate buffered saline

PCM–Peritoneal resident macrophage

PFU–Plaque forming unit

PI–Propidium iodide

PMA– Phorbol myristate acetate

PRR–Pattern recognition receptors

RA – Retinoic acid

RIG-I– Retinoic acid-inducible gene 1

RIP– Receptor-interacting serine/threonine-protein kinase

RLR–RIG-I-like receptors

ROS–Reactive oxygen species

SEM – Standard error of the mean

STAT– Signal transducer and activator of transcription

Sup – Supernatant

T3SS – type III secretion systems

TAK1– TGF-beta activated kinase 1

TCR–T cell receptor

TGF-β–Transforming growth factor β

Tg–Transgenic

Th–T helper

TIR–Toll/IL-1R

TLR–Toll-like receptor

TNFα–Tumor necrosis factor α

TRAF–TNF receptor associated factor

Treg–Regulatory T cell

TRIF– TIR domain containing adapter inducing interferon-β

VSV – Vesicular stomatitis virus

WCL – Whole cell lysate

WT – Wildtype

CHAPTER ONE
Introduction

OVERVIEW

Toll like receptors (TLRs) are innate immune receptors that recognize conserved microbial patterns and play profound roles in both innate and adaptive immunity. Upon activation, TLRs expressed by dendritic cells (DCs) trigger DC maturation, which involves upregulation of major histocompatibility complex (MHC) and costimulatory molecules, as well as production of various inflammatory cytokines and chemokines. These events play essential roles in activation of naïve T cells and directing their subsequent differentiation into effectors with distinct functions. T helper (Th) 17 cells, a highly inflammatory subtype of $CD4^+$ T cells, are involved in clearing extracellular bacteria that cannot be handled effectively by Th1 and Th2 cells. Th17 cells are also implicated in numerous autoimmune diseases. Ample progress have been made to understand the requirement and mechanism of Th17 differentiation *in vitro*. However, regulation of Th17 development by DCs and DC derived cytokines *in vivo* is still poorly understood.

Both commensals and pathogens harbor TLR ligands, thus making TLRs incapable of distinguishing commensal and pathogenic microbes. TLRs are transmembrane receptors that patrol the extracellular space and by extension, the endocytic, compartments. The function of TLRs are compensated by receptors in the cytosol that specifically detect pathogenic invasion. One of such cytosolic recognition systems is the inflammasome complex, which triggers caspase-1 activation, the protease required for the post translational activation of IL-1 family

members. It is therefore important to understand whether there is cooperation between the TLR and inflammasome pathways. TLR activation is known to induce upregulation of some inflammasome components, but whether TLR signaling plays a direct role in activating the inflammasome has not been studied.

TLRS AND THE CONTROL OF INNATE AND ADAPTIVE IMMUNITY

The ability to defend against foreign organisms is essential for multicellular organisms. The immune system evolved to recognize microbes, trigger host clearance mechanisms, and coordinate tissue repair afterwards. Based on its specificity and origin, immunity can be divided into two separate but related branches, innate immunity and adaptive immunity.

Innate immunity is present in all living organisms, and uses germline encoded receptors with fixed and limited varieties of specificities. Receptors for innate immunity recognize conserved molecules present broadly in microbes. These conserved microbial patterns, or pathogen associated molecular patterns (PAMPs), are recognized by the pattern recognition receptors (PRRs) including TLRs, nucleotide binding oligomerization domain-like receptor (NLRs), RIG-I like receptors (RLRs), C-type lectin like receptors (CLRs) and DNA sensors. These PRRs can be further classified into PRRs that monitor the extracellular compartment (TLRs and some CLRs) and PRRs that survey intracellular infections (RLRs, NLRs, and DNA sensors).

TLRs and control of innate immunity

TLRs are transmembrane PRRs consisting of the extracellular ligand binding leucine-rich repeats (LRRs) and the intracellular Toll/IL-1R (TIR) domain for signaling. There are 10 TLRs in humans and 13 TLRs in mice. The ligands for most TLRs have been identified. TLR2 dimerizes with TLR1 or TLR6 to recognize cell wall components from gram positive bacteria and yeast, as well as bacterial lipoproteins [2-4]. TLR4 recognizes lipopolysaccharide (LPS) [5], TLR5 recognizes bacterial flagellin [6], TLR3 and TLR7/TLR8 recognize double-stranded and single-stranded RNA, respectively [7-9], TLR9 recognizes unmethylated CpG motifs of DNA [10], mTLR11 and mTLR12 recognize profillin from the protozoa parasite *Toxoplasma gondii* [11, 12], and mTLR13 recognizes bacterial 23S ribosomal RNA [13] (Figure 1.1).

Upon ligand binding, the TIR domain of TLRs recruits downstream TIR domain containing signaling adaptors via homotypic interactions. At least six TIR signaling adaptors have been identified [14, 15]. Myeloid differentiation primary response 88 (MyD88) is the central signaling adaptor for most TLRs and receptors of IL-1 family members [16]. Recruitment of MyD88 initiates the recruitment of IL-1 receptor associated kinase (IRAK)-4, followed by IRAK-1 and IRAK-2, which contain TRAF6 binding motifs and therefore lead to its recruitment and activation [17-20]. TRAF6 processes ubiquitin E3 ligase activity, and is able to synthesize lysine 63 conjugated poly ubiquitin chains that facilitate the recruitment of TAK1 and the IKK complex [21, 22]. TAK1 phosphorylates and activates MAPKKs and IKK, which ultimately triggers activation of the transcription factors AP-1 and NF-κB, leading to the synthesis of proinflammatory genes [23]. Most TLRs use MyD88 as the sole signaling adaptor. However, TLR4 also uses an additional signaling adaptor TRIF [24-27], which recruits RIP1 and TRAF6 to mediate NF-κB activation [24, 28]. TRIF also has the unique

ability to recruit the adaptor TRAF3, which mediates the activation of IKKi/TBK1, leading to the activation of the transcription factor IRF3 and production of type I interferons (IFNs) [29-32],. TLR3 uses TRIF as its only signaling adaptor to activate a similar signaling pathway and IFN production [27] (Figure 1.1).

TLR engagement activates a variety of innate defense mechanisms. TLR-induced NF-κB and MAPK activation induces a host defense gene program including proinflammatory cytokines, chemokines and antimicrobial peptides, which can directly or indirectly contribute the pathogen clearance [33].

The TLR-MyD88-p38 pathway has been shown to promote bacterial phagocytosis through the induction of phagocytic genes such as scavenger receptors [34]. TLR activation also functions in gene induction-independent ways to promote phagocytosis. It has been shown that TLR2-MyD88-dependent signaling enhances the phagocytosis of *L. monocytogenes* by macrophages through activation of PI3K and the small Rho GTPase Rac1 [35]. Likewise, TLR recognition by TLR2/4 is required for phagocytosis of bacteria including *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus*, by regulating phagosome maturation [36]. While phagocytosis is essential for removal of extracellular organisms, the autophagy machinery, an emerging innate immune mechanism, mediates the destruction of organisms in the cytosol. Several groups have reported the induction of autophagy induced by TLR4, TLR7, TLR3, TLR2 and TLR5, and enhanced killing of the intracellular bacteria by the autophagy machinery [37-40]. MyD88 has been shown to interact with Beclin 1, a key protein for inducing autophagy [39]. The TRIF-RIP1-p38 pathway has also been suggested to regulate autophagy [40].

TLR signaling augments macrophage bactericidal activity through both cytosolic and mitochondrial reactive oxygen species (ROS). MyD88 is required for the assembly of NADPH oxidase, as it induces the phosphorylation of p47phox via p38. Consequently, MyD88-deficient macrophages are defective of NADPH oxidase-mediated production of cytosolic ROS, and exhibit a marked inability to kill Gram-negative bacteria [41]. Engagement of TLR1, TLR2 and TLR4 results in the recruitment of mitochondria to macrophage phagosomes and augments mitochondrial ROS production, in a manner dependent on TRAF6 and ECSIT, a protein implicated in mitochondrial respiratory chain assembly. ECSIT- and TRAF6-defeicient macrophages have decreased TLR-induced mitochondrial ROS and are impaired in their ability to kill intracellular bacteria [42].

TLRs and control of adaptive immunity

The adaptive immunity relies on T and B cell receptors generated through random genomic DNA recombination and hypermutation, therefore creating a much larger repertoire of receptors, with theoretically infinite possible specificities that can uniquely target one or a few microbes, thus enabling pathogen specific immunological memory. Due to the randomness of the DNA recombination and hypermunation processes, the resulting antigen receptors could be self-reactive. Although many self-reactive clones are eliminated during the development of T and B cells, the central tolerance mechanism is imperfect, as a breach of tolerance is seen in various autoimmune diseases. Moreover, all higher multicellular organisms are colonized with a complex microbiota, which provide essential benefits to the hosts. These commensals,

despite being nonself, must be immunologically tolerated to allow peaceful coexistence. More than two decades ago, Janeway postulated that the recognition of PAMPs by the innate immune system can provide an additional layer of self/nonself discrimination and renders the adaptive immune system able to react to pathogenic rather than innocuous antigens [43]. Increasing evidence have shown that innate immunity instructs adaptive immunity at multiple stages throughout the immune response.

PRRs expressed by the innate immune cells have the ability to instruct adaptive immune responses indirectly by inducing the upregulation of costimulatory molecules and the secretion of cytokines by the antigen-presenting cells (APCs). It is the crosstalk between APCs and T cells that shapes the subsequent T cell response. Priming of naive T cells can be induced only when TCR-MHC/peptide recognition ("signal 1") is accompanied by costimulatory signal ("signal 2") delivered by interactions between CD28 on T cells and CD80/CD86 on APCs [44]. Following activation, T cells differentiate into effector and memory T cells with properties best suited for clearing the pathogen. This process is directed by innate cytokines ("signal 3") generated by the PAMP-activated APCs. For instance, IL-12 is responsible for directing the T cell towards Thl cells [45]. Engagement of TLRs on dendritic cells (DCs) by PAMPs leads to up-regulation of both MHC and co-stimulatory molecules, a process termed DC maturation. Matured DCs then migrate to the draining lymph nodes and deliver all three signals to naive T cells to initiate pathogen-specific immune responses. In the absence of PAMP-induced activation of APCs, as what happens during homeostatic migration of DCs into the lymph nodes, the APCs present self-peptides to T cells, which results in tolerance of self-reactive T

cells [46, 47], or acquisition of properties of suppressor T cells [48, 49], which contribute to peripheral tolerance.

Although TLR is the first PRR studied in the context of initiation of adaptive immunity, all five classes of PRRs have been shown to link innate and adaptive immunity in different types of infections. TLRs alone can mediate innate control of adaptive immunity. However, TLRs often collaborate with other PRRs to achieve maximal adaptive immune responses. It has becoming increasingly clear that the type of PRR/PRR combinations stimulated by a particular pathogen or tissue damage is critical for the outcome of the immune response, such as the effector T cells generated, the mechanism the robustness of the memory response, and the type of immunoglobulin produced.

TLRs

TLRs are the best characterized PRRs in terms of studying innate control of adaptive immunity. TLRs promote phagocytosis, a process involving uptake of microbial antigens to allow them to be processed and presented on the surface of the APC [36, 50]. In addition, TLR activation is linked to the cytoskeleton network to promote transportation of MHC vesicles from the intracellular storage to the cell surface [51]. Also, TLR-induced NF-κB activation causes production of proinflammatory cytokines TNFα, IL-1, and IL-6, IL-12, IL-23, and so on. These mechanisms work in concert to initiate the adaptive T cell response.

It has been demonstrated, using mice lacking MyD88, that TLR-induced DC maturation during infection is essential for naive T cell activation. Mycobacterial extracts fail

to induce maturation of DCs derived from MyD88-deficient mice. Consequently, MyD88-deficient mice failed to induce T cell activation and IFN-γ production when immunized with antigens emulsified in complete Freund's adjuvant (CFA), which contains heat killed *Mycobacterium* [52]. TLR signaling via MyD88 has be shown to be essential for IL-12 production and Th1 immunity in response to bacterial pathogens such as *Mycobacterium avium* [53], viruses such as herpes simplex virus type 2 [54], fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus* [55], and protozoa pathogens such as *Leishmania major* and *toxoplasma gondii* [56, 57]. MyD88 has also been shown to be important for cross presentation and priming $CD8^+$ T cells for optimal IFN-γ production and killing activity [58].

Another mechanism by which DCs control T cell activation is by overcoming regulatory T cell (Treg) mediated suppression. DCs from MyD88-deficient mice can still undergo maturation in response to LPS through the TRIF pathway, but fail to produce proinflammatory cytokines [59]. These DCs are unable induce effective priming of $CD4^+$ T cells. Further study reveals that IL-6 produced by DCs upon TLR ligation is required to overcome the repression of Treg cells, and is required for inducing priming of $CD4^+$ cells [60].

CLRs

The most important CLRs implicated in innate control of adaptive immunity are Dectin-1, Dectin-2, mincle and Clec9. Dectin-1 recognizes β-(1,3)-glucans such as zymosan [61], and Dectin-2 recognize α-mannans, both of which are major component of the fungal cell wall [62]. Mincle recognizes mycobacterial cord factor, as well as necrotic cells and an

unknown ligand from the fungus *Malassezia* [63-65]. Clec9 recognize F-actin released by necrotic cells [66, 67].

CLRs use the adaptor protein tyrosine kinase Syk, which recruits and activates the CARD9/Bcl10/Malt1 complex and leads to activation of MAP kinases, NF-κB, and NFAT [68-72]. Like TLRs, activation of CLRs in DCs is sufficient for the induction of adaptive immunity. Dectin-1 activated DCs up-regulate costimulatory molecules, produce TNF-α, IL-6, IL-2, IL-10, and IL-23, and are able to prime $CD4^+$ T cells and instruct their differentiation [68, 73]. Dectin-1 activation in DCs appears to favor the induction of Th17 responses, as it induces IL-23 but little IL-12 [68]. During pulmonary infection with *Aspergillus fumigatus*, Dectin-1 signaling inhibits innate IFN-γ and IL-12p40 production, thus enhancing Th17 differentiation by repressing Th1 differentiation of *A. fumigatus*–specific $CD4^+$ T cells [74]. The mechanism by which Dectin-1 suppresses the Th1 inducing capacity of DCs is not entirely clear, but it has been shown that Dectin-1 can induce SOCS1, which down-regulates TLR signaling and hence IL-12 production [75]. Adjuvants that stimulate the Dectin-1 pathway also promote DCs to prime $CD8^+$ T cell responses [76].

Similar to Dectin-1, Dectin-2 also constitutes a major fungal PRR coupled to the Syk-CARD9 pathway to activate DCs and regulate adaptive immune responses. In a model of *Candida albicans* infection, blockade of Dectin-2 abrogates *Candida*-specific Th17 responses and, in combination with Dectin-1 deficiency, decreased *Candida*-specific Th1 responses [77].

Mincle recognition of mycobacterial cord factor controls the Th1/Th17 adjuvanticity of mycobacterial cord factor [78]. Recognition of mycobacterial cord factor by mincle via CARD9 is also necessary for CFA-induced Th17 polarization [79].

Clec9A activation in DCs favors cross-priming of $CD8^+$ T cells specific for antigens from necrotic cells [80]. Clec9A also regulates cross-presentation against cytopathic viruses, such as vaccinia virus, by detecting the Clec9A ligand in dying infected cells [81].

RLRs

RLRs comprise three members: RIG-I, MDA5 and LGP2. RIG-I and MDA5 serve as PRRs [82, 83], while LGP2 is thought to play a regulatory role [84]. Both RIG-I and MDA5 contain a helicase domain mediating the recognition of short and long double-stranded RNAs, respectively [83, 85]. RIG-I is also activated by single-stranded RNA with 5'-triphosphate [86, 87]. RIG-I and MDA5 signal via the mitochondrial localized adaptor MAVS, and induce activation of NF-κB as well as IRF3/7-dependent antiviral genes [88]. RIG-I can also trigger an MAVS-independent pathway via signaling adaptor ASC, and leads to the production of IL-1β by caspase-1 [89].

RNA is a PAMP that can be sensed by the TLR or RLR system in a cell type-specific manner. The TLRs system is primarily utilized in plasmacytoid DCs, while the RLR system is used by conventional DCs and fibroblasts [90]. TLRs and RLRs have overlapping yet not identical functions in terms of controlling adaptive immunity. Depending on the particular immune challenge, TLR or RLR alone is able to induce adaptive immunity. However, optimized adaptive immunity is often achieved via the cooperative effects between TLRs and RLRs. It has been demonstrated that the $CD8^+$ T cell response to lymphocytic choriomenigitis virus (LCMV) infection is entirely dependent on TLRs, with RLRs dispensable. This is

probably because LCMV is primarily recognized by plasmacytoid DCs via TLRs *in vivo*, as shown by the largely MyD88-dependent type I IFN production [91]. During influenza A infection, however, the virus-specific $CD4^+$ T cell response and antibody response are strictly dependent on TLRs instead of RLRs, whereas induction of virus-specific $CD8^+$ T cells is partially dependent on both TLRs and RLRs. Importantly, the TLR pathway is necessary long-term protection against reinfection [92]. When poly I:C, a double-stranded RNA analog, is used as an adjuvant, antigen-specific antibody production as well as $CD8^+$ T cell priming is dependent on both the TLR signaling adaptor TRIF and the RLR adaptor MAVS, suggesting that the adjuvant effects of poly I:C require cooperative activation of TLR and RLR pathways [93]. Interestingly, in the case of respiratory syncytial virus (RSV) infection, neither the TLR nor the RLR system is necessary for generation of $CD8^+$ T cell responses and protection against re-challenge [94]. The mechanism for TLR/RLR-independent CD8 priming during RSV infection remains unclear. One suggested mechanism is that the tissue damage triggered by replicating RSV can be sensed by other receptors, potentially Clec9A, to induce CD8 T cell activation independent of TLRs and RLRs (Hua Wang, personal communication).

NLRs

NLRs contain as C-terminal LRR domain, a nucleotide oligomerization domain, and one of four N-terminal effector domains that mediate downstream effects. With respect to innate control of adaptive immunity, the best characterized NLRs are NOD1 and NOD2, which recognize bacterial cell wall components γ-D-glutamyl-meso-diaminopimelic acid (meso-DAP)

and muramyl dipeptide (MDP), respectively [95-97]. Upon ligand binding, both NOD1 and NOD2 recruit RIP2 via their N-terminal CARD domains, which in turn results in the activation of MAPK and NF-κB and subsequent induction of inflammatory genes [98, 99]. In addition, NOD2 can also induce type I interferon in response to viral infections by activating the MAVS pathway [100].

NOD1 and NOD2 activated DCs upregulate costimulatory molecules, and produce inflammatory cytokines such as TNF-α, IL-6, IL-8, IL-10, and IL-12, rendering their ability to prime T cells [101, 102]. Consequently, NOD1 and NOD2 have been shown to promote Th1, Th17 and antigen-specific antibody responses. Innate immune sensing of peptidoglycan by NOD1 has been shown be required for optimal generation of T cell and antibody responses *in vivo*, when CFA is used as the adjuvant. NOD1 stimulation alone is sufficient to drive antigen-specific immunity with a predominant Th2 polarization profile. However NOD1 synergizes with TLRs to elicit Th1, Th2, as well as Th17 pathways [103]. Similarly, NOD2 activation alone triggers a Th2-dominated response, characterized by the induction of IL-4 and IL-5 by T cells and IgG1 antibody responses. Combined TLR and NOD2 activation, however, is able to induce both Th1 and Th2 type responses [104]. Another study shows that NOD2-deficient mice have impaired antibody responses to protein immunization when the NOD2 ligand MDP is used as the adjuvant [105]. Moreover, NOD1/NOD2 stimulation in DCs has been shown to facilitate cross-priming of antigen-specific $CD8^+$ T cells by $CD8^+$ DCs [102].

Another large group of NLRs are involved in the activation of the inflammasome, a protein complex responsible for activating caspase-1 and processing IL-1β and IL-18. IL-1β and IL-18 are pleiotropic cytokines for the instruction of adaptive responses. Therefore,

inflammasomes play an important role in innate control of adaptive immunity. These concepts will be discussed in the following sections.

DNA sensors

Cytosolic AT-rich DNA can be sensed indirectly by the RIG-I/MDA5 pathway upon transcription into RNA intermediates by RNA polymerase III [106]. More broadly, cytosolic DNA *per se* can be sensed by cytosolic DNA sensors. A number of DNA sensors have been identified, including DAI [107], RNA polymerase III [106], LRRFIP1 [108], hIFI16 (p204 in the mouse) [109], and DNA-dependent protein kinase [110]. The most potent DNA sensor is cyclic GAMP synthase (cGAS). Upon sensing DNA, cGAS synthesizes the secondary messenger cyclic GAMP, which activates the ER localized signaling molecule STING, leading to IRF3 activation and induction of IRF3 and NF-κB activation [111-113].

Adaptive immune responses to DNA vaccines are largely TLR9-independent, and are instead dependent on TBK-1 and type I IFN, suggesting that cytosolic DNA sensors play predominant role in mediating adaptive immune responses in response to DNA vaccines [114, 115]. Using mice deficient for cGAS, it has been demonstrated that cGAS required the adjuvant effect of double-stranded DNA for antigen-specific T cell activation and antibody production [116].

TH17 CELLS

Upon antigenic activation, naive $CD4^+$ T cells differentiate into different lineages of effector cells characterized by their distinct cytokine profiles and abilities to mediate particular types of immune responses. Initially, based on cytokine phenotypes, two effector T helper (Th) cell subsets were proposed: Th1 and Th2. IL-12 and IFN-γ promote the differentiation of Th1 cells that produce IFN-γ, which is important for fighting against intracellular pathogens. IL-4 induces differentiation of Th2 cells that produce IL-4, IL-5, and IL-13, which are important for immunity to parasitic infections and causing allergic diseases such as asthma [117]. In 2005, the IL-17 producing $CD4^+$ T cells, termed Th17 cells, were identified as a distinct subtype of T helper cells [118, 119]. Th17 cells produce the signature cytokines IL-17A (IL-17), IL-17F and IL-22, and are primarily responsible for clearance of extracellular pathogens not effectively handled by either Th1 or Th2 cells, as well as mediating tissue inflammation associated with autoimmune and autoinflammatory diseases [120].

Differentiation of Th17 cells

The combination of IL-6 and TGF-β induces the development of Th17 cells [121-124]. Several lines of evidence have highlighted the central role of TGF-β in Th17 differentiation. First, TGF-β transgenic animals develop exacerbated experimental autoimmune encephalomyelitis (EAE) owing to enhanced frequencies of Th17 cells [123]. Second, mice whose T cells are unresponsive to TGF-β are protected from EAE due to the lack of Th17 cells [125]. Similar observations are also made in mice defective of TGF-β production in T cells [126]. More recent studies show that TGF-β plays a dual role in Th17 development. At

lower concentrations, together with IL-6 or IL-21, TGF-β synergistically induces IL-23R and thus promotes Th17 cell differentiation in the presence of IL-23. However, at higher concentrations, TGF-β inhibits IL-23R, IL-22 and IL-17 expression and favors induction of Foxp3 and, thus, induce Treg differentiation [127].

In addition to IL-6, IL-21, IL-23 and IL-1 have also been shown to play a role in the differentiation of Th17 cells [124, 128-131]. IL-21 can, in combination with TGF-β, induce Th17 differentiation in the absence of IL-6, providing an alternative pathway for priming Th17 cells [128, 129, 131]. IL-21 is also required for optimal Th17 differentiation, as IL-21 receptor-deficient T cells show reduced Th17 differentiation in response to IL-6 and TGF-β [128]. IL-21 is induced by IL-6 signaling in $CD4^+$ T cells, and has been shown to induce RORγt ,IL-17A and IL-17F, thus amplifying the differentiating Th17 cells [132].

IL-23 p19-deficient mice lack Th17 cells and fail to develop EAE, suggesting an important role for IL-23 in the development of Th17 cells [133]. However, naïve $CD4^+$ T cells do not express the IL-23 receptor. Consequently, IL-23 fails to induce differentiation of naïve $CD4^+$ T cells towards the Th17 lineage *in vitro* [132]. Both IL-6 and IL-21 are strong inducers of IL-23R in T cells [132]. IL-23 signaling can also induce the expression of its own receptor [134]. As a result, IL-23 acts on already differentiated Th17 cells, and promotes their terminal differentiation and maintenance [135]. This notion is supported by the fact that Th17 cells generated with TGF-β1 and IL-6 produce IL-17 but do not readily induce autoimmune disease without further exposure to IL-23 [136]. IL-23 suppresses the secretion of IL-10 by Th17 cells [136], at least in part via the induction of TGF-β3, which induces Th17 cells that are

functionally and molecularly distinct from TGF-β1-induced Th17 cells, with a molecular signature similar to pathogenic effector Th17 cells in autoimmune disease [137].

Based on these findings, it has been proposed that three steps are required for Th17 development: induction, amplification and stabilization/maintenance. First, the differentiation is initiated by the combined actions of IL-6 and TGF-β1, which simultaneously induces IL-21 expression. Second, the amplification of the Th17 response is driven by IL-21 from developing Th17 cells. Lastly, the stabilization/maintenance of the Th17 phenotype is achieved by IL-23 acting on IL-23 receptor induced in the first two steps. In brief, a sequential involvement of IL-6, IL-21 and IL-23 leads to complete differentiation of Th17 cells (Figure 1.2).

The role for IL-1 in Th17 differentiation, however, is less clear. Mice defective of IL-1R1 signaling have been shown to be resistant to EAE due to a severe defect in the generation of Th17 cells [138], suggesting that IL-1 is important for mouse Th17 cell regulation *in vivo*. IL-1R1-deficient T cells have no defect in Th17 development in response to *in vitro* polarization with IL-6 and TGF-β [139]. Instead, it has been suggested that IL-1 expands and maintains Th17 cell program, as well as regulates Th17 cell cytokine production [139].

Transcriptional control of Th17 cells

In 2006, the orphan nuclear receptor RORγt was shown to be the key transcription factor regulating Th17 development [140]. Since then, at least six transcription factors have been shown to play important roles in Th17 development.

RORγt. RORγt is selectively expressed in Th17 cells, and introduction of RORγt into naive T cells induces the development of Th17 cells [140]. Conversely, loss of RORγt in T cells prevents the generation of Th17 cells and subsequent development of EAE or colitis [140, 141]. *Il17a* is a direct target gene of RORγt [142, 143]. Another ROR family member, RORα, is also sufficient to induce Th17 differentiation *in vitro*, although it only plays a minor role in Th17 differentiation *in vivo* [143].

STAT3. IL-6, IL-21 and IL-23 signaling all engage the JAK-STAT pathway and activate STAT3, suggesting that STAT3 plays an essential role in Th17 cell differentiation [144]. STAT3 is required for induction of RORγt by cytokines. STAT3 also binds to the *Il17* promoter directly and acts together with RORγt to induce maximal IL-17 expression [132, 145]. In STAT3-deficient $CD4^+$ T cells, induction of IL-21 and IL-23R by IL-6 and TGF-β is greatly diminished. Likewise, IL-17 expression induced either by IL-6 plus TGF-β or IL-21 plus TGF-β is also impaired [129, 132].

IRF4. IRF4-deficient $CD4^+$ T cells fail to differentiate into Th17 cells, and IRF4 KO mice are protected from EAE [146]. RORγt and RORα induction are impaired in IRF4-deficient T cells, but their forced expression can partially restore induction of IL-17, suggesting that IRF-4 may function upstream of the nuclear receptors [146, 147]. IRF-4 regulates the expression of IL-21 and IL-23R induced by IL-21, and is therefore essential for the self-amplifying function of IL-21 [146, 147].

BATF. BATF is an AP-1 family transcription factor. BATF-deficient mice have selective defect in Th17 differentiation, and are resistant to EAE [148]. BATF-deficient T cells fail to induce RORγt and IL-21 in response to Th17 polarizing conditions [148]. Furthermore,

addition of IL-21 or forced expression of RORγt cannot restore IL-17 production in BATF-deficient T cells [148]. It has been shown recently that IRF4 can cooperate with BATF to bind to AP1-IRF4 composite elements in $CD4^+$ T cells activated under Th17 polarizing conditions, and regulate important functional genes such as *Il17a*, *Il21* and *Il10* [149, 150].

c-Maf. Th17 cells been shown to express c-Maf, a transcription factor involved in regulation of Th2 cell differentiation. c-Maf-deficient $CD4^+$ T cells are defective in IL-21 production and IL-23R expression, and are less capable of differentiating into Th17 cells [151]. In Th17 cells, TGF-β regulates IL-22 and IL-17 differently. IL-6 induces the production of only IL-22, whereas the combination of IL-6 and high concentrations of TGF-β results in the production of IL-17 but not IL-22 from T cells. c-Maf induced by TGF-β serves as a direct repressor of *Il22* [152].

AhR. Aryl hydrocarbon receptor (AhR) is a transcription factor activated by its ligand such as dioxin or FICZ, a UV photoproduct of tryptophan [153]. AhR is required for IL-22 and, in part, IL-17 expression under Th17 polarizing conditions in the presence of its ligand [154]. However, the precise contribution of AhR to Th17 cell differentiation is unclear.

A regulatory network for Th17 cell specification has recently been proposed. In this model, early cooperative binding of BATF and IRF4 contribute to initial chromatin accessibility and, with STAT3, initiate a transcriptional program that is then globally tuned by the lineage-specifying TF RORγt, which plays a focal deterministic role at key loci. Unexpectedly, c-Maf in Th17 cells functions mainly as a negative regulator that suppresses the expression of pro-inflammatory loci (*Rora, Runx1*, *Il1r1*, *Ccr6*, *Tnf*) and globally repressing genes in pathways regulated by other core transcription factors [155].

Reciprocal relationship between iTreg and Th17 cells

TGF-β induces the Treg-specific transcription factor Foxp3 and hence the generation of induced Treg cells. However, addition of IL-6 to TGF-β inhibits the generation of Treg cells and induces Th17 cells. These observations suggest a reciprocal relationship between Treg cells and Th17 cells, and that IL-6 has a pivotal role in dictating the balance between these two cell populations [123]. Mechanistically, TGF-β induces both RORγt and Foxp3. Foxp3 physically interacts with RORγt and RORα and can thus inhibit their transcriptional activities. The inhibitory effect of Foxp3 is antagonized by inflammatory cytokines such as IL-6 or IL-21 [127]. Importantly, TGF-β regulates Th17 cell differentiation in a concentration-dependent manner. Low concentrations of TGF-β synergize with IL-6 and IL-21 to promote *Il23r* expression, thus favoring Th17 cell differentiation. At high concentrations, however, TGF-β represses *IL23r* expression and favor Treg cell development [127].

Other factors have also been implicated in tipping the balance between Tregs and Th17 cells. For instance, IL-2 signaling through Stat5 acts as a growth factor for Treg cells, and in the meantime inhibits the generation of Th17 cells [156]. Retinoic acid (RA), a vitamin A metabolite, can drive the generation of Treg cells by enhancing TGF-β signaling and enhancing Foxp3 promoter activity while suppressing the differentiation of Th17 cells through the inhibition of IL-6 signaling [157]. HIF1α expression by $CD4^+$ T cells promote the generation of Th17 cells, and HIF1α-deficient $CD4^+$ T cells differentiate into Treg cells instead of Th17 cells under Th17 polarizing conditions [158].

Effector functions of Th17 cells

The IL-17 family includes six members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F, all of which are 20 to 30 kDa cytokines with four conserved C-terminal cysteine residues. IL-17A and IL-17F share the highest sequence homology (50%), whereas IL-17B, IL-17C and IL-17E differ considerably from IL-17A and IL-17F in their N-terminus, with IL-17E being the least homologous to IL-17A (16% identity) [159]. Although IL-17A and IL-17F are often coexpressed in Th17 cells, there are also T helper cells that express only IL-17A, IL-17F or IL-17A–IL-17F heterodimer. Both IL-17A and IL-17F homodimers, as well as the IL-17A–IL-17F heterodimer signal through the IL-17RA/IL-17RC heterodimer, and share overlapping functions. They induce the production of proinflammatory cytokines (TNF, IL-1, IL-6, G-CSF, and GM-CSF), chemokines (CXCL1, CXCL5, IL-8, CCL2, and CCL7) and metalloproteinases (MMP1, MMP3, and MMP13), and trigger granulopoiesis as well as the recruitment of neutrophils [120]. IL-17A and IL-17F could also display different functions. Using *Il17a*$^{-/-}$, *Il17f*$^{-/-}$, and *Il17a*$^{-/-}$*Il17f*$^{-/-}$ mice, it has been shown that IL-17F play marginal roles in delayed-type and contact hypersensitivities, EAE, and rheumatoid arthritis. In contrast, IL-17F and IL-17A both contribute to host defense against *Staphylococcus aureus* and *Citrobacter rodentium* infections [160]. IL-17B and IL-17C can also induce the expression of proinflammatory cytokines and neutrophil recruitment, and play similar roles as IL-17A and IL-17F in certain diseases, although their source is less clear [161]. Unlike other IL-17 family members, IL-17E is mainly involved in promoting eosinophilia and

regulating type 2 immune responses [162, 163]. Th17 are not the major source IL-17B, IL-17C and IL-17E. IL-22, another Th17 effector cytokine and an IL-10 family member, acts primarily on epithelial cells to induce production of antimicrobial peptides and promote tissue repair [164].

Th17 cells are important for host defense against primarily extracellular pathogens and fungi. Studies using IL-17 depletion have demonstrated important roles of Th17 cells in mediating host protection against various pathogens, including *Mycobacterium tuberculosis* [165], the Gram-positive bacteria *Propionibacterium acnes* [166], the Gram-negative negative bacteria *Citrobacter rodentium* [121], *Klebsiella pneumonia* [167], *Bacteroides fragilis* [168] and *Borrelia burgdorferi* [169], and fungi such as *Candida albicans* [170]. As discussed before, the PRRs Dectin-1 or NOD1 combined with TLR favor the activation of Th17 responses [68, 103].

Th17 cells in autoimmune diseases

Th17 cells have diverse pro-inflammatory effects and induce tissue damage during the course of various autoimmune diseases. IL-17-deficient mice develop attenuated collagen-induced arthritis and EAE [171]. Increased levels of IL-17 have been shown to associate with rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and psoriasis [172-175]. Similar to IL-17, IL-22 is overexpressed in psoriasis, and induces keratinocyte proliferation [176]. IL-22 is also required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation [177]. Additionally, IL-22 produced by Th17 cells mediates IL-23-

induced dermal inflammation and acanthosis [178]. These data indicate that Th17 cells are potent inducers of autoimmunity through the promotion of tissue inflammation and the mobilization of the innate immune system.

INFLAMMASOME: A SPECIALIZED DETECTOR OF PATHOGENIC MICROBES

The inflammasome was originally identified as a molecular platform mediating the post-translational activation of the IL-1β and IL-18 [179], and later shown to exert broader roles including the secretion of leaderless peptides [180], release of lipid mediators [181], and causing a programmed necrotic cell death termed pyroptosis [182]. IL-1β and IL-18 are synthesized as inactive precursors in the cytosol, and depend on proteolytic processing to be converted to their respective active forms. Several proteases have been shown to cleave IL-1β and IL-18, but the cysteine protease caspase-1 is the most common and effective one [183-186]. Caspase-1 exists in the in the cytosol in its inactive form, and requires signal-dependent activation to gain its catalytic activity. The inflammasome is a multiprotein complex responsible for activating caspase-1 [179]. The inflammasome is composed of an upstream sensor, an adaptor protein ASC (with the exception of NLRC4 inflammasomes), and the effector caspase-1. Most of the upstream sensors belong to the NLR family, comprised of a nucleotide binding domain (NBD), leucine-rich repeats (LRRs) and an effector domain (CARD or pyrin) [187]. The LRR domain senses variable insults, which causes the assembly of NLRs into an oligomerized state via the NBDs. This oligomerization is followed by the recruitment and oligomerization of Caspase-1, either directly through the homotypic

interactions between the CARD domains of the NLR and caspase-1, or indirectly through the pyrin domain of NLR and the CARD-pyrin containing adaptor protein ASC. Oligomerized Caspase-1 then undergo trans autoproteolysis and generate two fragments, p10 and p20. The active form of caspase-1 is a tetramer composed of two p10 and two p20 subunits, which together form the catalytic site [187]. A number of inflammasomes have been identified, including the NLRP3, NLRP1, NLRC4, NLRP6, NLRP7, and AIM2 inflammasomes. NLRP3 senses a broad spectrum of stimuli, both microbial and non-microbial, which will be discussed in the next section. NLRP1 senses the lethal toxin (LeTx) from *Bacillus anthracis*, the causative agent of anthrax [188]. NLRC4 is activated by bacterial flagellin or components of the bacterial type III secretion systems (T3SS) [189, 190]. The specificity of ligand recognition by NLRC4 is determined by differential pairing with another class of NLRs termed the NIAPs. In the mouse, NIAP5/6 facilitate recognition of flagellin, while NIAP1/2 assist recognition of T3SS components, such as PrgJ from *Legionella* [1, 191]. The NLRP6 inflammasome detects unknown ligands that stimulate host defense program against *Prevotellaceae* [192]. The NLRP7 inflammasome recognizes microbial acylated lipopeptides [193]. The NLRP12 inflammasome recognize unknown ligands from *Yersinia pestis* [194]. The AIM2 inflammasome is activated by cytosolic DNA, such poly dA:dT [195]. (Figure 1.3)

The NLRP3 inflammasome

Activators of NLRP3 inflammasome

The NLRP3 inflammasome senses a variety of stimuli. The activators of NLRP3 inflammasome include molecules that accumulate during tissue damage and sterile inflammation, bacterial toxins, and numerous other microbial insults.

(1) ATP, nigericin, maitotoxin

ATP activates the ATP-gated cation channel P2X7 receptor; and leads to the opening of larger pore involving pannexin-1, a hemichannel that associates with P2X7 [196]. These events trigger K^+ efflux and lead to activation of NLRP3. Nigericin and matotoxin, two marine toxins, can directly forms pores on the cell surface and induce similar K^+ efflux and subsequent NLRP3 activation [197].

(2) DNA/RNA

Although cytosolic DNA delivered by transfection activates the AIM2 but not NLRP3 inflammasome, internalized adenoviral DNA induces NLRP3-dependent caspase-1 activation [198]. Furthermore, oxidized mitochondria DNA (mtDNA) released by damaged mitochondria activates the NLRP3 inflammasome as well [199]. Bacterial RNA also activates the NLRP3 inflammasome [200, 201]. Cytosolic RNA sensing upstream of NLRP3 is mediated by the helicase DHX33, which interacts with NLRP3 and form the inflammasome complex following stimulation with RNA [202].

(3) Crystalline substances

Many crystals, including asbestos, silica, monosodium urate crystals, calcium pyrophosphate dehydrate crystals, β-amyloid and alum have been shown to activate the NLRP3 inflammasome [203-205]. NALP3 activation by these crystals require phagocytosis,

and this uptake subsequently leads to lysosomal damage and rupture, which ultimately induces NLRP3 activation [203].

(4) Cell volume

A recent study has shown that macrophage swelling upon exposure to hypotonic solutions is interpreted as a damage signal by the host, which triggers a K^+-dependent conformational change NLRP3 and, together with stimulation of the TRP channels TRPM7 and TRPV2, induces NLRP3 inflammasome activation [206].

(5) Endogenous proteins

Necrotic cells can activate the NLRP3 inflammasome and contribute to sterile inflammatory response [207]. Necrotic cells are known to release DAMPs such as uric acid crystals and ATP [208, 209]. Additionally, histones released by necrotic cells can also to activate the NLRP3 inflammasome in a manner depending on reactive oxygen species (ROS) production [210].

(6) Bacterial toxins and effector proteins, viral/ fungal infection

Many pathogenic microbes have been shown to be able to activate the NLRP3 and other inflammasomes through various mechanisms. Table 1.1 summarizes the pathogens that activate the inflammasome. Mice deficient in inflammasome sensors, the inflammasome adaptor ASC and caspaseo-1 exhibit altered susceptibility and/or pathology during infection, underscoring the importance of the inflammasome in host response to these pathogens.

Mechanisms of NLRP3 inflammasome activation

The NLRP3 inflammasome assembles in response to extracellular ATP, toxins, or crystals only in the presence of proinflammatory stimuli such as TLRs, NLRs and proinflammatory cytokines (TNF or IL-1). It has been suggested that the proinflammatory stimuli prime cells to induce NLRP3 expression through NF-κB activation, which is necessary but not sufficient for NLRP3 activation, and a second stimulus such as ATP- or crystal-induced damage is required for NLRP3 activation [211].

Given the variety of NLRP3 agonists, it is generally accepted that they do not act as direct ligands for NLRP3 but instead all converge on a few forms of change in host cell physiology that are sensed by the NLRP3 inflammasome. Lysosomal damage, ROS generation, K^+ efflux and $Ca2^+$ flux have all been suggested as common mechanisms in NLRP3 inflammation activation. However, a universal mechanism for NLRP3 activation remains unknown.

The mitochondria have been implicated to be essential for activation of the NLRP3 inflammasome. Previous studies have suggested that NLRP3 translocates from the cytosol to the mitochondria during NLRP3 inflammasome activation, which appears to be essential for optimal NLRP3 inflammasome activation [212]. The mitochondrial protein MAVS has been shown to promote this process and is therefore required for optimal NLRP3 inflammasome activation [212]. A separate study shows that NLRP3 translocation is dependent on acetylated α-tubulin and the inactivation of the NAD^+-dependent α-tubulin deacetylase SIRT2 [213]. The mitochondrial protein mitofusin 2, a mediator of mitochondrial fusion, is required for NLRP3 inflammasome activation after RNA virus infection [214]. The mitochondrial lipid cardiolipin directly binds to NLRP3 and facilitates NLRP3 inflammasome activation [215]. Together these

data suggest mitochondria as an ideal platform for assembly of the NLRP3 inflammasome complex. Mitochondria are also the source of ROS and mtDNA, which induce activation of the NLRP3 inflammasome [199, 216]. Conversely, the autophagy/mitophagy pathway mediated by LC3B, RIPK2/ULK1 preserves mitochondrial integrity and dampens activation of caspase-1[217, 218].

In the case of crystalline stimuli, endocytosis of the crystals leads to lysosomal damage and rupture, resulting in cytosolic release of lysosomal enzymes, such as cathepsin B, that somehow activate NLRP3 [203].

A second mechanism of NLRP3 inflammasome activation involves the generation of ROS. ROS are generated by ATP signaling through the P2X7 receptor [219], by NADPH oxidase during phagocytosis of particulate stimuli [205], or by the mitochondria [216]. Many studies suggest that ROS generated by damaged mitochondria directly activates the NLRP3 inflammasome [216], although others suggest that ROS are required for the priming phase, as opposed to directly activating NLRP3 [220]. The mechanism of ROS-dependent NLRP3 oligomerization is not completely understood. One report suggests that ROS can dissociate thioredoxin interacting protein (TXNIP) from thioredoxin, allowing TXNIP to act as a ligand for NLRP3 [221].

The third mechanism for NLRP3 activation is efflux of intracellular K^+. All NLRP3 activators including bacterial pore-forming toxins, nigericin, ATP, and particulate matter cause reduction of the intracellular K^+ concentration, which has been shown to be sufficient to activate NLRP3 [222]. Moreover, NLRP3 activation by all agonists can be blocked by high

extracellular K^+ that blocks K^+ efflux [223]. Collectively, these data suggest that K^+ efflux may be the common step that is necessary and sufficient for NLRP3 activation.

The forth mechanism involves the mobilization of Ca^{2+}. This notion is first supported by the fact that voltage-dependent anion channels (VDAC), through the uptake of Ca^{2+} from the ER into the mitochondria to promote mitochondrial metabolic activity, is essential for NLRP3 inflammasome activation [216]. Consistently, molecules that regulate Ca^{2+} release from the ER, such as C/EPB homologous protein (CHOP), facilitate the activation of NLRP3 [224]. In addition, two other studies have identified CASR and GPRC6A, two G protein-coupled Ca^{2+}-sensing receptors that upregulate cytosolic Ca^{2+}, as positive regulators in NLRP3 activation [225, 226]. By contrast, inhibition of mitochondrial Ca^{2+} uptake by silencing mitochondrial Ca^{2+} uniporter abolishes NLRP3-dependent IL-1β release [227]. It is not clear whether Ca^{2+} influx occurs upstream or downstream of other suggested mechanisms (mitochondira damage, ROS/mtDNA production) for NLRP3 activation. One study shows that NLRP3 inflammasome activation by ATP mobilizes Ca^{2+}, which precedes mROS and mtDNA release to cause NLRP3 inflammasome activation [224]. Liposomes and crystal substances, however, induce Ca^{2+} flux in a ROS-dependent manner via the TRPM2 channel [228].

Recent studies have reported two additional inflammasome regulators: guanylate-binding protein 5 (GBP5) [229] and double-stranded RNA-dependent protein kinase (PKR) [230]. GBP5 belongs to the 65kD GBP gene family and promotes NLRP3 inflammasome activation by ATP, nigericin, and bacteria, but not crystalline agents. In contrast to the selective effect of GBP5 on NLRP3, PKR has been shown to contribute to the activation of all known inflammasomes, including NLRP1, NLRP3, NLRP4, and AIM2 inflammasomes. The

mechanism of how these two molecules facilitate NLRP3 activation remains elusive. Considering the complexity of the activation process, it is very likely that additional players are needed for NLRP3 inflammasome activation, and it would be informative to identify more molecules involved in NLRP3 inflammasome activation.

During inflammasome activation, the NLR protein undergoes a conformational change from an auto-inhibitory state to an open state, leading to the exposure of the NBDs and allowing the oligomerization and assembly of the inflammasome complexes. Increasing evidence have suggested that the conformational change is permitted by post-translational modifications of the NLR protein. It has been demonstrated that cleavage of NLRP1 is necessary for activation of NLRP1 inflammasome activation [231]. Phosphorylation of NLRC4 by PKCδ is crucial for NLRC4 inflammasome activation [232]. NLRP3 has been shown to be constitutively ubiquitinated, and deubiquinination of NLRP3 by the deubiquitinase BRCC3 is necessary for the activation of NLRP3 inflammasome [233].

Non-canonical inflammasome

Recently, Kayagaki *et al.* identified an alternative mechanism for caspase-1 activation, termed the non-canonical inflammasome pathway [234], which activates caspase-11, another inflammatory caspase. Caspase-11 is not required for caspase-1 activation to the canonical NLRP3 inflammasome activators, such as ATP, but is necessary for caspase-1 activation and subsequent IL-1β and IL-18 secretion in response to a group of non-canonical inflammasome activators, including cytosolic LPS and several gram negative bacteria, such as *Escherichia*

coli, *Citrobacter rodentium*, *Shigella flexneri*, *Vibrio cholera*, *Salmonella typhimurium*, *Legionella pneumophila* and *Yersinia pseudotuberculosis* [234-238]. Interestingly, caspase-11 alone, independent of caspase-1, is responsible for pyroptosis and the release of alarmins such as IL-1α and HMGB1, during non-canonical inflammasome activation, indicating that caspase-11 orchestrates both caspase-1-dependent and -independent outputs. Caspase-1 activation by non-canonical stimuli requires NLRP3 and ASC, but caspase-11 processing and cell death does not, suggesting that there is a distinct activator of caspase-11. Cytosolic LPS, particularly penta- and hexa-acylated lipid A, has been shown to activate the non-canonical inflammasome [238, 239], although the actual sensor for cytosolic LPS remains unknown. The non-canonical pathway is activated by cytosolic, but not vacuolar, bacteria [237, 240]. Therefore, specific *Salmonella typhimurium* or *Legionella pneumophila* mutants (sifA and sdhA, respectively) that escape from the vacuoles to enter the cytosol trigger caspase-11. *Burkholderia* species that naturally invade the cytosol also triggered caspase-11, which protected mice from lethal challenge with *B. thailandensis* and *B. pseudomallei* [240].

Caspase-11 is not constitutively expressed by macrophages, but is instead induced upon TLR4 recognition of LPS. Caspase-11 induction is primarily mediated by feedback of type I IFNs synthesized via the TRIF-IRF3 pathway [235, 241], with Myd88 also playing some roles [235, 236]. Therefore, activation of the non-canonical inflammasome require two signals: a priming signal that upregulates caspase-11 and the cytosolic LPS receptor, and the actually activator that engages the LPS receptor can triggers caspase -11 activation.

Inflammasome and the control of adaptive immunity

The inflammasome, through the production of IL-1β and IL-18, plays pivotal roles in both the initiation and reactivation of adaptive immune responses. IL-1 promotes T cell proliferation and Th17 differentiation, as discussed before. Thus, activation of the inflammasome enhances antigen-specific Th17 immunity. IL-1 produced upon inflammasome activation is important for Th17 differentiation in various immunization and infection models, including CFA (NLRP3 inflammasome) [79], uric acid crystals (NLRP3 inflammasome) [242], *Candida albicans* (NLRP3 inflammasome) [243], *Legionella pneumophila* (NLRC4 inflammasome) [244], and *Schistosoma mansoni* (NLRP3 inflammasome) [245]. Conversely, APCs from mice carrying a constitutively active NLRP3 mutant produce excessive amounts of IL-1β, which augments Th17 cell differentiation, neutrophil infiltration to the skin and cutaneous inflammation [246]. IL-18 promote IFN-γ production by Th1 cells [247]. It is likely that IL-18 production following inflammasome activation plays an analogous role in enhancing Th1 immunity.

The role of the inflammasome in activating other arms of adaptive immunity has also been demonstrated. Respiratory infection with influenza virus induces $CD4^+$ and $CD8^+$ T cell responses, as well as mucosal IgA secretion and systemic IgG responses, all of which require ASC- and caspase-1-dependent IL-1 production [248]. NLRP3 has been shown to be required for optimal antibody production when alum is used as an adjuvant [249]. West Nile virus infection induces production of IL-1β via the NLRP3 inflammasome, which is essential for generating quality $CD8^+$ T cell responses [250]. Necrotic tumor cells generated by chemotherapeutic agents, which cause inflammasome activation through release NLRP3

inflammasome activators such as ATP, uric acid and histones, act as effective adjuvants to prime antitumor $CD8^+$ T cell[251].

IL-1 and IL-18 have also been shown to be essential for the non-cognate reactivation of differentiated effector and memory T cells. It has long been known that memory $CD8^+$ T cells can produce IFN-γ in response to IL-12 combined with IL-18 in the absence of cognate antigen[252]. Recent findings highlight the inflammasome's importance in mediating this "innate" form of IFN-γ release, through the production of IL-18. Activation of NLRC4 inflammasome in DCs by *Salmonella Typhimurium* cause IL-18 release that can stimulate IFN-γ production from splenic memory $CD8^+$ T cells of unrelated specificity. Similarly, memory $CD8^+$ T cells produced innate IFN-γ secretion following *Listeria monocytogenes* and *Pseudomonas aeruginosa* infection, under the control of IL-18 derived from inflammatory monocytes and subcapsular sinus macrophages, respectively, where NLRP3 inflammasome is activated by these two pathogens. It has also been shown that IL-1β synergizes with IL-23 to stimulate IFN-γ as well as IL-17 secretion from CD4+ T cells independent of TCR engagement [138].

CONCLUSION

TLRs play prominent roles in directing both innate and adaptive immunity. The role of TLRs in regulating the Th1 responses has been well demonstrated. However, the role of TLRs in regulating Th17 differentiation remains unclear. Th17 cells have drawn intense research interest due to their role in autoinflammatory and autoimmune diseases. Although the cytokine

requirements and transcriptional regulation of Th17 cells have been elucidated using *in vitro* generated Th17 cells, *in vivo* regulation of Th17 cell development, especially in the context of innate control of adaptive immunity, is largely unknown. Both IL-6 and IL-1 contribute to Th17 differentiation *in vitro*, but *in vivo* evidence regarding the relative contributions of these two cytokines remains to be clarified. The current work investigates the *in vivo* roles of IL-6 and IL-1 in regulating Th17 lineage differentiation, and presents evidence that Th17 differentiation *in vivo* is regulated via tissue-specific mechanisms. Although IL-1 is ubiquitously required, IL-6 is required in a tissue-specific manner, for generating Th17 cells. Preliminary mechanisms for the tissue specific regulation of Th17 differentiation are also presented. Because IL-1 plays an indispensable role in Th17 priming, we go on to investigate how TLR regulates the biogenesis of IL-1, particularly the activation of the inflammasome complex. This part of the work demonstrates a direct role of TLR signaling pathway, via the signaling protein IRAK-1, in regulating the activation of the NLRP3 inflammasome. The physiological relevance of IRAK-1-dependent inflammasome activation is also explored. These works provide unique insights to TLR mediated host defense mechanism.

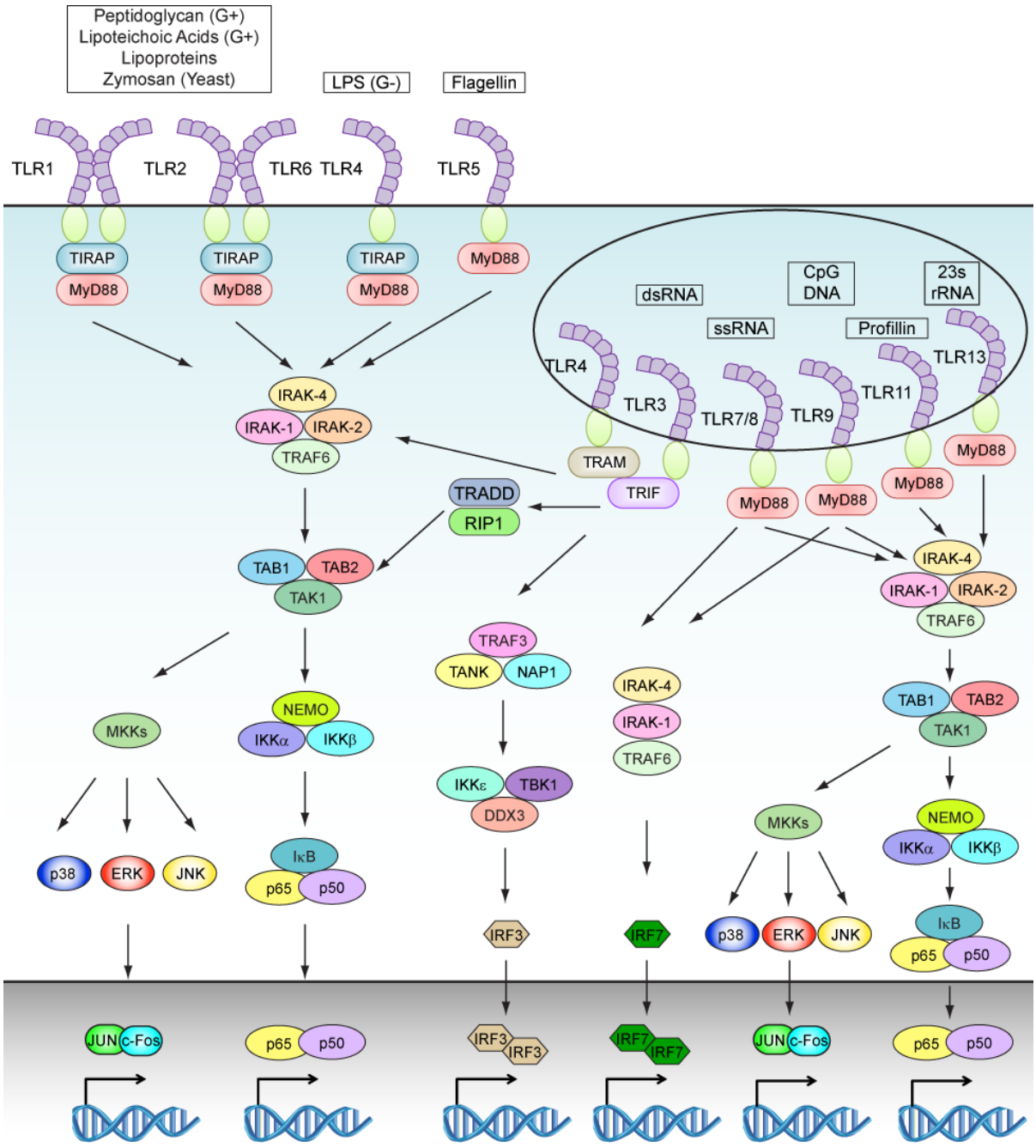

Figure 1.1. TLR signaling pathways.

TLRs can be classified into suface TLRs (TLR1, 2, 4, 5, 6) and endosomal TLRs (TLR 3,7,8, 11,12,13). TLR activation indces a MyD88 dependent pathway and MyD88 independent pathway through TRIF. Some surface TLRs also require the sorting adaptor TIRAP to recruit MyD88. TLR4 recruit TIRAP and MyD88 from the cell surface, and subsequently translocates to the endosome with the help of the sorting adaptor TRAM, where the TRIF pathway is initiated.

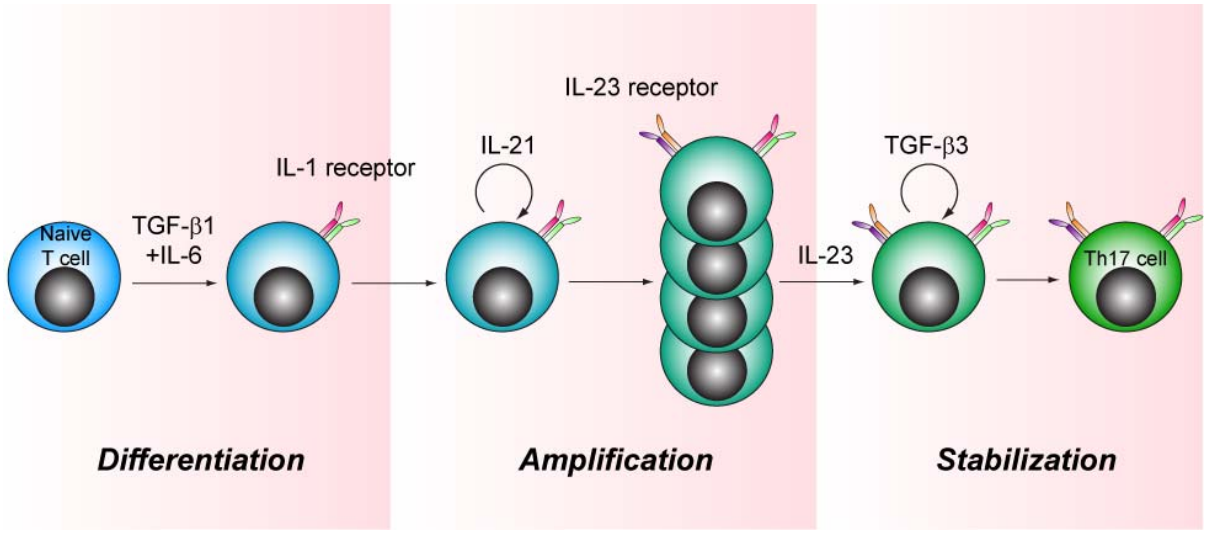

Figure 1.2. Th17 differentiation pathway.

Initial encounter to IL-6 and TGF-β1 induces differentiation of Th17 cells from naive T cells. IL-21, which is produced by IL-6/TGF-β1-acitivated Th17 cells, acts in a positive feedback loop to amplify Th17 cells. IL-6 and IL-21 also upregulate the IL-23 receptor, which allows IL-23 to expand and stabilize Th17 cells. IL-23 induces the production of TGF-β3, which acts in an autocrine manner to induce fully pathogenic Th17 cells.

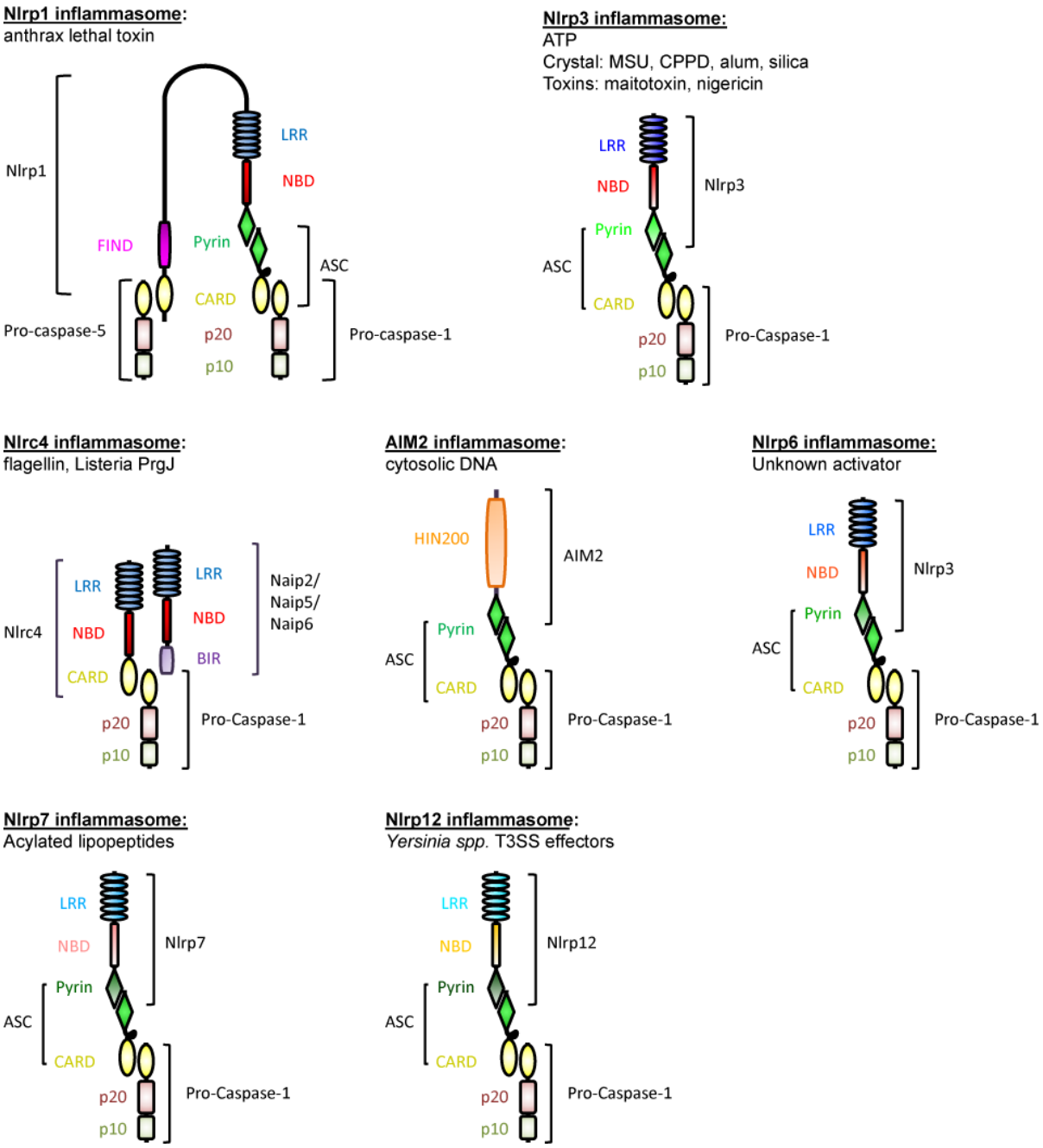

Figure 1.3. Inflammasomes and their activators.

All known inflammasomes and their activators are shown. With the exception of NLRC4, all inflammasome complexes engage caspase-1 via the adaptor protein ASC.

Table 1.1. Inflammasome activation by microbes.

Bacteria	*Inflammasome(s)*	*Mechanism*	*Reference*
Aeromonas hydrophila	NLRP3/NLRC4	Aerolysin	[253]
Bacillus anthracis	NLRP1b	Anthrax leathal toxin	[231]
Brucella abortus	NLRP3/AIM2	mROS/DNA	[254]
Burkholderia pseudomallei	Non-canonical/NLRC4	Cytosolic LPS/BsaK (T3SS component)	[240, 255]
Candida albicans	NLRP3	secreted aspartic proteas	[256]
Chlamydia pneumoniae	NLRP3	Oxidized mtDNA	[199, 257]
Citrobacter rodentium	Non-canonical	Cytosolic LPS	[234, 238]
Clostridium difficile	NLRP3/Unknown	TcdA and TcdB (toxins)	[258]
Encephalomyocarditis virus	NLRP3	Viroporin 2B-$Ca2^{+}$ influx	[259]
Enterohemorrhagic Escherichia coli	Non-canonical	Cytosolic LPS	[234, 238]
Francisella tularensis	AIM2	DNA	[260]
Gambierdiscus toxicus	NLRP3	Maitotoxin	[197]
Helicobacter pylori	NLRP3	CagL	[261]
Legionella spp.	NLRC4	Flagellin/PrgJ	[1, 191, 262]
Listeria monocytogenes	NLRP3/AIM2/NLRC4	Listeriolysin O/ gDNA/Flagellin	[263, 264]
Mycobacterium tuberculosis	NLRP3	ESAT-6, cytosolic Ag85	[265]
Mycoplasma spp.	hNLRP7	Acylated lipopeptides	[193]
Neisseria gonorrhoeae	NLRP3	Gonococcal lipooligosaccharide	[266]
Porphyromonas gingivalis	NLRP3/AIM2	ATP release, lysosomal damage/DNA	[267]
Pseudomonas aeruginosa	NLRC4	Pilin, PscI (T3SS component)	[190]
Rabies virus	NLRP3	Undetermined	[268]
Salmonella typhimurium	NLRC4	Flagellin, PrgJ (T3SS component)	[189, 190]
Shigella flexneri	NLRC4	MxiI (T3SS component)	[269]
Staphylococcus aureus	NLRP3	α-, β-, γ-Hemolysin, Panton-Valentine leukocidin (PVL)	[270, 271]
Streptococcus pneumoniae	NLRP3/AIM2	Pneumolysin/gDNA	[272, 273]
Streptomyces hygroscopicus	NLRP3	Nigericin	[197]
Toxoplasma gondii	NLRP1b	Undetermined	[274]
Treponema denticola	NLRP3	Td92- integrin α5β1-ATP release	[275]
Yersinia pestis	NLRP3/NLRC4/NLRP12	T3SS/Undertermined	[194, 276]

CHAPTER TWO
Materials and Methods

Mice

Mice genetically deficient for MyD88, TRIF, MyD88/TRIF, TLR2/TLR4, TLR7, IRAK-1, IRAK-2, IRAK-1/2, Rag1, NLRP3, Caspase-1, IL-6, IL-1R1 and IL-18R, as well as CD11c-MyD88 Tg, CD2-DNgp130 Tg, OT-I (CD45.1background) Tg, and OT-II Tg (CD45.1background) transgenic mice were bred and maintained at the animal facility of UT Southwestern Medical Center. All mice are on C57BL/6 background. Control C57BL/6 mice were obtained from UT Southwestern mouse breeding core facility. IRAK-4 kinase dead (KD) knock-in mice [277] and littermate controls were bred and housed at the animal facility of Cleveland Clinic. IRAK-1 KD knock in mice [278] and their littermate controls were bred and housed at the animal facility of University of Dundee, Scotland. TRAF6fl/fl and TRAF6fl/fl x Mx-1 Cre Tg mice [279] were bred and maintained at the animal facility of the University of Cologne, Germany. For experiments studying LPLs, mice of different strains were co-housed at least two weeks prior to analysis. For most *in vivo* and *in vitro* experiments, all mice were used at 8-12 weeks of age. For some experiments, mice were aged to 16-20 weeks before use. All mouse experiments were done as per protocols approved by Institutional Animal Care and Use Committee (IACUC) at UT Southwestern Medical Center. All strains were on C57BL/6 background.

Generation of CD2-DNgp130 Tg mice

A dominant negative gp130 described previously[280] was cloned into XmaI-digested VA-CD2 plasmid[281]. The construct was linearized with KpnI and XbaI. The fragment containing the dominant negative gp130 was purified and injected into C57BL/6 blastocysts by UT Southwestern transgenic and knockout core facility. Transgenic founders were identified by PCR and bred to C57BL/6 mice.

Reagents and antibodies

TLR ligands and inflammasome inducers

LPS was purchased from *Escherichia coli* 0111:B4 was purchased from Sigma-Aldrich. CpG was purchased from Keck facility at New Haven, CT. Pam3CSK4, poly I:C (high molecular weight) and R837 were purchased from Invivogen. ATP was purchased from Sigma-Aldrich. Poly dA:dT, nigericin, monosodium urate (MSU) and silica dioxide crystals were purchased from Invivogen. Fla-tox and Anthrax toxin protective antigen (PA) [1] were gifted by Dr. Russell Vance (UC Berkeley).

Flow cytometry reagents

Purified anti-CD16/32, FITC anti-CD4, FITC anti-CD44, PE anti-CD44, PE anti-IFN-γ, PerCP anti-CD4, PerCP anti-CD3, APC anti-CD45.1, Alexa Fluor 647 anti-CCR6, APC

anti-IL-17A, biotin anti-CD62L, biotin anti-CD44 were all purchased from BD Biosciences. FITC anti-IA/IE, FITC Annexin V, FITC anti-CD45.1, Alexa Fluor 488 anti-Foxp3 (clone 150D), PerCP anti-CD8, PE-Cy7 anti-CD11c, APC anti-CD11c, Alexa Fluor 647 anti-T-bet, Alexa 647 anti-F4/80, biotin anti-CD103, biotin anti-NK1.1, PerCP streptavidin, APC streptavidin and Foxp3 Staining Buffer Set were purchased from Biolegend. PE anti-RORγ and Fixable Viability Dye eFluor® 660 were purchased from eBioscience. Propidium iodide solution was purchased from Sigma-Aldrich.

Western blot and immunofluoresce reagents

Antibodies for ASC (rabbit), IRAK-1 (mouse or rabbit), IRAK-2 (rabbit), IRAK-4 (goat), IκBα, HMGB-1, IL-18, IL-1β, STAT3 and GAPDH were obtained from Santa Cruz Biotechnology. Anti-phospho STAT3 antibody was purchased from BD Biosciences. Anti-NLRP3 (goat, clone Cryo-2) was purchased from Adipogen. MyD88 antibody (goat) was purchased from R&D Systems. Phospho-Smad Antibody Sampler Kit (contain anti-Smad2, anti-Smad3, anti-pSmad2 and anti-pSmad3), phospho-IκBα and β-Tubulin antibodies were purchased from Cell Signaling. Anti-caspase-1 p20 rat monoclonal antibody was kindly provided by Dr. V. Dixit (Genentech).

Secondary antibodies conjugated with horseradish peroxidase (HRP), $F(ab')_2$ fragment of donkey secondary antibodies conjugated with fluorochromes (FITC (fluorescein isothiocyanate), TRITC (tetramethyl rhodamine isothiocyanate), cy5 (cyanine dye 5), and normal donkey serum (NDS) were from Jackson ImmunoResearch Laboratories. Vectashield

mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories.

Recombinant cytokines, inhibitors and neutralizing antibodies

Recombinant mouse IL-2 was from Biolegend. Recombinant IL-1β, IL-6, TNF-α and TGF-β were from Peprotech. Recombinant IL-23 was purchased from eBioscience. Purified anti-TGF-β, the respective isotype control antibody (mouse IgG_1) and recombinant IL-1 receptor antagonist (IL-1RA) were from R&D Systems. LEAF™ Purified anti-IL-6 was from Biolegend. Cycloheximide, BAY 11-7082, retinoic acid and SB431542 were purchased from Sigma-Aldrich. Diphenyleneiodonium Chloride (DPI) and IRAK-1/4 inhibitor (CAS 509093-47-4) were from EMD Millipore. NG-25 was provided by Dr. Philip Cohen (University of Dundee).

Cell culture reagents and others

With the exception of the Th17 polarization experiment, T cells or DC-T cell co-cultures were performed in complete RPMI-1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate (all from Hyclone), and 50μM β-mercaptoethanol (Sigma Aldrich). Differentiated BMDMs were cultured in complete RPMI-1640 supplemented with 10% or 1% FCS, 2mM L-glutamine, 1mM sodium pyruvate, and 50μM β-mercaptoethanol. 100 U/ml penicillin and 100μg/ml

streptomycin was added to the media with the exception of antibiotic-free media. For the Th17 polarization experiment, cells were cultured in X-VIVO 20 media supplemented with 10% FCS, 2mM L-glutamine, 1mM sodium pyruvate, and 50μM β-mercaptoethanol.

Purified anti-CD25 antibody (clone PC61) was from BD Biosciences. Ovalbumin, incomplete Freund's adjuvant (IFA) were from Sigma.

T cell purification

Anti-CD8 (TIB 105 and TIB 150), anti-Class II (Y3JP), anti-MAC-1 (TIB-128), anti-B220 (TIB 164 and TIB 146), anti-CD4 (GK1.5) anti-NK1.1 (HB191) and anti-Thy1 (Y19) monoclonal antibodies were used as hybridoma supernatants. For total T cell isolations, single cell suspensions were incubated with anti-Class II (Y3JP), anti-MAC-1 (TIB-128) and anti-B220 (TIB-146 and TIB-164), and anti-NK1.1 (HB191) hybridoma supernatants. For $CD4^+$ T cell isolations, anti-CD8 hybridoma (TIB-105 and TIB150) supernatants were added to the above cocktail. For $CD8^+$ T cell isolations, anti-CD4 hybridoma (GK1.5) supernatants were added to the above cocktail. Antibody-labeled cells and B cells were depleted using BioMag goat anti-mouse IgM (Polysciences), goat anti-mouse IgG and goat anti-rat IgG beads (Qiagen). For isolating $CD4^+CD62L^{hi}CD44^{lo}$ and $CD4^+CD62L^{lo}CD44^{hi}$ T cells, negatively selected $CD4^+$ T cells were incubated with biotin anti-CD62L followed by anti-biotin microbeads and sorted using the AutoMACS sorter (Miltenyi Biotec.). The negative fraction was enriched for $CD62L^{lo}$ $CD44^{hi}$ cells and the positive fraction was enriched for $CD62L^{hi}$ $CD44^{lo}$ cells.

B cell purification

Splenocytes were incubated with a cocktail of anti-CD4, anti-CD8, anti-Thy1 and anti-Mac-1 hybridoma supernatants on ice for 30 minutes, followed by rabbit complement (Cedarlane Laboratories, Ontario, Canada) for 60 minutes at 37°C. Cells were washed twice after complement lysis.

T cell proliferation assay

Purified $CD4^+$ T cells ($2x10^5$) from draining lymph nodes were cultured in flat bottom 96 well plates with *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ B cells ($3 x10^5$) and titrating doses of antigen for 72 to 84 hours. Proliferation of T cells was determined by incorporation of ^{3}H-thymidine for the last 12-16 hours of the culture.

Dendritic cell preparation

Splenic DCs were prepared as follows: Single cell suspension of spleen was incubated with anti-Thy1 (Y19) and anti-NK1.1 (HB191) hybridoma supernatants. Antibody-labeled cells as well as B cells were depleted using BioMag goat anti-mouse IgM, goat anti-mouse IgG and goat anti-rat IgG beads. The enriched DCs were further purified using rabbit complement at 37ºC for 60 minutes followed by three washes. LP DCs were purified from LP mononuclear cells using the following protocol: LP mononuclear cells were incubated with anti-Thy1 (Y19)

and anti-NK1.1 (HB191) hybridoma supernatants. Antibody-labeled T cells, NK cells and B cells were depleted using BioMag goat anti-mouse IgM, goat anti-mouse IgG and goat anti-rat IgG beads.

Enzyme linked immunosorbent assay (ELISA)

Paired antibodies against IL-6, IL-23 (p19 specific), IFN-γ and IL-17A were purchased from Biolegend to perform cytokine specific ELISAs. IL-1β and IL-18 were measured using antibody pairs purchased from R&D Systems.

Isolation of lamina propria lymphocytes

Lamina propria lymphocytes (LPL) were isolated as described previously [282] with modifications. Intestines were harvested and flushed with ice-cold PBS. And but open lengthwise, then into ~1.5 cm pieces. These tissue pieces were washed 5 times in ice-cold PBS and suspended in Hank's balanced salt solution containing 3% FCS, 1mM DTT, and 1mM EDTA, followed by incubation for 30 minutes at 37°C under shaking at 250 RPM. Tissues were vortexed vigorously for 2 minutes. Supernatant was discarded and the remaining tissue pieces were washed 5 times in ice-cold PBS. Next, the tissue fragments were suspended in RPMI containing 5% FCS, 4.17mM $NaHCO_3$, 0.1mg/ml collagenase from *Clostridium histolyticum* (Sigma, cat no. C2139), and 0.1mg/ml DNase I (Roche) followed by incubation for 60 minutes at 37°C under shaking at 250 RPM. After digestion, LPL containing

supernatants were strained through 100 micron cell strainers, washed twice and suspended in 8 ml of 40% Percoll (GE Healthcare Life Sciences). The cells in 40% Percoll were overlayed onto 70% Percoll and subjected to centrifugation at 800xG for 20 minutes with no brake. Cells were collected from the gradient interphase and washed two times using FCS-containing medium. For enrichment of $CD4^+$ T cells, LPLs were incubated with anti-Class II (Y3JP), anti-MAC-1 (TIB-128) anti-B220 (TIB-146 and TIB-164) and anti-CD8 (TIB150) hybridoma supernatants followed by rabbit complement (Cedarlane laboratories, Ontario, Canada). Cells were washed and the purity of $CD4^+$ T cells was confirmed by staining with an anti-CD4 antibody.

Isolation of mononuclear cells from the liver and lung

The liver or lung was cut into small pieces. Single cells from the liver were prepared by pushing the tissue through 70μm cell strainers. Single cells from the lung were obtained by digesting the tissue at 37°C under shaking in RPMI medium supplemented with 5% FCS, 0.1mg/ml DNase I and 1mg/ml collagenase for 60 minutes followed by passing through 70μm cell strainers. Cells were then pelleted and washed then applied to 40%-70% Percoll gradient centrifugation to obtain mononuclear cells. Cells were collected from the gradient interface, washed, and used for subsequent analysis.

Staining and flow cytometry

For surface stains, cells were Fc-blocked with anti-CD16.32 and stained with relevant antibodies for 30 minutes on ice and were washed. For intracellular staining of cytokines, cells were stimulated with 50ng/ml phorbol myristate acetate (PMA) and 1μM ionomycin in the presence of 1μg/ml brefeldin A for 4 hours, followed by surface staining, fixed with 4% paraformaldehyde, permeabilized with 0.3% saponin, and stained for intracellular cytokines. For transcription factor and innate IFN-γ staining, the Foxp3 Staining Buffer Set from Biolegend was used for fixation and permeabilization of freshly isolated cells. The stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, inc).

Annexin V staining

Freshly isolated LPLs were stained for CD4, washed three times with serum/protein-free, azide-free PBS and stained with fixable viability dye (eBioscience) for 30 minutes on ice. Subsequently, cells were washed two times with flow stain buffer and once with Annexin V binding buffer (10mM Hepes/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and stained with Annexin V for 15 minutes at room temperature in Annexin V binding buffer. Cells were then fixed and permeabilized immediately with Foxp3 Staining Buffer Set and stained for RORγt. For data analysis, dead cells were excluded based on positive stain for fixable viability dye.

Quantitative reverse transcribed polymerase chain reaction (RT-PCR)

Freshly sorted cells were lysed immediately in TRIzol Reagent (Invitrogen) and RNA was extracted following manufacturer's instructions. cDNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen). *Tbx21* and *Rorc* (specific for the RORγt isoform) transcripts were measured with DyNAmo SYBR Green qPCR Kit (Finnzymes). Primer sequences are listed in supplemental information. *Cd4* transcripts were used for normalization.

Tbx21 Forward: 5'- CAACAACCCCTTTGCCAAAG-3'

Tbx21 Reverse: 5'- TCCCCCAAGCAGTTGACAGT-3'

Rorc Forward: 5'-TTCACCCCACCTCCACTG-3'

Rorc Reverse: 5'- GTGCAGGAGTAGGCCACATT-3'

Cd4 Forward: 5'- AGGAAGTGAACCTGGTGGTG-3'

Cd4 Reverse: 5'- TCCTGGAGTCCATCTTGACC-3'

Ex vivo T cell stimulation

Tissue culture plates were coated with 0.5μg/ml of anti-CD3 and 0.5μg/ml of anti-CD28 at 37ºC for 2 to 3 hours and washed 3 times before use. Purified splenic $CD4^+$ T cells were plated at $5x10^5$ cells/well in 48 well plates and LP $CD4^+$ T cells were plated at ~$3x10^4$ cells/well. Cells were stimulated for 48 hours and culture supernatants were assayed for cytokine production.

Cell transfer

$5x10^7$ mononuclear cells isolated from the spleen and lymph nodes from young naïve donors (6 weeks of age) were transferred into *Rag1*$^{-/-}$ recipients through the tail vein. Differentiation of the donor cells in multiple organs was analyzed on day 7 post-transfer.

Bacterial culture

Citrobacter rodentium (strain ICC168) was provided by Dr. Vanessa Sperandio at UT Southwestern Medical Center. *Salmonella typhimurium* (strain SL1344) was provided by Dr. Denise Monack at Stanford University. Bacteria were first grown on LB agar plates, and a single colony was inoculated to LB broth and cultured at 37˚C overnight under shaking. Bacteria were subcultured in fresh LB broth under shaking until the OD reaches between 0.6 and 1. The bacterial culture was diluted in PBS for infection.

LM 10403 serotype 1 [283] was provided by Dr. James Forman at UT Southwestern Medical Center and had been repeatedly passaged through B6 mice to maintain virulent stocks. Bacteria were first grown on LB agar plates, and a single colony was inoculated to LB broth and cultured at 37˚C overnight without shaking. For *in vitro* infection, the bacterial culture was added directly to the cells without washing. For *in vivo* infection, the bacterial culture was diluted in PBS.

Infections

In vivo Citrobacter and Salmonella infections

Mice were infected with 1-2x10^9 colony forming units (CFU) *Citrobacter rodentium* suspended in 100μl PBS by oral gavage, or 1000 CFU of *Salmonella typhimurium* suspended in 500μl PBS by intraperitoneal injection.

In vivo Listeria infection

Mice were injected with 1x10^7 CFU of *L. monocytogenes* intraperitoneally. After 20 minutes, peritoneal cells were stained with anti-CD45 and anti-F4/80 on ice for 10 min, washed once and resuspended in FACS buffer containing 0.5 μg/ml PI and analyzed by flow cytometry immediately. Alternatively, mice received 1000 OT-I T cells (CD45.1 background) through the intravenous route. The next day, mice were infected intravenously with 2x10^6 PFU vesicular stomatitis virus expressing OVA (VSV-OVA). Primary expansion of OT-I cells were confirmed in the peripheral blood on day 7 post infection. 45 days after the primary infection, mice were injected with 1x10^6 CFU of non-OVA expressing *L. monocytogenes* intravenously. In experiments measuring the response of polyclonal memory $CD8^+$ T cells and NK cells, mice were directly infected with *L. monocytogenes* intravenously without prior exposure to VSV-OVA. 12 hours after Listeria infection, sera were collected via cardiac puncture and cytokine concentrations were measured by ELISA. Splenocytes were cultured at 2x10^6 cells/ml for 6 hours in the presence of 1μg/ml brefeldin A and 20μg/ml gentamicin. IFN-γ production by memory $CD8^+$ T cells and NK cells was assayed by intracellular staining using the Foxp3 staining buffer set from Biolegend.

In vitro E. coli and Citrobacter infection

BMDMs were cultured at $1x10^6$ cells/ml in 12 well plates. Cells were infected with *Citrobacter* or *E. coli* for 1h, and then incubated with 100μg/ml gentamicin for 1h. Cells were then washed and the media was replaced with 1% FCS containing media supplemented with 20μg/ml gentamicin for 12 hours. Supernatants were analysed as described in the pyroptosis assay.

***In vitro* T cell priming**

Purified naïve $CD62L^{hi}CD44^{lo}$ $CD4^+$ T cells ($3x10^5$/well) were cultured with splenic DCs ($6x10^4$/well) and anti-CD3 (10ng/ml) in 48-well plates in the presence or absence of TLR ligands (100ng/ml Pam3CSK4, 100ng/ml LPS, or 1μM CpG). Alternatively, purified naïve $CD62L^{hi}CD44^{lo}$ $CD4^+$ T cells ($1.5x10^5$/well) were cultured with enriched LP DCs ($1.5x10^5$/well) or sorted LP DCs ($3x10^4$/well) and anti-CD3 (10ng/ml) in 96-well plates in the presence or absence of TLR ligands. Supernatants were analyzed for cytokines after 5 days of culture.

Th17 polarization

$3x10^5$ naïve $CD4^+$ T cells were cultured in X-VIVO 20 medium (Lonza) with plate-bound anti-CD3 (5μg/ml) and anti-CD28 (5μg/ml) in 96-well plates. Anti-IFN-γ (clone XMG1.2, 10μg/ml), anti-IL-4 (clone 11B11, 10μg/ml), and recombinant mouse IL-2 (Biolegend, 50U/ml) were added to the culture. The following cytokines were used to induce Th17 differentiation: IL-6 (20ng/ml), TGF-β (5ng/ml), IL-1β (10ng/ml), IL-23 (100ng/ml). After 4 days, culture supernatants were collected and assayed for IL-17 secretion by ELISA. Cells were restimulated with 50ng/ml PMA and 1μM ionomycin in the presence of 1μg/ml brefeldin A for 4 hours and stained for intracellular cytokines.

Preparation of bone marrow derived macrophages (BMDMs)

Bone marrow cells from the femurs and tibias were prepared. Erythrocytes were lysed, and the white cells were seeded in tissue culture dishes in media containing 10% FCS and 10% conditioned media from L929 cells. The remaining suspension cells were harvested the next day, and plated at $4x10^6$ cells/dish in fresh petri plates for selection by growth and adhesion for four additional days. For preparation of TRAF6 KO BMDMs, mice with homozygous loxP-flanked *Traf6* alleles were crossed to mice carrying the Mx1-Cre transgene and cells were isolated after poly(I:C)-mediated induction of recombination as previously described [279]. All mice received two intraperitoneal injections of poly(I:C) (250μg/250μl sterile PBS/ mouse, GE Healthcare # 27-4732-01) with two days interval. Bone marrow cells were isolated three days after the second poly(I:C) injection and BMDMs were cultured following the protocol described above.

Preparation of peritoneal resident macrophages (PCMs)

Peritoneal cells were collected by flushing the peritoneal cavity with 10 ml of ice-cold HBSS and plated at 1 x 10^6 cells per well in 12-well plates. After 2 hours, cells in suspension were removed and the remaining adherent cells (PCMs) were washed and used for caspase-1 activation.

Caspase-1 activation assay

BMDMs were plated in 12-well tissue culture plates at 5 x 10^5 cells per well and allowed to attach. For acute caspase-1 activation studies, BMDMs were stimulated simultaneously with TLR ligands and ATP (5 mM) for 30 min. For delayed activation studies, BMDMs were incubated with LPS (100 ng/ml) or R837 (5 μg/ml) for 2 to 4 h and then pulsed with ATP (5 mM) during the last 30 min of incubation. In some cases, cells were pre-incubated with cycloheximide or Bay11-7082 for 1 h prior to stimulation with TLR ligands. After stimulation, cells were washed with cold PBS, SDS-sample buffer was then added directly to each well, and cell lysates were harvested, centrifuged, and incubated at 95°C for 40 min. The cell lysates were then subjected to western blot analysis using rat anti-mouse caspase-1 monoclonal antibody, followed by HRP-conjugated secondary antibody. The immunoblots were visualized with ECL detection reagent (Perkin-Elmer).

Immunofluorescence microscopy

BMDMs were plated in 60-mm dishes containing sterile glass cover slips the day before the experiments. Cells were treated with ATP (5 mM) and LPS (100 ng/ml) for 15 minutes, followed by two washes with PBS. Cells were fixed with ice-cold methanol for 10 min at -20 °C, and then methanol was removed and cells left to air-dry. Cells were then washed three times with PBS. Cells used for staining of actin and HMGB-1 were fixed with 4% PFA for 15 min at room temperature followed by three washes with PBS. Cover slips were first incubated with 20% normal donkey serum (NDS) in PBS containing 1 μg/ml of mouse BD Fc Block (BD Pharmingen) for 30 min to suppress non-specific binding of IgG. Primary antibodies to IRAK-1 (rabbit or mouse), ASC (rabbit), NLRP3 (goat or mouse), MyD88 (goat), IRAK-2 (rabbit), IRAK-4 (goat) and HMGB-1 (mouse) diluted in PBS containing Fc Block and 10% NDS were added to the cover slips and incubated for 60 min at room temperature. Following washes with PBS, cover slips were incubated with appropriate $F(ab')_2$ fragment of donkey secondary antibodies conjugated with fluorochromes for 60 min at room temperature. After three washes with PBS, cover slips were mounted on glass slides with Vectashield mounting medium containing DAPI. The signal was examined by fluorescent microscopy at the appropriate wavelength for the secondary antibody on an IX81 Olympus microscope or a Zeiss LSM 510 meta confocal microscope, and images were obtained using a Hamamatsu Orca digital camera with a DSU confocal unit using Slidebook software (Intelligent Imaging Innovations (3i)). The images were then processed with ImageJ software (National Institutes of Health, http://imagej.nih.gov/ij/, 1997-2012) followed by Adobe Photoshop.

Pyroptosis Assays

BMDMs were harvested and suspended at $1x10^6$ cells/ml in 1% FCS containing RPMI media. Cells were stimulated with LPS (100 ng/ml) and ATP (5 mM) or *Listeria monocytogenes* ($1x10^7$ CFU) for 1 h. Cells were spun down at 1,500 rpm for 5 min, resuspended in FACS buffer (0.5% (w/v) BSA in PBS) containing 0.5 μg/ml propidium iodide (PI, Sigma), and analyzed for PI incorporation using a FACS Calibur flow cytometer. LDH activity in the supernatants was measured using the LDH Cytotoxicity Detection Kit (Clontech) according to the user manual. For western blots, 500 μl of supernatant was concentrated to 20 μl using the Vivaspin 500 centrifugal filter units (10K) (Bioexpress), and subjected to western blot analysis for HMGB-1 and Caspase-1. Ponceau S Staining Solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) was used to verify equal loading.

CHAPTER THREE
Priming Microenvironments Dictate Cytokine Requirements for T Helper 17 Cell Lineage Commitment

Work presented in this chapter has been published in *Immunity*, volume 35, pages 1010-22.

INTRODUCTION

Activation of the innate immune system is critical for inducing priming of antigen specific naïve $CD4^+$ T cells [43, 284]. Dendritic cells (DCs) are equipped with a broad array of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) [285], retinoic acid inducible gene I (RIG-I)-like receptors [286], nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors (NLRs) [287] and C-type lectin receptors [288], all of which sense pathogen associated molecular patterns (PAMPs) and trigger DC maturation. Maturation of DCs is characterized by high expression of major histocompatibility complex (MHC) and costimulatory molecules, as well as the production of inflammatory cytokines and chemokines, which play critical roles in activation of naïve T cells [289].

In addition to naïve T cell priming, cytokines secreted by DCs following PRR engagement govern the fate of activated $CD4^+$ T cells, and regulate their survival and lineage commitment [117]. Cytokines such as IL-12 and IL-18 initiate or promote T helper-1 (Th1) cell commitment of primed T cells, which protect the host against various bacterial and viral pathogens [45, 247]. A newly defined lineage of T cells, called T helper-17 (Th17) cells, has

been shown to be critical for protection against certain bacterial and fungal infections, and also to be responsible for several autoimmune diseases [290]. The orphan nuclear receptor RORγt has been shown to be both necessary, and sufficient, for Th17 cell differentiation [140, 143]. A combination of IL-6 and TGF-β, *in vitro,* leads to induction of the transcription factor RORγt and differentiation of murine naïve T cells into Th17 lineage cells [121-124]. Other studies have demonstrated that IL-1 enhances Th17 cell differentiation induced by a combination of IL-6, TGF-β, IL-23 or IL-21 [128-132, 291]. Furthermore, interleukin-1 receptor-deficient mice have been shown to be resistant to experimental autoimmune encephalomyelitis (EAE) [138] and, most recently, IL-6 has been shown to control Th17 cell differentiation through regulation of IL-1R on $CD4^+$ T cells [139]. However, the relative contributions of IL-1 and IL-6 in Th17 cell differentiation are not completely understood.

Multicellular organisms constantly encounter microbial stimuli, both from commensals as well as invading pathogens. Most microbes invade their hosts through the mucosal surfaces such as the intestine, the respiratory tract, uro-genital tract, as well as the skin. Adaptive immune responses to pathogens are generated in the draining lymph nodes of the site of infection. However pathogens also penetrate into the blood stream and cause systemic infection and adaptive immune responses to such pathogens are generated in the spleen. The mucosal immune system, the cutaneous immune system, and the systemic immune system face unique challenges in dealing with infectious agents. The former two are in constant contact with commensal micro-organisms while the spleen is largely a sterile environment. In addition, unique DC populations reside in different tissues. Broadly, DCs can be categorized into tissue-resident and lymphoid organ-resident DCs [292]. The former reside in peripheral tissues such

as mucosa, skin, and non-lymphoid organs, and migrate from peripheral tissues into the corresponding lymph nodes, through the afferent lymphatics [293], both at steady state and during infection. Lymphoid organ-resident or blood DCs enter the spleen and lymph nodes as bone-marrow derived precursors and develop within those organs without trafficking through peripheral tissues [294]. The spleen contains exclusively blood-derived DCs; whereas, the lymph nodes in addition to blood-derived DC subsets contain migratory DCs coming from peripheral tissues [295]. While we understand that DCs play a critical role in activation of naïve T cells, the importance of anatomical location of DCs for T cell differentiation remains largely unexplored.

In the current study we discovered that Th17 cell lineage commitment has differential cytokine requirements depending on the site of priming. We have shown that IL-6, which is thought to be a necessary differentiation factor for Th17 cell lineage development, was critical in the mucosal tissues of the gut and lungs as well as in the skin draining lymph nodes but not in the spleen. However, IL-1R signaling was critical for induction of IL-17 secreting T cells in all tissues. We have further demonstrated that DC populations resident in the mucosal tissues, skin and spleen were responsible for the differential cytokine requirements for Th17 cell priming. This study provides important insights into how priming microenvironments guide the cytokine requirements for development of Th17 lineage cells and demonstrates that DC populations from sterile microenvironments and commensal inhabited microenvironments impose different rules for Th17 cell priming.

RESULTS

MyD88 adaptor signaling in T cells is critical for inducing antigen specific Th17 cells

Depletion of $CD4^+CD25^+$ regulatory T (Treg) cells in MyD88 knockout (KO) mice restores otherwise defective antigen specific $CD4^+$ T cell priming as well as Th1 cell commitment [296]. We investigated whether such priming would also restore Th17 cell lineage commitment in MyD88 KO mice. As demonstrated before, immunization of MyD88 KO mice using antigen mixed with TLR4 ligand LPS, after *in vivo* depletion of Treg cells, led to priming of interferon-γ (IFN-γ) producing T cells (Figure 3.1). Intriguingly, $CD4^+$ T cells from Treg cell depleted MyD88 KO mice failed to secrete detectable IL-17A (IL-17) (Figure 3.1). To explore the possibility that a lack of IL-6 and IL-23 secretion by DCs could be the cause of defective Th17 cell priming in MyD88 KO mice, we used a transgenic mouse expressing MyD88 only in DCs and macrophages under the control of the Cd11c promoter [297] (CD11c-MyD88 Tg). DCs from the CD11c-MyD88 Tg mice had normal TLR-induced IL-6 and IL-23 secretion (Figure 3.2 A). Strikingly, these mice had normal Th1 cell priming but defective Th17 cell priming (Figure 3.2 B), demonstrating that restoring TLR signaling in DCs was insufficient to induce antigen specific Th17 cell priming.

In our *in vitro* priming assays, WT T cells differentiated into both Th1 and Th17 cell lineages (Figure 3.3). However, MyD88 KO T cells differentiated into IFN-γ producing Th1 cells but not IL-17 producing Th17 cells (Figure 3.3). Since MyD88 is an adaptor molecule shared by TLRs and the IL-1 receptor [298], we tested the ability of IL-1R1 KO T cells to

differentiate into Th17 cells and found that, IL-1R1 KO $CD4^+$ T cells behaved like MyD88 KO $CD4^+$ T cells in terms of IL-17 production (Figure 3.3). This is consistent with previously suggested role for IL-1R signaling in $CD4^+$ T cells for Th17 cell differentiation [138, 139]. A very surprising result however was that IL-6 KO DCs were able to induce Th17 cell differentiation comparable to their WT counterparts (Figure 3.4). These data suggest that IL-1 is critical and IL-6 is dispensable for Th17 cell lineage commitment. Since the current understanding is that IL-6 is critical for Th17 cell priming [290], these results led us to revisit the paradigm and further investigate *in vivo* requirements for Th17 cell lineage commitment.

Th17 cells are present in the peripheral secondary lymphoid organs but not in the intestinal lamina propria of IL-6 KO mice

Since our *in vitro* data suggested that IL-6 is dispensable for Th17 cell lineage commitment, we investigated the status of Th17 cells in IL-6 KO mice. In the spleens of IL-6 KO mice, we found that $CD62L^{lo}CD44^{hi}$ memory $CD4^+$ T cells secreted IFN-γ when activated *ex vivo,* similar to their WT counterparts. Surprisingly, memory cells derived from the spleens of IL-6 KO mice also secreted IL-17 comparable to the quantities secreted by WT $CD4^+$ T cells (Figure 3.5). The percentage of memory $CD4^+$ T cells making IL-17 was also comparable between WT and IL-6 KO mice (Figure 3.6). In agreement with our *in vitro* data, memory $CD4^+$ T cells from both MyD88 KO and IL-1R1 KO mice were defective in the secretion of IL-17 (Figure 3.7). We next measured the expression of the Th17 cell -lineage master regulator RORγt, and found that memory T cells obtained from both WT and IL-6 KO mice expressed

similar quantities of this transcription factor, both at the mRNA (Figure 3.8 A) and the protein (Figure 3.8 B) level. The RORγt-expressing memory $CD4^+$ T cells from IL-6 KO mice also express CCR6, a chemokine receptor known to be preferentially expressed by Th17 cells [299] (Figure 3.8 C). Overall, IL-6 KO mice seem to have Th17 cells that are phenotypically indistinguishable from those in the WT mice in the spleen.

These data differ from earlier findings demonstrating that IL-6 plays a non-redundant role in Th17 cell lineage commitment [123, 140]. However, many earlier reports have used recombinant cytokine cocktails for *in vitro* differentiation assays. Likewise, *in vivo* studies have dealt mainly with analyzing cytokine commitment in the $CD4^+$ T cells derived from the lamina propria (LP) of the intestines. We therefore decided to investigate Th17 cell commitment of $CD4^+$ T cells derived from the LP. Of note, because certain components of the intestinal microflora have been shown to favor the induction of Th17 cells [300], mice were co-housed for at least two weeks to ensure homogenous microflora populations between different genotypes. In contrast to our findings in the spleen, we found that Th17 cell lineage commitment was defective in LP $CD4^+$ T cells in IL-6 KO mice (Figure 3.9). In MyD88 KO and IL-1R1 KO mice, $CD4^+$ T cells from the LP behaved like $CD4^+$ T cells in the spleen and were defective in secretion of IL-17 (Figure 3.7).

It has been previously reported that TGF-β drives expression of the transcription factor Foxp3 and leads to generation of peripherally-induced Treg cells [123, 301, 302]. However, IL-6 antagonizes Foxp3-mediated inhibition of RORγt, thus preventing the generation of Treg cells and promoting the induction of Th17 lineage cells [127]. Since we found that IL-6 KO mice had normal Th17 lineage cells that express RORγt in the spleen, but both were defective

in the LP of the gut, we wanted to determine if the proportion of $Foxp3^+$ Treg cells was influenced in these tissues in IL-6 KO mice. We saw a similar proportion of $CD4^+$ T cells from the spleens of WT and IL-6 KO mice expressing Foxp3 (Figure 3.10). However, when we examined LP lymphocytes, we found that IL-6 KO mice had approximately double the proportion of $Foxp3^+$ $CD4^+$ T cells when compared to WT mice (Figure 3.10). These data suggest that IL-6 affects the lineage choice between $Foxp3^+$ Treg cells and Th17 lineage cells only in the LP of the intestines but not in the spleen.

Differential requirement of IL-6 for Th17 cell differentiation in secondary lymphoid organs and intestinal lamina propria

The above observations raised the interesting possibility that IL-6 could be required for Th17 cell lineage commitment in a tissue-specific manner. To test this hypothesis, we generated mice that express a dominant negative form of gp130, the signaling subunit of the IL-6 receptor complex [303, 304], in T cells under the control of a Cd2 promoter (CD2-NDgp130 Tg) [281] . $CD4^+$ T cells from these mice had diminished phosphorylation of STAT3 in response to IL-6 (Figure 3.11 A) and failed to show an enhanced response to a combination of IL-1β and IL-6 in a proliferation assay with anti-CD3 stimulation (Figure 3.11 B). Analysis of T cells from the spleen and LP of CD2-NDgp130 Tg mice revealed a phenotype similar to IL-6 KO mice, where IL-17 secreting T cells were present in the spleen and lymph nodes, but were reduced in the LP (Figure 3.11 C, D).

In order to look at the differentiation of Th1 and Th17 cells in various tissues, we transferred naïve cells pooled from the spleen and lymph nodes of CD2-NDgp130 Tg mice into Rag1 KO recipients. Commensal microflora cross the intestinal barrier in Rag1 KO mice and this approach would allow us to assess Th1 and Th17 cell differentiation in various organs. We analyzed T cells from the spleen, liver, LP and lung of the recipients seven days post transfer for the presence of IFN-γ- and IL-17-producing $CD4^+$ T cells. Intracellular staining suggested that both WT and CD2-NDgp130 Tg $CD4^+$ T cells committed to Th17 lineage cells in the spleen and the liver (Figure 3.12 A). In contrast, CD2-NDgp130 Tg $CD4^+$ T cells were defective in Th17 cell commitment in the LP and the lungs (Figure 3.12 A). CD2-NDgp130 Tg $CD4^+$ T cells from the spleens of Rag1 KO recipients secreted appreciable quantities of IL-17, whereas CD2-NDgp130 Tg $CD4^+$ T cells from the LP of Rag1 KO recipients failed to secrete detectable amounts of IL-17 (Figure 3.12 B, C). In contrast, IL-1R1 KO $CD4^+$ T cells recovered from the Rag1 KO hosts had defective IL-17 production in all the organs that were examined (Figure 3.13).

Lack of Th17 cells selectively in the gut in IL-6 KO and CD2-NDgp130 Tg mice may suggest a requirement of IL-6 for the survival of Th17 cells in the intestine. We tested this possibility by staining intestinal $ROR\gamma t^+$ $CD4^+$ T cells with Annexin V, which marks early apoptotic cells. There was indeed a small proportion of $ROR\gamma t^+$ Th17 cells in the gut of IL-6 KO and CD2-NDgp130 Tg mice (Figure 3.9) and the proportions of these cells that stained positive with Annexin V was comparable to those of WT mice (Figure 3.14). These results argue that IL-6 signaling is not specifically required for the survival of Th17 cells in the gut, and favors the hypothesis that IL-6 is a tissue-specific priming factor for Th17 cells.

IL-6-independent priming of Th17 cells in the spleen following systemic infection

Since our data suggest a selective requirement of IL-6 for Th17 cell commitment in mucosal tissues (the intestines and lungs), but not in spleen both at steady state and during differentiation under lymphopenic conditions (Rag1 KO transfer model), we investigated the generation of Th17 lineage cells during infection. Oral infection using *Citrobacter rodentium* led to priming of *Citrobacter*-specific Th17 cells in the LP of WT mice, but Th17 cell priming was defective in both IL-6 KO mice as well as IL-1R1 KO mice (Figures 3.15A, B). We were also not able to detect any *Citrobacter*-specific Th17 cell response in the mesenteric lymph nodes of IL-6 KO mice (Figure 3.15 C), indicating that the lack of antigen-specific Th17 cells in the LP is due to a priming defect rather than defective migration of activated cells from the site of priming to the peripheral tissue. Importantly, when mice were infected with *S. typhimurium* by the intraperitoneal route causing a systemic infection, comparable *Salmonella*-specific Th17 cell priming was observed in WT and IL-6 KO mice in the spleen (Figure 3.16 A). However, there was defective priming of *Salmonella*-specific Th17 cells in IL-1R1 KO mice (Figure 3.16 B). These data demonstrate that IL-1 is required for generating IL-17-secreting $CD4^+$ T cells, irrespective of the route of infection, whereas IL-6 is important for inducing Th17 cells in the LP of the intestines during oral infection, but is not required for Th17 cell priming in the spleen during a systemic infection.

Subcutaneous immunization fails to generate Th17 cells in the absence of IL-6

Because of the similarities between the mucosal and cutaneous immune systems, we sought to determine whether our findings in the intestine are applicable to the cutaneous immune system. Although IL-17 secreting $CD4^+$ T cells are present in the skin-draining lymph nodes in both WT and IL-6 KO mice (Figures 3.17), it is possible that these cells are primed in the spleen and subsequently traffic to the peripheral lymph nodes. Therefore, we tested whether IL-6 is required to generate fresh antigen-specific Th17 cell responses during immune responses initiated from the skin. We transferred OT-II T cells into WT and IL-6 KO mice and recipients were immunized in the footpads with ovalbumin and LPS emulsified in incomplete Freund's Adjuvant (IFA) (Figure 3.18 A). OT-II T cells from the immunized WT recipients secreted both IFN-γ and IL-17 (Figure 3.18 B). However, OT-II T cells recovered from IL-6 KO recipients secreted only IFN-γ but no IL-17 suggesting that IL-6 is required for Th17 cell priming during subcutaneous immunization (Figure 3.18 B). Consistently, CD2-NDgp130 Tg mice immunized in the footpads generated a much reduced antigen-specific Th17 cell response (Figure 3.18 C), further supporting the notion that IL-6 is required for generating Th17 cells in the skin draining lymph nodes.

Importantly, $CD4^+$ T cells from the gut of did not appear to have an intrinsic defect in becoming Th17 cells, as naïve $CD4^+$ T cells from the mesenteric lymph nodes (where gut homing T cells are primed) could be polarized towards the Th17 cell lineage, with IL-6 and TGF-β as well as IL-1, IL-23 and TGF-β, as efficiently as splenic $CD4^+$ T cells (Figure 3.19). In addition, lack of IL-6 during development did not affect the ability of $CD4^+$ T cells to become Th17 cells, because Th17 cell polarizing conditions induced similar amounts of IL-17 producing cells from splenic and mesenteric lymph node $CD4^+$ T cells from IL-6 KO mice

comparing to their WT counterparts (Figure 3.19). In the context of *in vitro* priming with WT splenic DCs, when splenic and mesenteric lymph node $CD4^+$ T cells from WT and IL-6 KO mice were compared, they produced very similar amounts of IFN-γ as well as IL-17 (Figure 3.20). Consistent with our earlier observation, IL-6 neutralization did not affect IL-17 production by all four groups of T cells (Figure 3.20 A); however IL-1 receptor blockade with IL-1 receptor antagonist completely abolished IL-17 secretion in the cultures (Figure 3.20 B). Using IL-6 KO splenic DCs in the *in vitro* culture system showed similar results as IL-6 neutralization, further confirming that IL-6 is not required for priming of Th17 cells when splenic DCs are used (Figure 3.20 C)**.**

Resident DCs in priming micro-environments dictate cytokine requirements for Th17 cell differentiation

To explore the possibility that DC populations resident in the spleen and LP could primarily be responsible for the differentially controlled lineage choices made by activated T cells in their milieu, we examined the necessity of IL-6 for Th17 cell priming induced by DCs from the spleen and LP. We purified DCs from the spleens and LP of WT and IL-6 KO mice and tested their ability to prime naïve WT $CD4^+$ T cells into Th1 and Th17 cell lineages *in vitro*. Splenic DCs from both WT and IL-6 KO mice induced differentiation of T cells that secreted IFN-γ as well as IL-17 (Figure 3.21 A), thus validating that Th17 cell lineage development induced by splenic DCs does not require IL-6. However, IL-6 KO LP DCs failed to induce differentiation of IL-17 secreting T cells, but induced differentiation of IFN-

γ secreting T cells (Figure 3.21 B). WT LP DCs induced both IFN-γ and IL-17 from activated $CD4^+$ T cells (Figure 3.21 B). These data demonstrate that while both splenic DCs and LP DCs induce differentiation of naïve T cells into Th17 cells, splenic DCs do so independent of IL-6, whereas LP DCs depend on IL-6 to induce Th17 lineage cells.

$CD103^+$ DCs determine the dependency on IL-6 for Th17 cell differentiation

In order to understand the differences in the abilities of spleen and LP DCs to induce Th17 cell priming we began to investigate the differences in DC populations that reside in these tissues. Most strikingly, we noticed that $CD103^{hi}$ DCs are absent in the spleen but comprise ~20% of intestinal DCs (Figure 3.22 A). These $CD103^{hi}$ DCs (hereafter referred to as $CD103^+$ DCs) were also present at a low percentage in the skin-draining lymph nodes but accumulated upon subcutaneous LPS injection (Figures 3.22 B, C). We sorted out $CD103^+$ DCs from the intestine and tested their ability to induce $CD4^+$ T cell differentiation. Both WT and IL-6 KO LP $CD103^+$ DCs failed to induce detectable IFN-γ or IL-17 production from *in vitro* activated $CD4^+$ T cells (Figure 3.22 D), consistent with a previous report showing that these DCs preferentially induce Treg cells [305]. We further explored the possibility that $CD103^+$ DCs might impose the requirement of IL-6 for Th17 cell priming. We depleted $CD103^+$ DCs from LP DCs and used the $CD103^-$ DC population from IL-6 KO mice for *in vitro* priming assays. Consistent with our prediction, the $CD103^-$ DC population from the LP resembled the splenic DCs. WT and IL-6 KO $CD103^-$ LP DCs induced similar quantities of IFN-γ as well as IL-17, demonstrating that $CD103^+$ DCs in the gut are dominant over $CD103^-$

DCs and prevent IL-6-independent priming of Th17 lineage cells (Figure 3.22 D). Also, we found that lymph node resident DCs from naïve mice, which have a very small $CD103^+$ population, were able to prime Th17 cells *in vitro* in the absence of IL-6 (Figure 3.23). However, when $CD103^+$ DCs were recruited to the skin draining lymph nodes, following subcutaneous immunization (Figure 3.22 B, C), IL-6 was required for Th17 cell priming (Figure 3.18).

It has been previously reported that $CD103^+$ DCs in the gut make high quantities of TGF-β and retinoic acid (RA), both of which have been shown to impact Th17 cell differentiation. Low concentration of TGF-β promotes Th17 cell differentiation while high concentration of TGF-β inhibits it [124]. RA suppresses Th17 cell differentiation yet enhances inducible regulatory T cell (iTreg) generation [157, 306]. We first tested the possibility that IL-6 could be required to overcome the suppressive effects of TGF-β. If this were true, neutralization of TGF-β would lead to IL-6-independent priming by LP DCs. However neutralization of TGF-β and inhibition of TGF-β signaling led to total abrogation of Th17 cell priming (Figure 3.24), even when WT DCs were used for priming. These experiments suggest that TGF-β is an absolute requirement for Th17 cell priming. We also tested the possibility that RA made by $CD103^+$ DCs was responsible for dictating the requirement of IL-6 for Th17 cell priming in the gut. Addition of RA abrogated Th17 cell priming induced by both WT and IL-6 KO DCs without significantly affecting Th1 cell lineage development (Figure 3.25). However, when a combination of TGF-β and RA was added to *in vitro* priming cultures, there was total abrogation of Th17 cell priming induced by IL-6 KO $CD103^-$ DCs but not by WT DCs (Figure 3.26 A). Similarly, TGF-β and RA also inhibited priming of Th17 cell lineage

cells by splenic DCs from IL-6 KO mice (Figure 3.26 B). RA has been demonstrated to enhance Smad3 phosphorylation and TGF-β signaling [157, 306]. Consistent with previous studies, IL-6 diminished the ability of TGF-β to induce Smad3 phosphorylation in $CD4^+$ T cells (Figure 3.27). Our data therefore suggest that in the presence of $CD103^+$ DCs that make both TGF-β and RA, IL-6 is required to overcome their effects to allow Th17 cell priming in the gut. In the absence of $CD103^+$ DCs, as is the case in the spleen, Th17 cell priming is independent of IL-6.

DISCUSSION

This study reveals several insights into how different priming microenvironments determine the cytokine requirements for Th17 cell lineage differentiation. Our previous work has shown that MyD88 KO mice fail to mount Th1 cell responses when LPS is used as an adjuvant [296]. However $CD25^+$ Treg cell depletion restores Th1 cell priming in MyD88 KO mice [296]. Since several studies show reciprocal regulation of Treg and Th17 cell lineages [123, 128], we were interested in exploring whether depletion of Treg cells restores or enhances Th17 cell priming in MyD88 KO mice. Our investigations of T cell differentiation in MyD88 KO mice revealed normal Th1 cell priming, but defective Th17 cell commitment in the absence of Treg cells. This is an important result since it suggests that, in the absence of MyD88 signaling, the lack of Treg cells is permissive for Th1 cell priming but not Th17 cell priming. Our results from transgenic mice that express MyD88 only in DCs and macrophages, but not in T cells, demonstrate that MyD88 in T cells is absolutely required for Th17 cell commitment. Requirement for MyD88 signaling in $CD4^+$ T cells is explained by the critical role for IL-1 in priming of Th17 lineage cells. Several studies have demonstrated that IL-6 is a master inducer of Th17 cell differentiation [122, 123, 132, 140, 291]. It has also been argued that IL-6 regulates the induction of Th17 lineage cells by enhancing the expression of IL-1R on $CD4^+$ T cells [139]. Since we observed normal Th17 cell priming *in vitro* while using IL-6 KO DCs for priming of naïve T cells, we decided to further investigate the exact roles of IL-1 and IL-6 in Th17 cell lineage commitment *in vivo*. As mice age, they develop a substantial proportion of $CD44^{hi}$ $CD62L^{lo}$ cells in the spleen, representing antigen experienced T cells. As revealed

in our results, $CD44^{hi}CD62L^{lo}$ $CD4^+$ T cells from WT, MyD88 KO, and IL-6 KO mice secreted IFN-γ. However, $CD44^{hi}CD62L^{lo}$ $CD4^+$ T cells from MyD88 KO and IL-1R1 KO mice failed to secrete appreciable quantities of IL-17. Surprisingly, $CD44^{hi}CD62L^{lo}$ cells from IL-6 KO mice made similar quantities of IL-17 as WT T cells. WT and IL-6 KO mice also had similar proportions of IL-17 secreting cells, as measured by intracellular staining. More importantly, the memory cell pool from IL-6 KO mice expressed RORγt. These results suggest induction of the transcription factor RORγt, and subsequent Th17 cell lineage commitment, is not dependent on IL-6 in $CD4^+$ T cells of the spleen.

Several reports suggest that IL-1 and IL-6, along with TGF-β, play a major role in the induction of Th17 cells. It has also been shown previously that LPLs from IL-6 KO mice make reduced IL-17 as assayed by intracellular staining as well as quantitative polymerase chain reaction (PCR) [140]. In agreement with this, we found that $CD4^+$ T cells from the LP of IL-6 KO mice did not make detectable IL-17. We also found that $CD4^+$ T cells from the LP of MyD88 KO and IL-1R1 KO mice secreted substantially lower or non-detectable quantities of IL-17 when compared to WT mice. These results along with our infection experiments argue that both IL-1 and IL-6 are required for generation of Th17 cells in the LP of the gut while IL-1 is critical for Th17 cell lineage development in the spleen. It is also remarkable that when naïve T cells are transferred into Rag1 KO mice, cells that are defective for gp130 signaling secrete IL-17 when isolated from the spleen and liver, but fail to do so in the lungs and the LP of the intestines. This unique differentiation pattern suggests that sterile and commensal bacteria-rich microenvironments impose different rules for Th17 cell lineage commitment.

It is also important to note here that skin draining lymph nodes have Th17 lineage cells, but subcutaneous immunization fails to induce Th17 cell priming in IL-6 KO mice. The presence of Th17 lineage cells in the lymph nodes can be explained by the possibility that following splenic priming, Th17 cells migrate from the spleen to these lymph nodes. However upon subcutaneous immunization, IL-6 was required for local priming of Th17 lineage cells in the lymph nodes. It has been recently proposed that IL-6 induces Th17 cell lineage commitment through upregulation of IL-1R [139]. Since IL-6 KO mice have defective Th17 cell lineage priming only in the lamina propria and skin draining lymph nodes, IL-6 could be controlling Th17 cell lineage commitment through regulation of the IL-1R only in the LP of the gut and skin draining lymph nodes but not in the spleen.

There are well known differences in the populations of the DCs residing in the gut and peripheral secondary lymphoid organs [307]; however our results provide further insights into the priming abilities of LP DCs. While splenic DCs induce normal Th17 cell priming, LP DCs fail to do so in the absence of IL-6. $CD103^+$ DCs are of interest because of some unique abilities that have been ascribed to them. $CD103^+$ DCs in the intestine have been shown to imprint T cells with gut-homing molecules such as the chemokine receptor CCR9 [308, 309]. They are also responsible for inducing regulatory T cells in the intestine through the production of TGF-β and retinoic acid [305], thereby contributing to the control of colitis [310]. Our findings have ascribed an additional function to $CD103^+$ DCs, of regulating the requirement for Th17 cell priming in the gut and the skin draining lymph nodes. We have demonstrated that $CD103^-$ cells are responsible for IL-6-independent priming of Th17 lineage cells. This conclusion is strongly supported by the fact that splenic DCs are all $CD103^-$ and also by the fact that $CD103^-$ DC

populations from the lamina propria could induce Th17 cell priming in the absence of IL-6. There was normal presence of Th17 lineage cells in the lymph nodes of IL-6 KO mice and that lymph node resident DCs could prime Th17 lineage cells *in vitro*, however subcutaneous challenge with TLR ligands led to accumulation of $CD103^+$ DCs in the draining lymph nodes and these DCs seemed to determine the requirement of IL-6 for Th17 cell priming. Interestingly, $CD103^+$ DCs did not seem to play a role in regulating Th1 cell priming. $CD103^+$ DCs are known to induce generation of $Foxp3^+$ iTreg cells [305] and our study supports the notion that these DCs impose a requirement of IL-6 for Th17 cell priming by $CD103^-$ DCs. Our data also demonstrate that TGF-β is absolutely essential for driving Th17 cell differentiation by DCs. However a combination of TGF-β and RA, which provides a more physiological microenvironment since they are both made by $CD103^+$ DCs, had an inhibitory effect on Th17 cell priming in the absence of IL-6, both by splenic and LP DCs. These data are consistent with earlier studies showing that RA enhances phosphorylation of Smad3 in response to TGF-β treatment [306] and that activation of STAT3 by IL-6 leads to desensitization of TGF-β signaling [311]. Our data therefore suggest that when $CD103^+$ DCs are part of the DC population involved in T cell priming, TGF-β and RA made by these DCs have a dominant role in determining the outcome of T cell differentiation. Consequently, in the absence of IL-6, $CD103^+$ DCs play a dominant role leading to generation of $Foxp3^+$ iTreg cells. An earlier study demonstrated that in mice with DCs incapable of making active TGF-β, the proportion of Foxp3 positive $CD4^+$ T cells is reduced in the LP, but not in the spleen [312]. Conversely, our results demonstrate that the absence of IL-6 enhanced the proportion of Foxp3 positive $CD4^+$ T cells only in the LP but not in the spleen.

In the systemic immune system, which is generally sterile, IL-1 mediated signaling seems to be sufficient to support Th17 cell lineage differentiation. In contrast, the mucosal and cutaneous surfaces are constantly exposed to commensal microorganisms and appear to have stringent requirements to induce Th17 cell priming. Since Th17 cells are pathogenic and can cause auto-immunity, it is particularly important for the mucosal and cutaneous immune systems to maintain a balance between immunity and tolerance. As IL-6 is highly pro-inflammatory, the mucosal and cutaneous systems may interpret the presence of IL-6 as a critical signal implicating pathogen invasion, and differentiate active infection from steady-state sampling of the commensal microflora and self-antigens from the tissues. IL-6 seems to act as an additional checkpoint for inducing Th17 cells in the gut as well as in the skin draining lymph nodes, guiding the lineage choice between inhibitory iTreg cells and inflammatory Th17 cells. It has been reported that $CD103^+$ DCs change their suppressive behavior in colitic mice [313] and it is possible that there could be transient Th17 cell priming in the guts of lL6-deficient mice when $CD103^+$ DCs are functionally shut down and it would be worthwhile to explore the role of IL-6 in survival and maintenance of Th17 cells generated under such conditions.

In summary, our study provides important insights into how priming microenvironments guide the cytokine requirements for the development of Th17 lineage cells. In commensal-bearing sites such as the intestine and the skin, IL-1 is critical, but IL-6 acts as a second checkpoint for Th17 cell lineage commitment. More importantly our study discovers that DC populations in the spleen, lymph nodes and LP of the intestines are responsible for determining the cytokine requirements for Th17 cell priming in respective tissues. These

findings could have important implications for designing therapies for systemic and mucosal autoimmunity, as well as for choosing routes of vaccination for inducing protective Th17 cell responses.

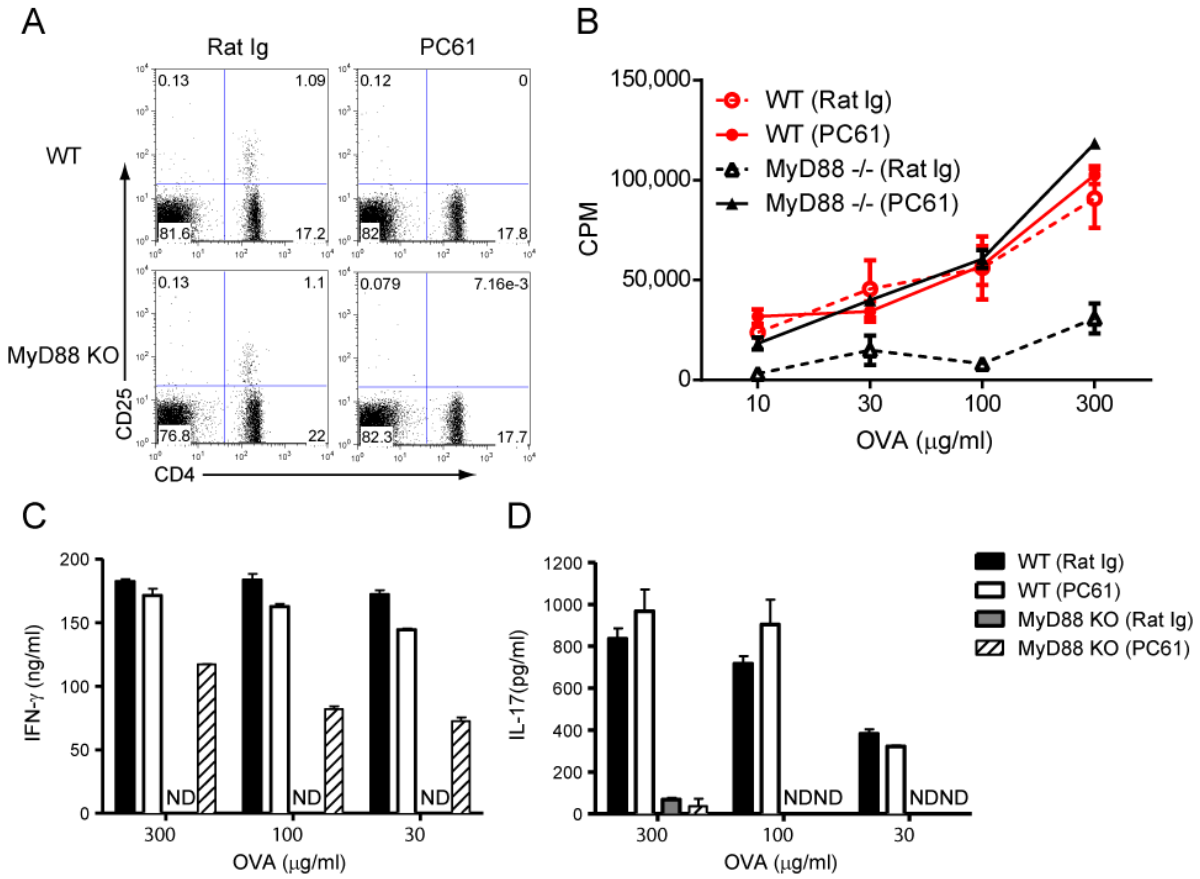

Figure 3.1. Treg depletion restores Th1 but not Th17 priming in Myd88-deficient mice.

WT and MyD88 KO mice received rat anti-mouse CD25 antibody (PC61) or control antibody (Rat Ig) by intravenous route (25μg/mouse). Three days later, peripheral blood was stained for CD4 and CD25 to confirm Treg depletion (A) and mice were immunized in the fp with OVA (50μg/fp) and LPS (5μg/fp) emulsified in IFA. CD4+ T cells ($2x10^5$) were purified from draining lymph nodes at day 7 post-immunization and cultured with TLR2/TLR4 DKO B cells ($3x10^5$) as APCs in the presence of titrating doses of OVA for 72 hours. Proliferation of CD4+ T cells was measured by ^{3}H-thymidine incorporation (B). IFN-γ (C) and IL-17 (D) concentrations in the culture supernatants were determined by ELISA. Line graph and bar graph represent mean ± SEM. ND, not detectable. Data are representative of four independent experiments.

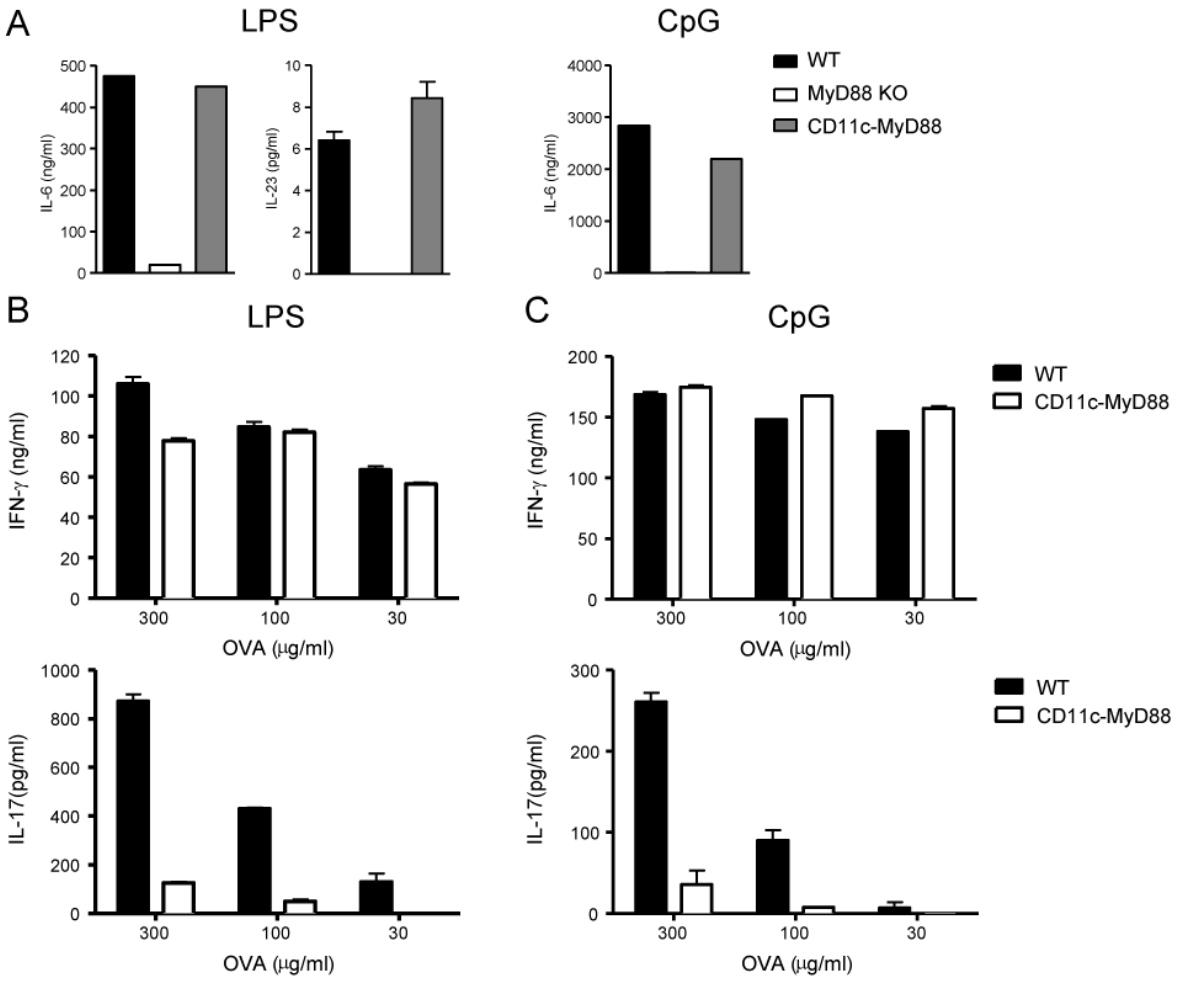

Figure 3.2. Restoring MyD88 expression in DCs fails to restore Th17 priming.

(A) Bone marrow derived dendritic cells from WT and CD11c-MyD88 Tg mice were stimulated with the TLR4 ligand, LPS (100ng/ml) or the TLR9 ligand, CpG (1μM) for 16 hours. Supernatants were assayed for IL-6 (upper panels) or IL-23 (lower panel) by ELISA. (B-C) WT and CD11c-MyD88 Tg mice were immunized in the fp with OVA (50μg/fp) and LPS (5μg/fp) (B) or OVA (50μg/fp) and CpG (5μg/fp) (C) emulsified in IFA. $CD4^+$ T cells were activated to measure OVA-specific IFN-γ (upper panels) and IL-17 (lower panels) as described in Figure 3.1. Bar graphs represent mean $\pm$ SEM. Data are representative of three independent experiments.

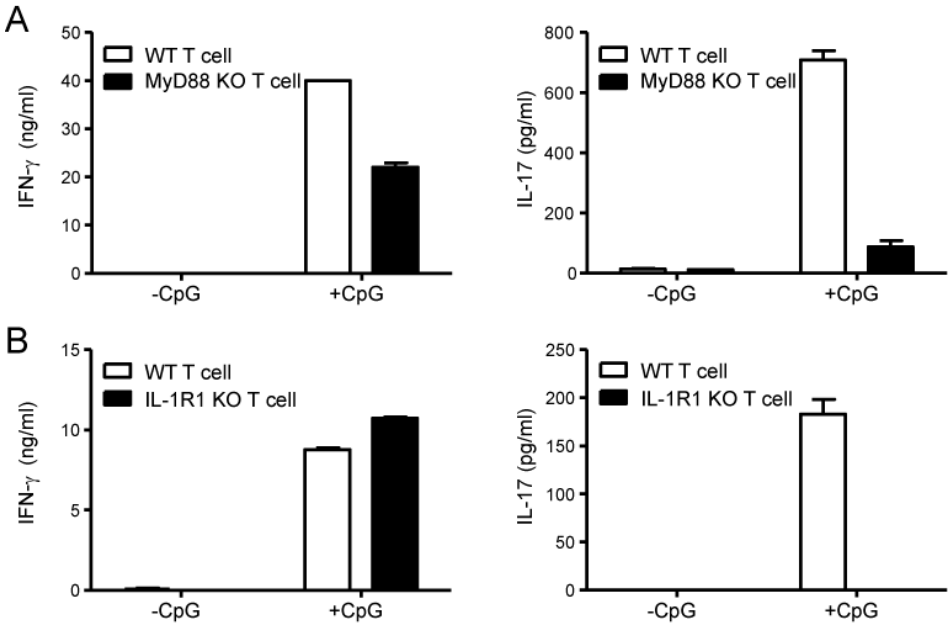

Figure 3.3. MyD88 dependent IL-1R signaling is required for Th17 priming *in vitro*.

Purified naïve CD4+ T cells ($3x10^5$) from the indicated strains were cultured in the presence of purified WT splenic DCs ($6x10^4$) and anti-CD3 (10ng/ml) for 5 days in the presence or absence of the TLR ligands. Culture supernatants were assayed for IFN-γ (right panels) and IL-17 (left panels) by ELISA. Data are representative of three to four independent experiments. Bar graphs represent mean ± SEM.

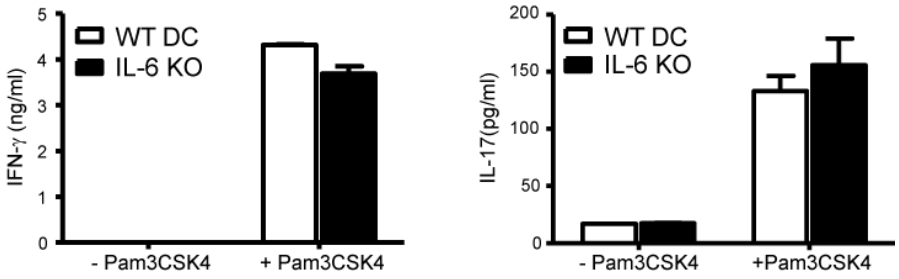

Figure 3.4. IL-6 is dispensable for Th17 priming *in vitro*.

Purified naïve WT CD4+ T cells ($3x10^5$) from the indicated strains were cultured in the presence of purified WT of IL-6 KO splenic DCs ($6x10^4$) and anti-CD3 (3ng/ml) for 5 days in the presence or absence of the TLR ligands. Culture supernatants were assayed for IFN-γ (right panels) and IL-17 (left panels) by ELISA. Data are representative of three to four independent experiments. Bar graphs represent mean $\pm$ SEM.

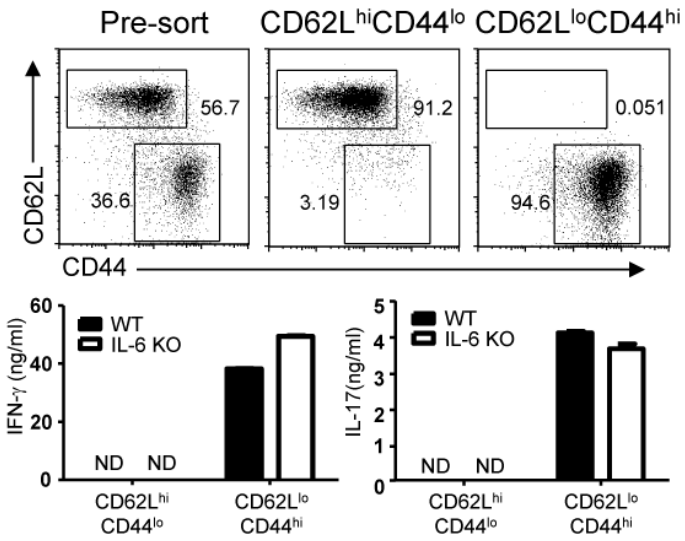

Figure 3.5. Th17 cells are present in the spleen of IL-6-deficient mice.

$CD4^+$ T cells from the spleens of slightly aged (16-20 weeks) WT and IL-6 KO mice were sorted to obtain $CD62L^{hi}CD44^{lo}$ naïve and $CD62L^{lo}CD44^{hi}$ memory populations. The sorted cells were stimulated with plate-bound anti-CD3 and anti-CD28 for 48 hours and culture supernatants were assayed for IFN-γ and IL-17 by ELISA. Bar graphs represent mean + SEM. Data are representative four independent experiments.

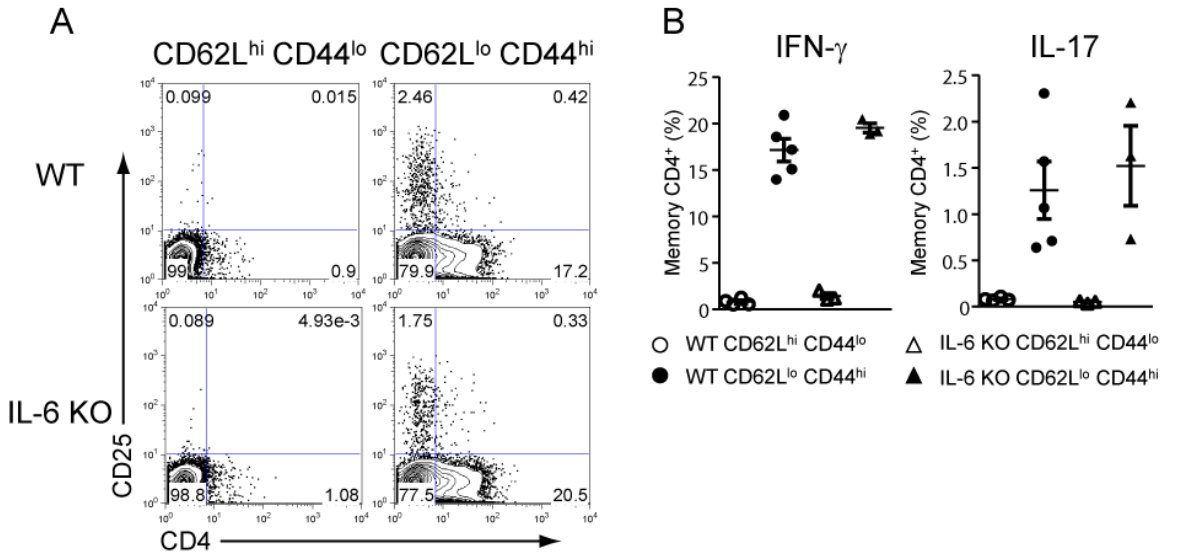

Figure 3.6. IL-17 secreting cells are present in the spleen of IL-6-deficient mice.

$CD62L^{hi}CD44^{lo}$ naïve and $CD62L^{lo}CD44^{hi}$ memory $CD4^+$ T cells were sorted from the spleen of WT and IL-6 KO mice as described in Figure 3.5 and stained for intracellular IFN-γ, and IL-17. Representative plots (A) and combined data (B) of IFN-γ and IL-17 secreting $CD4^+TCR\beta^+$ cells from several mice are shown. Scatter plot represents mean $\pm$ SEM.

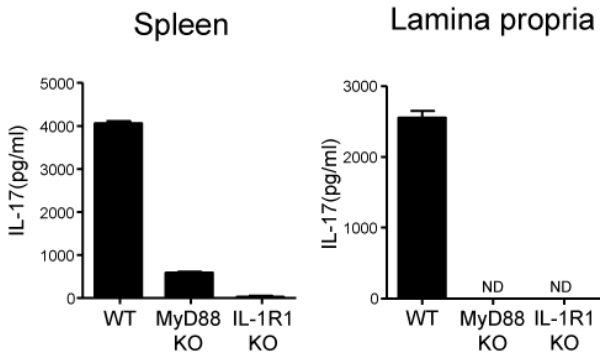

Figure 3.7. CD4$^+$ T cells from both the spleen and LP of MyD88 KO and IL-1R1 KO mice are defective in IL-17 production.

CD62L^{lo}CD44^{hi} memory CD4$^+$ T cells from the spleen or intestinal lamina propria CD4$^+$ T cells of WT, MyD88 KO or IL-1R1 KO mice were stimulated with plate-bound anti-CD3 and anti-CD28 for 48 hours and culture supernatants were assayed for IL-17 by ELISA. Bar graphs represent mean $\pm$ SEM. Data are representative three independent experiments.

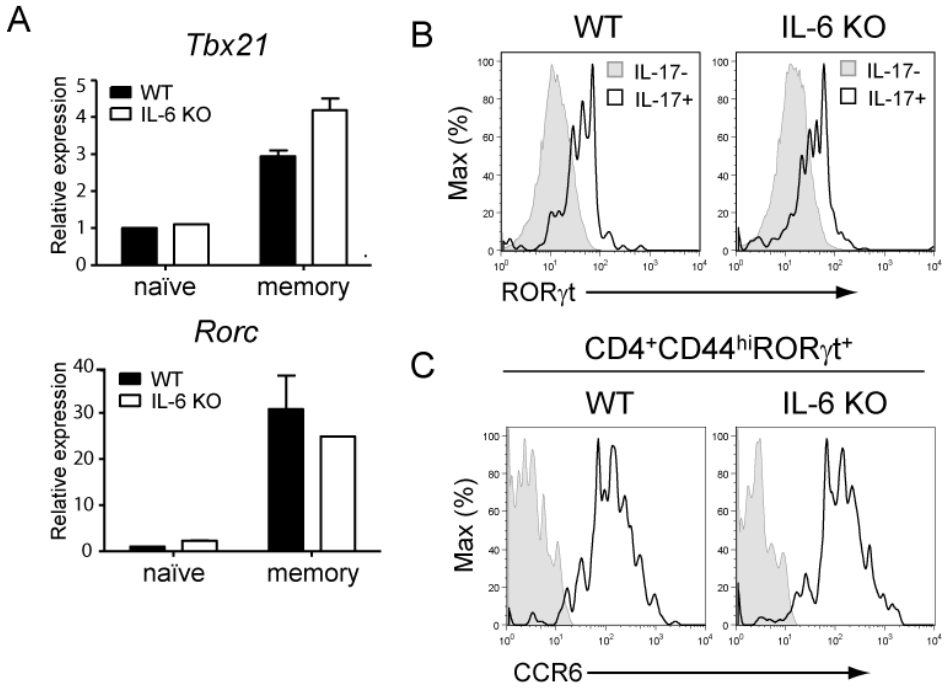

Figure 3.8. Splenic Th17 cells from IL-6-deficient mice are phenotypically identical to the WT counterparts.

(A-B) CD62L^{hi}CD44^{lo} naïve and CD62L^{lo}CD44^{hi} memory CD4^{+} T cells were sorted from the spleen of WT and IL-6 KO mice as described in Figure 3.5. (A) The transcript levels of *Tbx21* and *Rorc* were measured by quantitative RT-PCR. (B) CD62L^{lo}CD44^{hi} memory populations were stimulated with PMA and ionomycin and stained for IL-17 and RORγt. Histograms show the expression of RORγt by CD4^{+}TCRβ^{+}IL-17^{+} and CD4^{+}TCRβ^{+}IL-17^{-} cells. (C) Splenocytes from WT and IL-6 KO mice were stained for surface CD4, CD44, CCR6 and intracellular RORγ. Open histograms show the expression of CCR6 on CD4^{+}CD44^{hi}RORγt^{+} cells. Shaded histograms, unstained control. Bar graphs represent mean ± SEM. Data are representative three independent experiments.

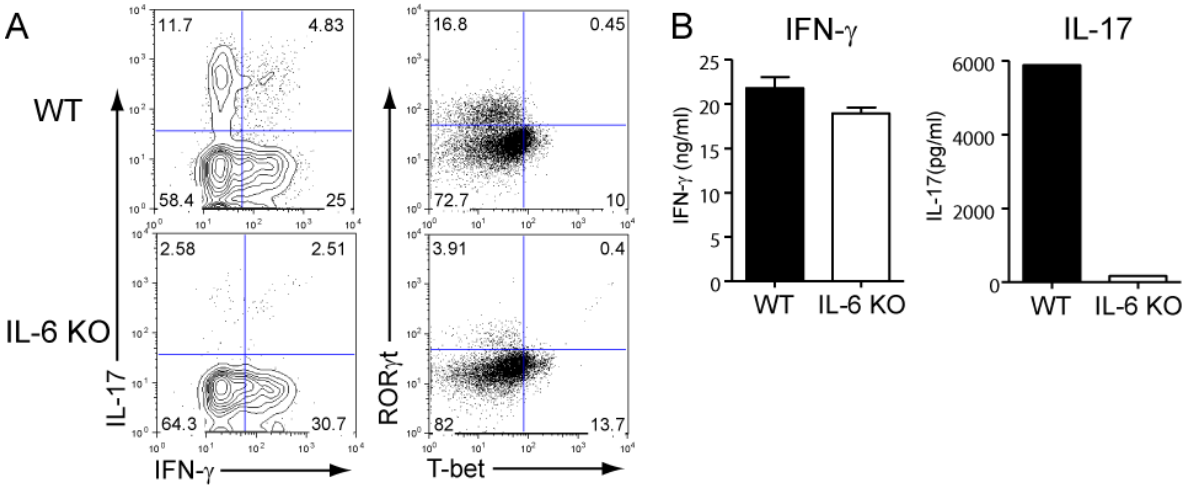

Figure 3.9. Th17 cells are absent in the intestine of IL-6-deficient mice.

(A) Lamina propria lymphocytes were isolated from indicated mice after at least two weeks of cohousing and stained for IFN-γ and IL-17 following stimulation, or RORγt and T-bet directly. (B) Enriched $CD4^+$ T cells from LP of indicates strains of mice were stimulated (30,000 $CD4^+$ T cells/well) with plate-bound anti-CD3 and anti-CD28 for 48 hours and culture supernatants were assayed for IFN-γ IL-17. Bar graphs represent mean $\pm$ SEM. Data are representative of three to five independent experiments.

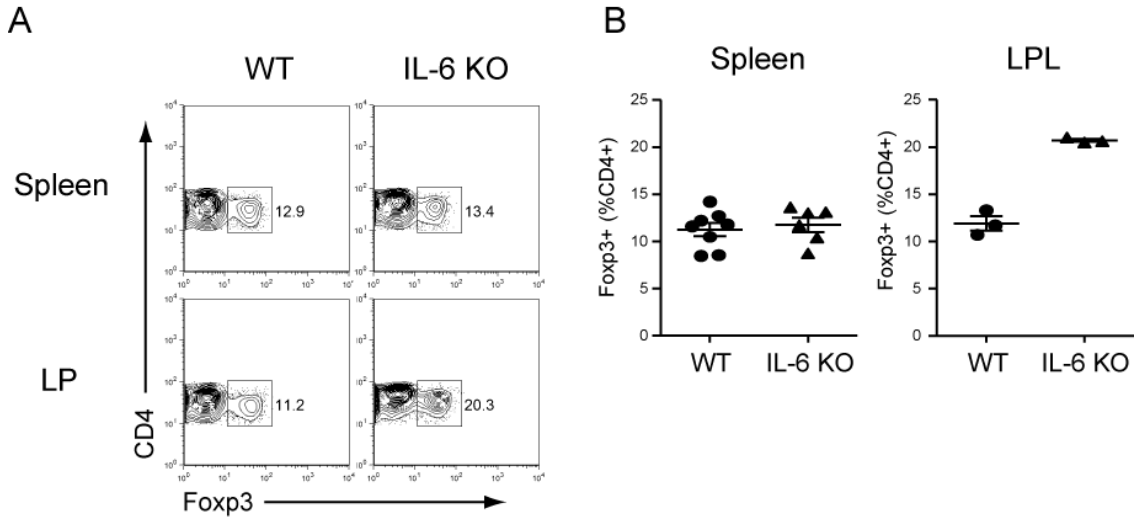

Figure 3.10. The absence of IL-6 enhances the proportion of Foxp3 positive CD4$^+$ T cells only in the LP, but not in the spleen.

Splenocytes and LPLs from WT and IL-6 KO mice were stained for surface CD4 and intracellular Foxp3. (A) Representative plots show cells positive for surface CD4 expressing intracellular Foxp3. (B) Combined results from several mice are shown as the percentage Foxp3 positive cells among the total CD4$^+$ T cell pool in the spleen and LP. Scatter plots represent mean $\pm$ SEM.

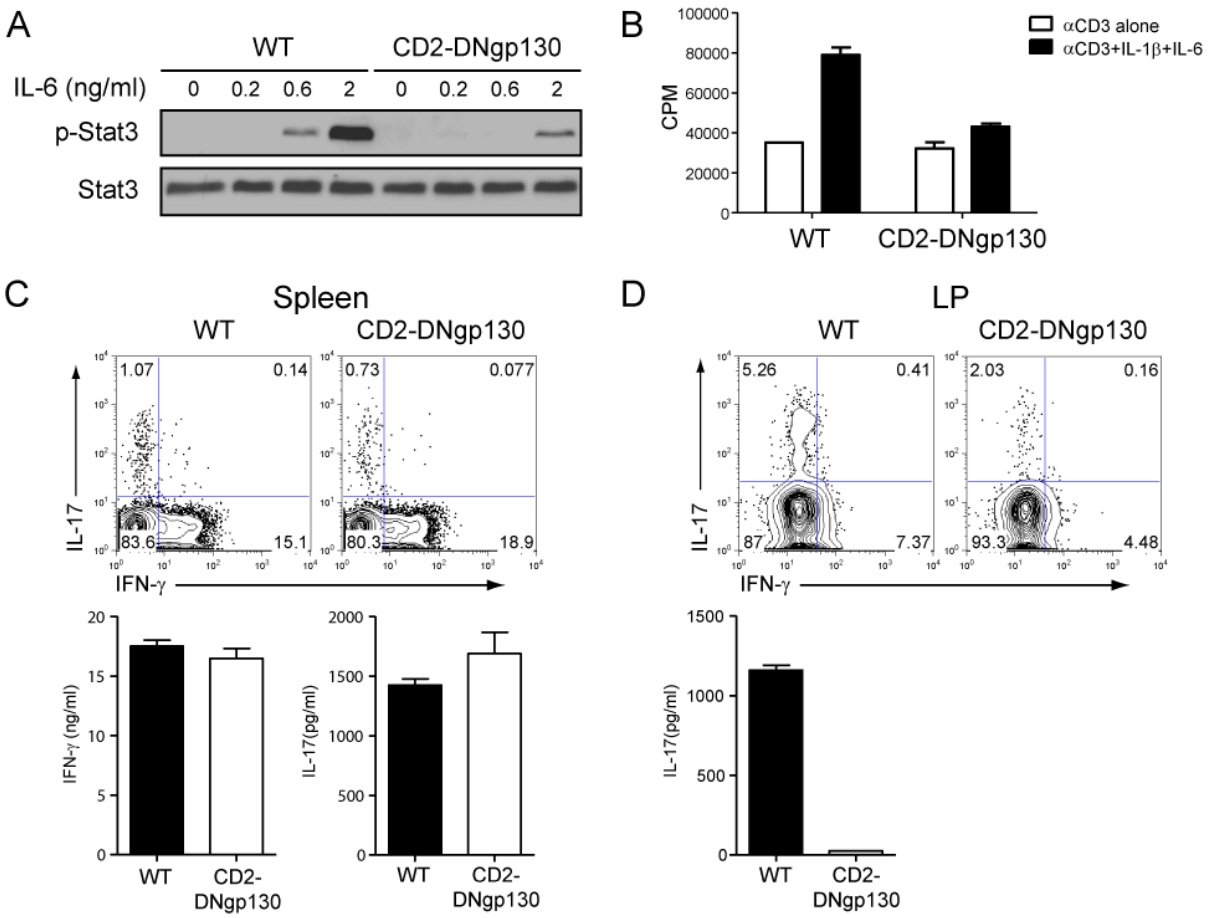

Figure 3.11. T cell intrinsic IL-6 signaling determines tissue-specific requirement for IL-6 in Th17 differentiation.

(A) $CD4^+$ T cells from the spleen and lymph nodes and stimulated for 15 minutes with indicated concentrations of IL-6 and analyzed for phospho STAT3 by western blot. (B) Purified $CD4^+$ T cells from WT and CD2-DNgp130 Tg mice were stimulated with anti-CD3 (10ng/ml) and MyD88/Trif DKO splenic DCs as APCs in the presence of 2ng/ml of IL-6 and 2ng/ml of IL-1β. Proliferation of $CD4^+$ T cells was measured by ^{3}H-thymidine incorporation. IL-6 and IL-1β synergize to enhance proliferation of WT T cells but fail to do so for T cells expressing the dominant negative gp130. (C-D) Splenic memory $CD4^+$ T cells (C) or LPLs (D) from WT and CD2-DNgp130 Tg mice were stained for intracellular IFN-γ and IL-17, or stimulated with plate bound anti-CD3 and anti-CD28 for 48 hours and culture supernatants were assayed for IFN-γ and IL-17. Bar graphs represent mean ± SEM. Data are representative of two independent experiments.

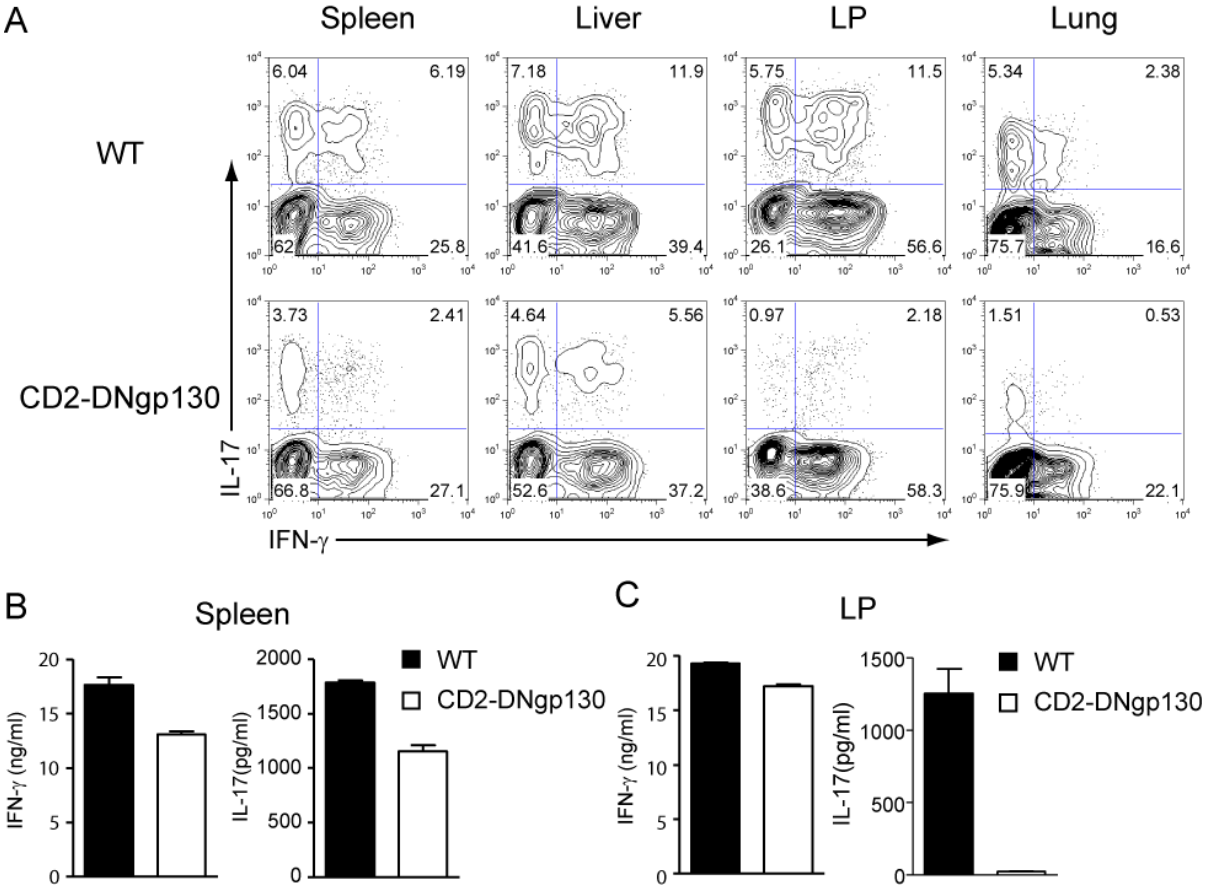

Figure 3.12. Differential requirement of IL-6 for Th17 cell lineage commitment in different priming microenvironments under lymphopenic conditions.

Cells from the spleen and lymph nodes of WT or CD2-DNgp130 Tg mice were transferred intravenously into Rag1 KO recipients. After 7 days, mononuclear cells (MCs) were isolated from the spleen, liver, LP and lung. (A) MCs from the indicated organs were stimulated with PMA and ionomycin and stained for intracellular IFN-γ and IL-17. $CD4^{+}TCR\beta^{+}$ positive cells expressing intracellular IFN-γ and IL-17 are shown. (C-D) $CD4^{+}$ T cells from indicated organs were stimulated with plate-bound anti-CD3 and anti-CD28 for 48 hours and analyzed for IFN-γ and IL-17 secretion in the culture supernatants by ELISA. Bar graphs represent mean ± SEM. Data are representative of three independent experiments.

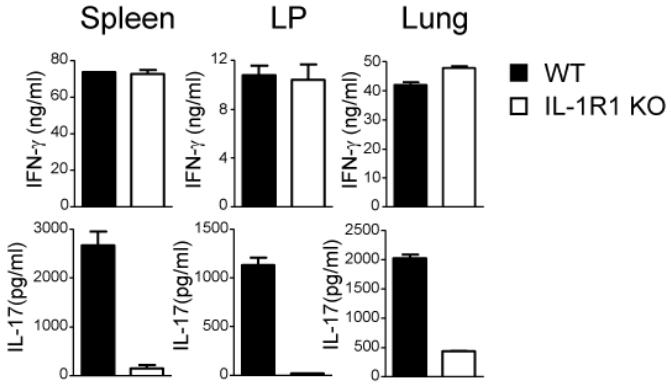

Figure 3.13. Ubiquitous requirement of IL-1 for Th17 lineage commitment in different priming microenvironments under lymphopenic conditions.

Cells from the spleen and lymph nodes of WT and IL-1R1 KO mice were transferred intravenously into Rag1 KO recipients. After 7 days, $CD4^+$ T cells isolated from the spleen, LP and lung were stimulated with plate-bound anti-CD3 and anti-CD28 for 48 hours and analyzed for IFN-γ and IL-17 secretion in the culture supernatants. Bar graphs represent mean ± SEM. Data are representative of three independent experiments.

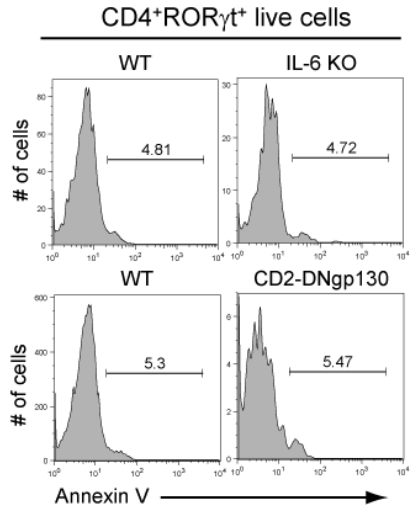

Figure 3.14. Lack of IL-6 signaling does not selectively affect the survival of intestinal Th17 cells.

Freshly isolated LPLs from WT and IL-6 KO mice (upper panel) or WT and CD2-DNgp130 Tg mice were stained immediately with anti-CD4, anti-RORγt, Annexin V, and fixable viability dye. The percentages of early apoptotic cells (Annexin V+) among all Th17 cells ($CD4^+ROR\gamma t^+$) are shown.

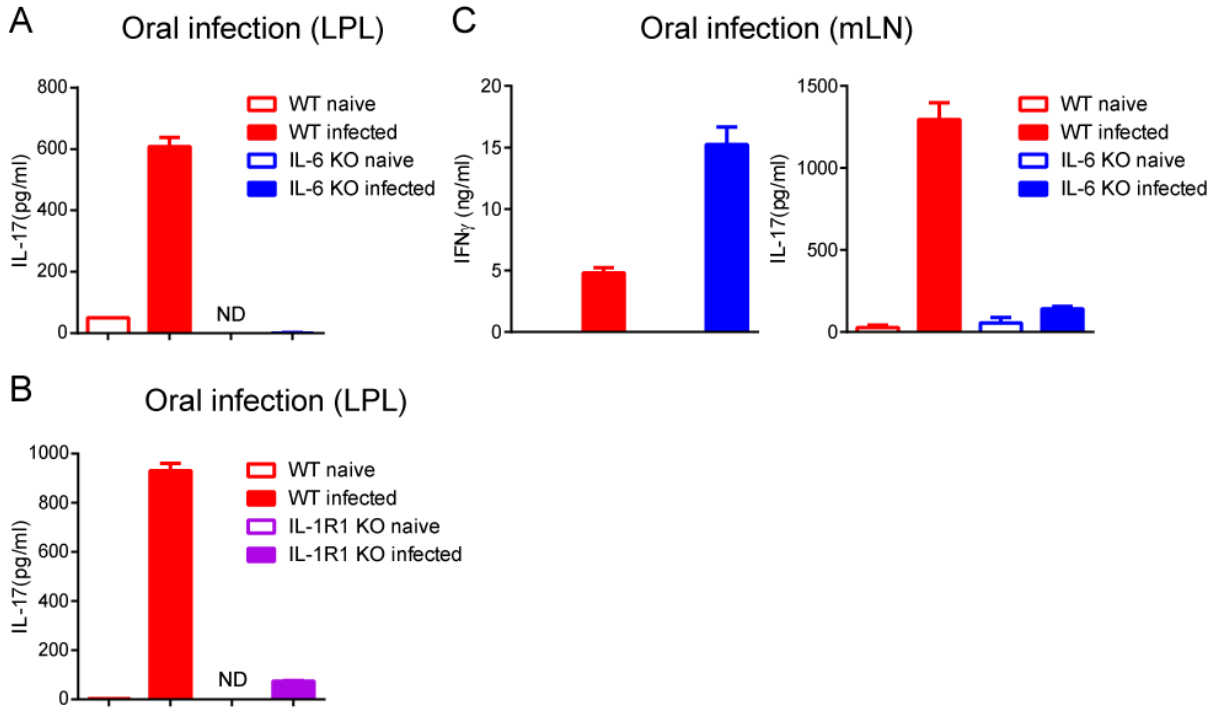

Figure 3.15. IL-6 is necessary for Th17 cell lineage commitment during oral infection.

Mice of indicated genotypes were infected orally with ~ 1-2 x 10^9 CFU of *Citrobacter rodentium*. $CD4^+$ T cells were purified from the LP or mLNs on day 8 post-infection and stimulated with 3μg/ml of *Citrobacter rodentium* sonicated lysates for 72 hours in the presence of naïve WT B cells as APCs. IFN-γ and IL-17 concentrations in the culture supernatants were then measured by ELISA. *Citrobacter*-specific IFN-γ was not detectable in the cultures of LPLs. Bar graphs represent mean ± SEM. Data are representative of three independent experiments.

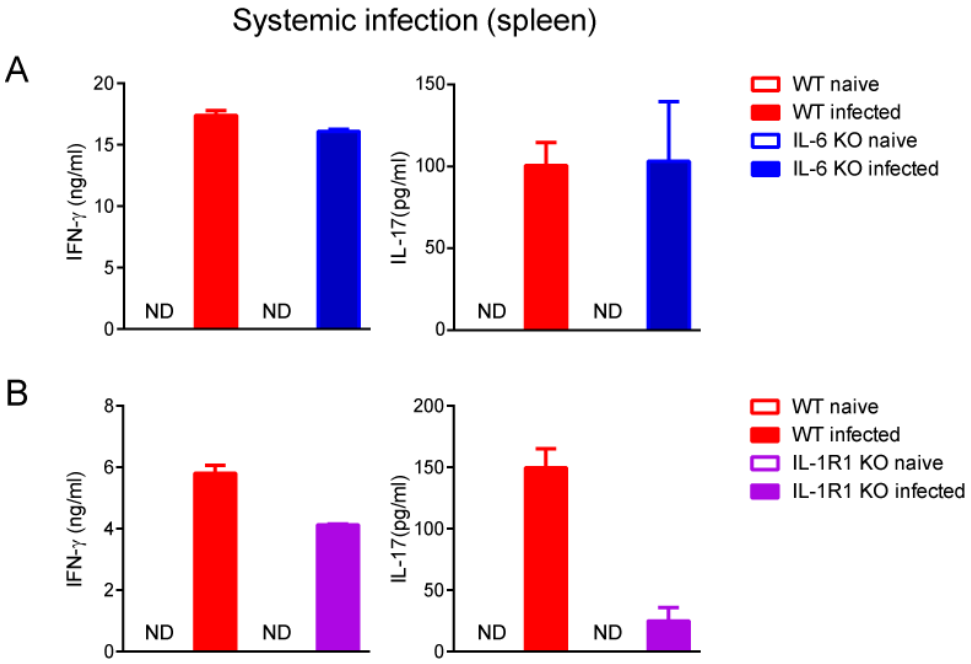

Figure 3.16. IL-6 is dispensable for Th17 cell lineage commitment during systemic infection.

Mice of indicated genotypes were infected intraperitoneally with 10^3 CFU of *Salmonella typhimurium*. $CD4^+$ T cells from the spleen were isolated on day 7 post infection and stimulated with 3μg/ml of *Salmonella typhimurium* sonicated lysates for 72 hours in the presence of naïve B cells as APCs. IFN-γ and IL-17 concentrations in the culture supernatants were then measured by ELISA. Bar graphs represent mean ± SEM. Data are representative of three independent experiments.

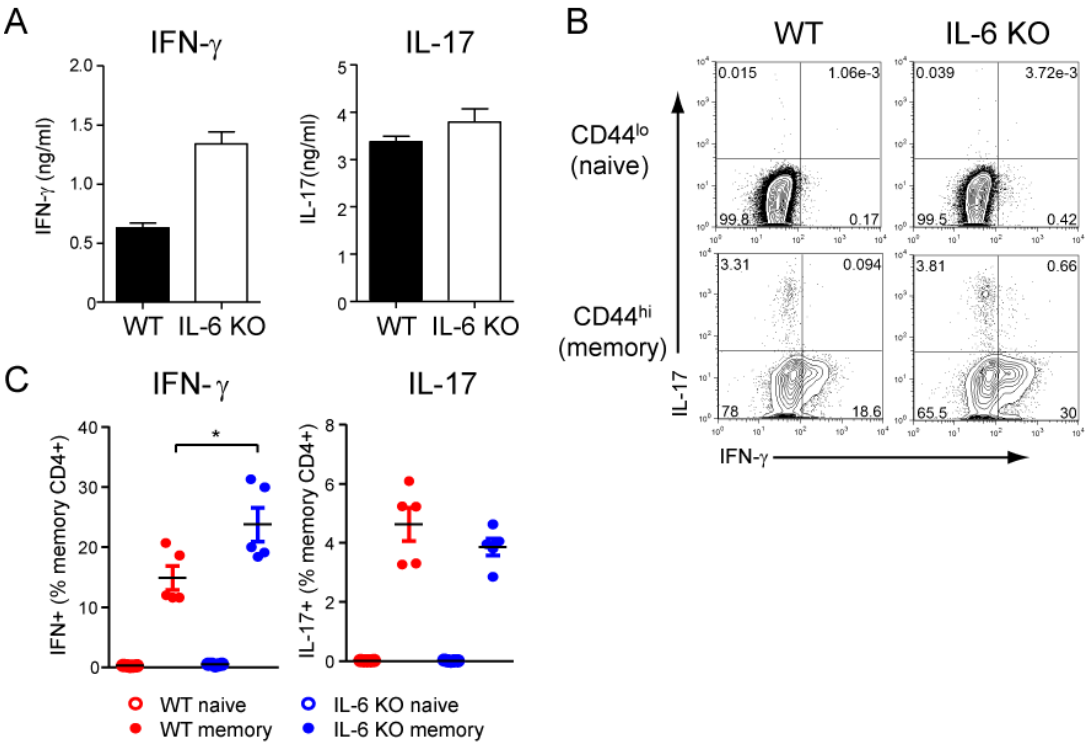

Figure 3.17. Th17 cells are present in the skin-draining lymph nodes of IL-6 KO mice.

(A) $CD4^+$ T cells from the inguinal, popliteal, brachial, and axillary lymph nodes of WT and IL-6 KO mice were stimulated with plate-bound anti-CD3 and anti-CD28 for 48 hours and cytokine production was assayed in the culture supernatants. (B-C) Cells from the above mentioned lymph nodes of WT and IL-6 KO mice were stimulated with PMA and ionomycin in the presence of brefeldin A and $CD4^+$ T cells were stained for intracellular IFN-γ and IL-17. Representative plots (B) and combined data (C) show IFN-γ and IL-17 positive cells among $CD4^+CD44^{lo}$ (naïve) and $CD4^+CD44^{hi}$ (memory) cells (n=5). Bar graphs and scatter plots represent mean ± SEM. *p<0.05, one-tailed t test.

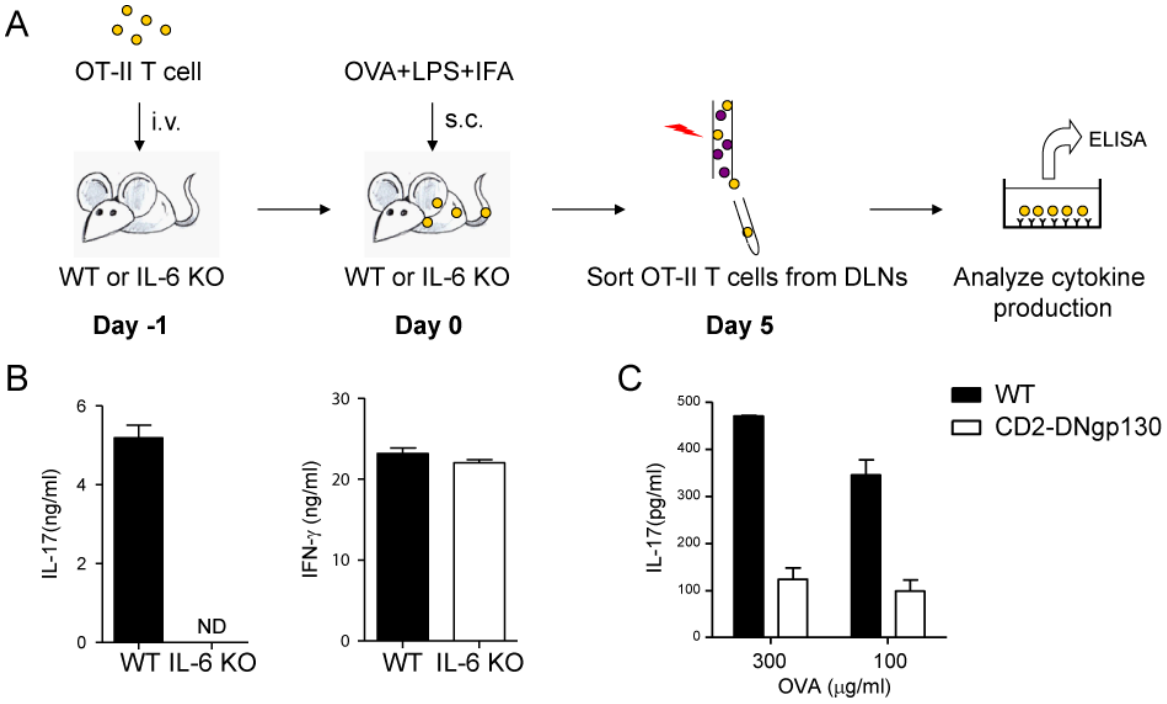

Figure 3.18. The cutaneous immune system primes Th17 cells in an IL-6-dependent manner.

(A) $2.5x10^6$ purified CD45.1 congenic OT-II T cells were transferred intravenously into WT or IL-6 KO mice (CD45.2 background). The recipients were then immunized in the footpads with 50μg/fp of OVA and 5μg/fp of LPS emulsified in IFA. On day 5 post-immunization, $CD4^+CD45.1^+$ OT-II T cells were recovered from draining lymph nodes by cell sorting followed by stimulation with plate-bound anti-CD3 and anti-CD28 for 48 hours. (B) Cytokine secretion was analyzed by ELISA in the culture supernatants. (C) WT and CD2-DNgp130 Tg mice were immunized in the fp with OVA and LPS emulsified in IFA. On day 7 post immunization, $CD4^+$ T cells from the draining lymph nodes were restimulated with MyD88/TRIF DKO dendritic cells as APCs and indicated doses of OVA for 72 hours. IL-17 concentrations in the culture supernatants were determined by ELISA. Data are representative of two or three independent experiments. Bar graphs represent mean $\pm$ SEM.

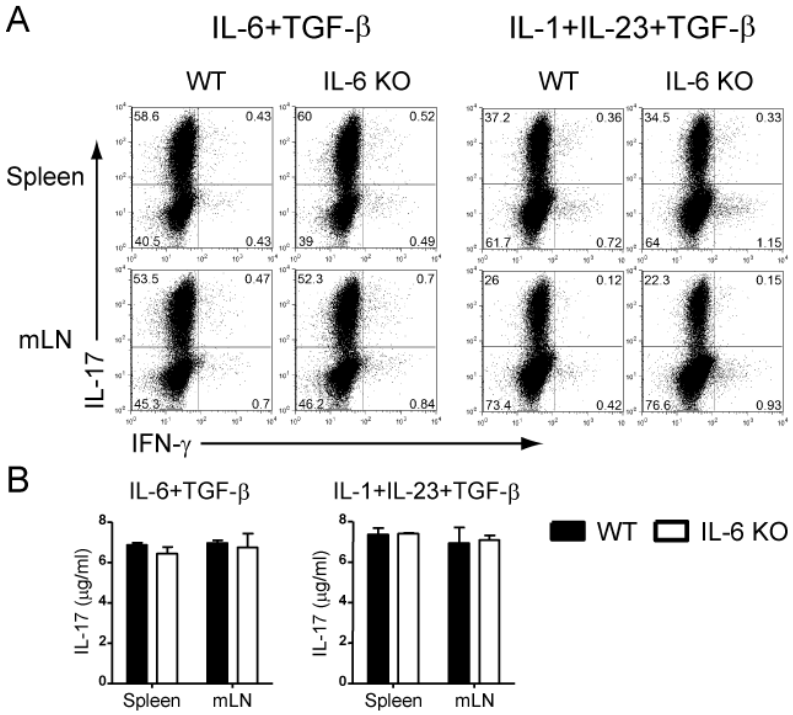

Figure 3.19. Splenic and mesenteric lymph node T cells from WT and IL-6 KO mice are equally responsive to Th17 polarization *in vitro*.

Naïve $CD4^+$ T cells from the spleen or mesenteric lymph nodes of WT or IL-6 KO mice were polarized with plate-bound anti-CD3 and anti-CD28 in the presence of IL-6 (20ng/ml) and TGF-β (5ng/ml) or IL-1β (10ng/ml), IL-23 (100ng/ml) and TGF-β (5ng/ml). After 4 days, cells were subjected to intracellular staining for IFN-γ and IL-17 following restimulation with PMA and ionomycin in the presence of brefeldin A (A), and culture supernatants were analyzed for IL-17 secretion by ELISA (B). Bar graphs represent mean $\pm$ SEM. Data are representative of two independent experiments.

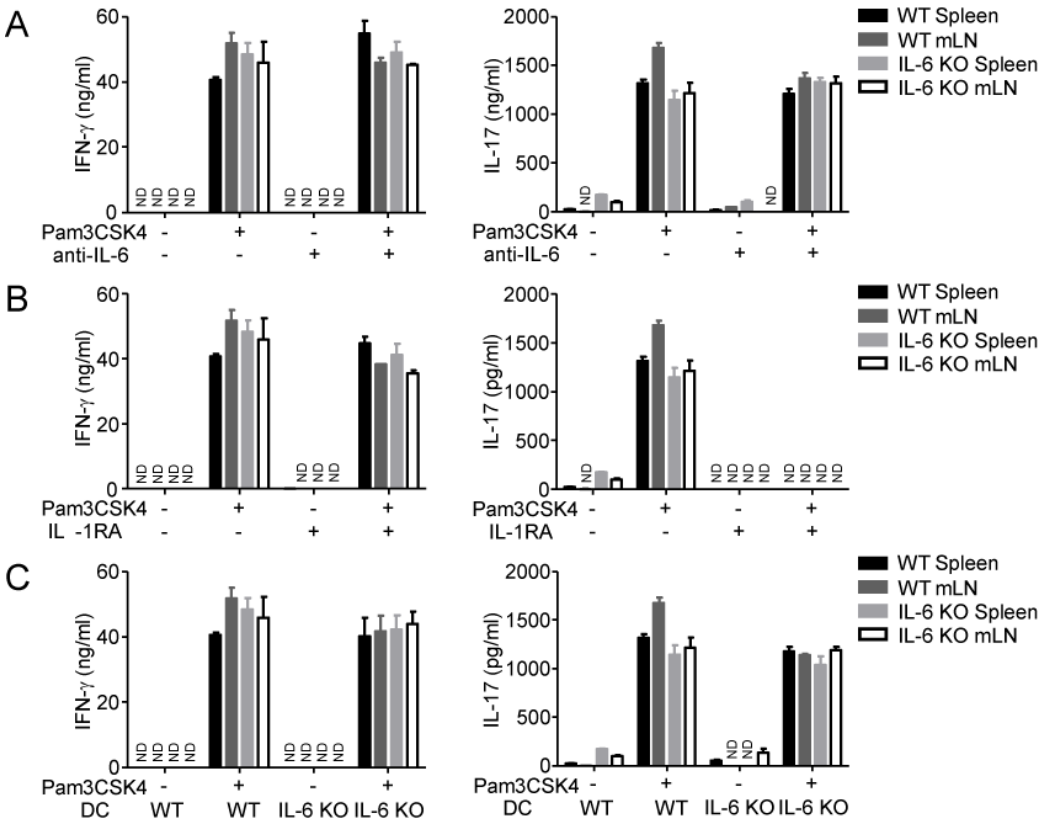

Figure 3.20. Splenic and mesenteric lymph node T cells from WT and IL-6 KO mice have the same cytokine dependency for Th17 differentiation.

Naïve $CD4^+$ T cells from the spleen or mesenteric lymph nodes of WT or IL-6 KO mice were primed with WT splenic DCs and anti-CD3 (3ng/ml) for 5 days in the presence or absence of the TLR2 ligand Pam3CSK. Anti-IL-6 (10μg/ml) (A) and recombinant IL-1 receptor antagonist (0.5μg/ml) (B) were added to the culture to block IL-6 and IL-1, respectively. Five days later, culture supernatants were analyzed for IFN-γ (left) and IL-17 (right) by ELISA. (C) Naïve $CD4^+$ T cells from the spleen or mesenteric lymph nodes of WT or IL-6 KO mice were primed with splenic DCs from indicated strains and anti-CD3 (3ng/ml) for 5 days in the presence or absence of Pam3CSK4. Five days later, culture supernatants were analyzed for IFN-γ (left) and IL-17 (right) by ELISA. Bar graphs represent mean ± SEM. Data are representative of three independent experiments.

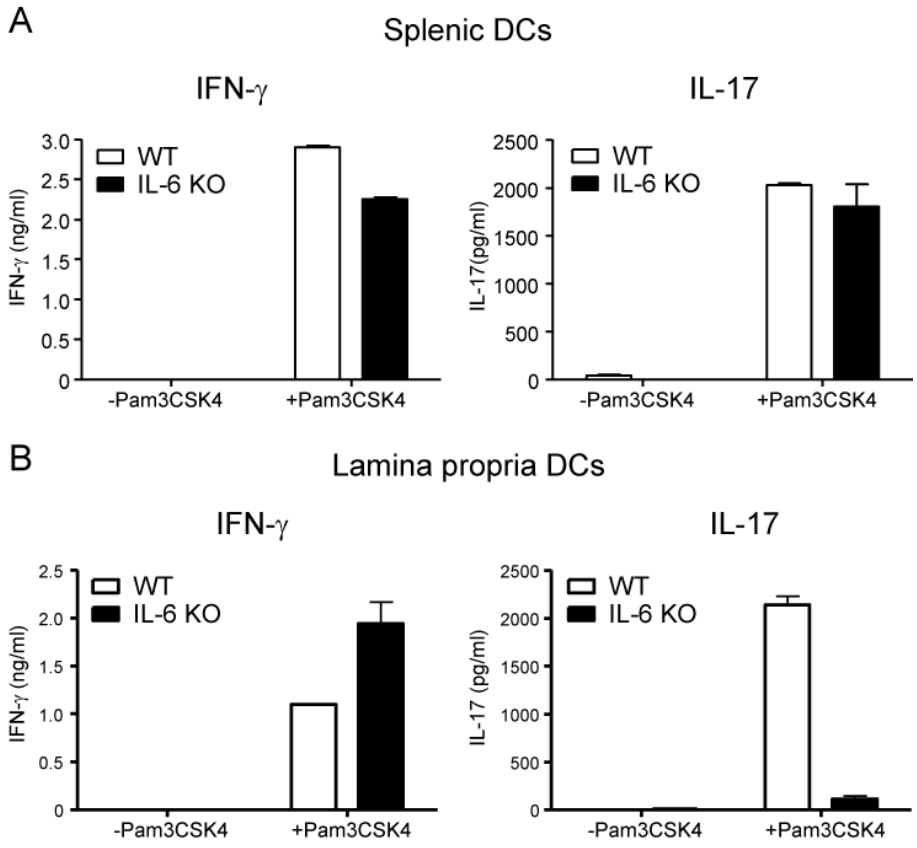

Figure 3.21. DCs from the spleen and LP prime Th17 cells in an IL-6-independent and dependent manner respectively.

Purified naïve WT $CD4^+$ T cells were cultured in the presence of purified splenic (A) or lamina propria (B) DCs from indicated strains and anti-CD3 (10ng/ml) for 5 days in the presence or absence of the TLR2 ligand Pam3CSK4 (100ng/ml). Five days later, culture supernatants were collected and analyzed for IFN-γ and IL-17 by ELISA. Bar graphs represent mean $\pm$ SEM. Data are representative of three independent experiments.

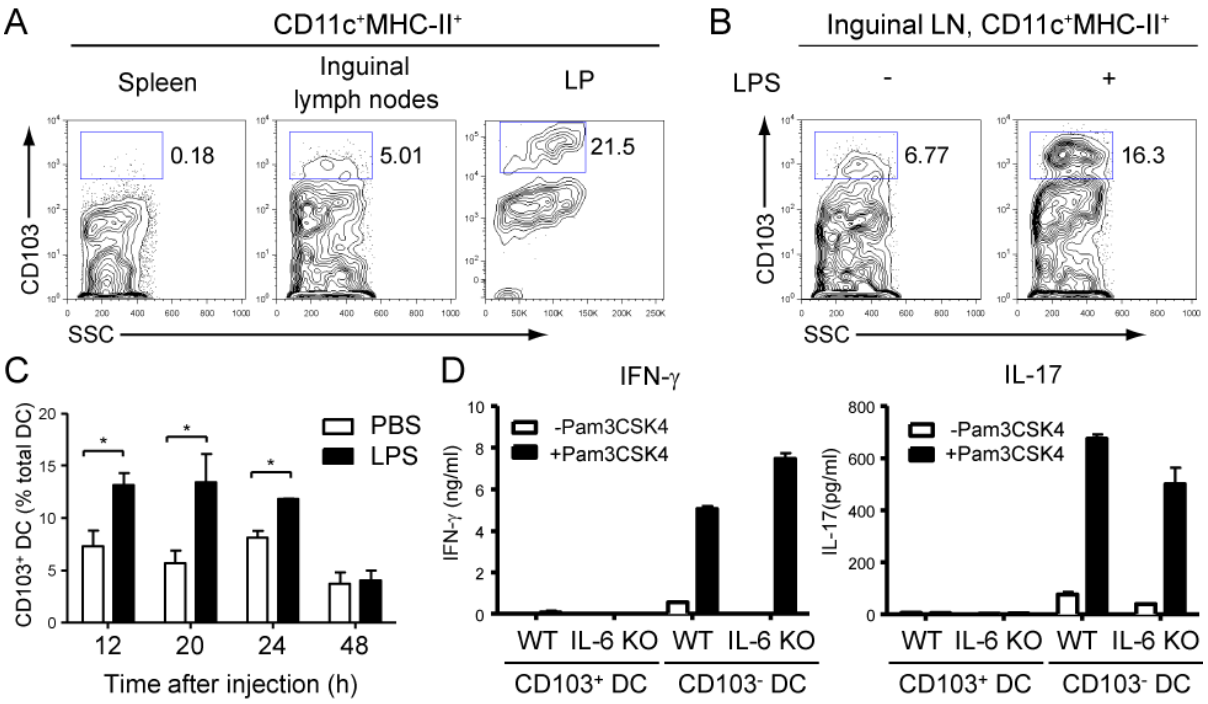

Figure 3.22. CD103$^+$ DCs in the lamina propria supresses IL-6 independent Th17 priming.

(A) Percentages of CD103$^+$ DCs in the spleen, the inguinal lymph nodes and the LP. (B-C) WT mice were injected with 10μg of LPS in the left footpad and PBS in the right footpad. Percentages of CD103$^+$ DCs to the inguinal lymph nodes were analyzed by flow cytometry. Representative data from 21 hours post injection (B) and combined data throughout the time course (C) are shown. (D) Purified naïve WT CD4$^+$ T cells were cultured in the presence of purified CD103$^+$ and CD103$^-$ LP DCs from indicated strains and anti-CD3 (10ng/ml) for 5 days in the presence or absence of Pam3CSK4 (100ng/ml). Five days later, culture supernatants were collected and analyzed for IFN-γ and IL-17 by ELISA. Bar graphs represent mean $\pm$ SEM. Data are independent of two or three independent experiments.

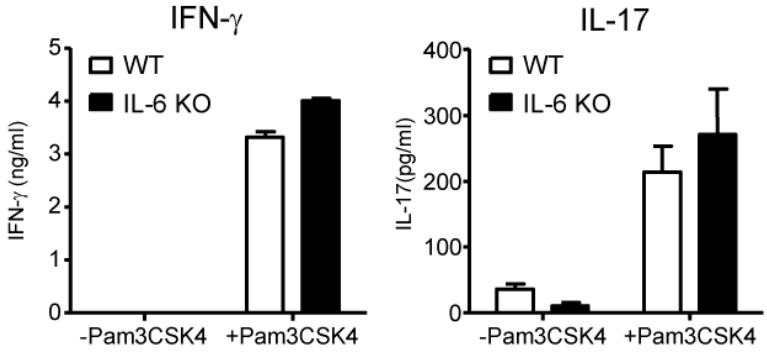

Figure 3.23. Th17 cells can be induced by lymph node-resident DCs independent of IL-6.

DCs were purified from the inguinal, popliteal, brachial, and axillary lymph nodes of WT and IL-6 KO mice. Purified naïve WT $CD4^+$ T cells were cultured in the presence of purified lymph node DCs from indicated strains and anti-CD3 (10ng/ml) for 5 days in the presence or absence of Pam3CSK4 (100ng/ml). Five days later, culture supernatants were collected and analyzed for IFN-γ and IL-17 by ELISA. Bar graphs represent mean $\pm$ SEM. Data are representative of two independent experiments.

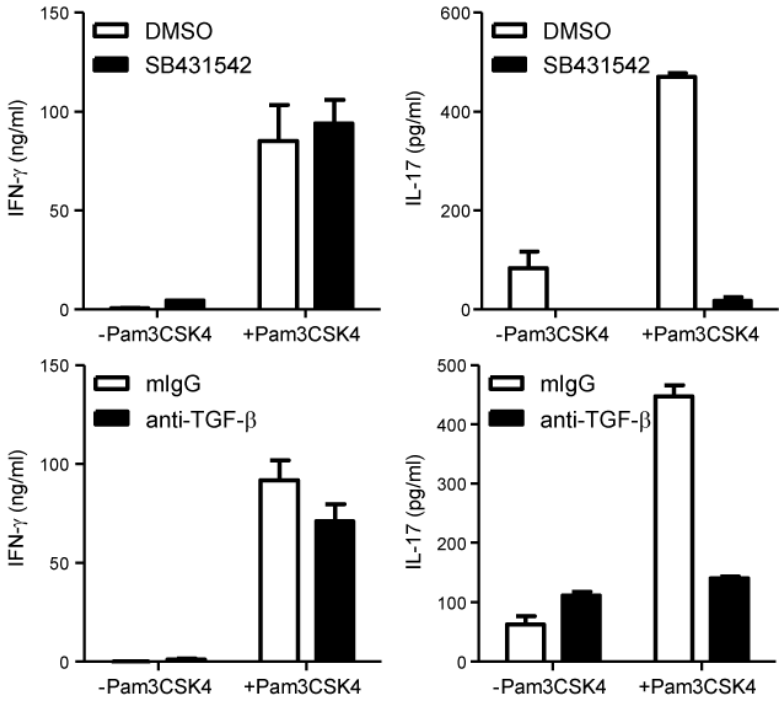

Figure 3.24. Complete TGF-β blockade abrogates Th17 differentiation.

Purified naïve WT $CD4^+$ T cells were cultured in the presence of purified WT DCs and anti-CD3 (10ng/ml) for 5 days in the presence or absence of the TLR2 ligand Pam3CSK4 (100ng/ml). SB153142, an inhibitor of TGF-β receptor (10μM, upper panels), or neutralizing antibody against TGF-β (40μg/ml, lower panels) was added into the culture as indicated. Five days later, culture supernatants were collected and analyzed for IFN-γ (left) and IL-17 (right) by ELISA. Data are representative of two independent experiments. Bar graphs represent mean ± SEM.

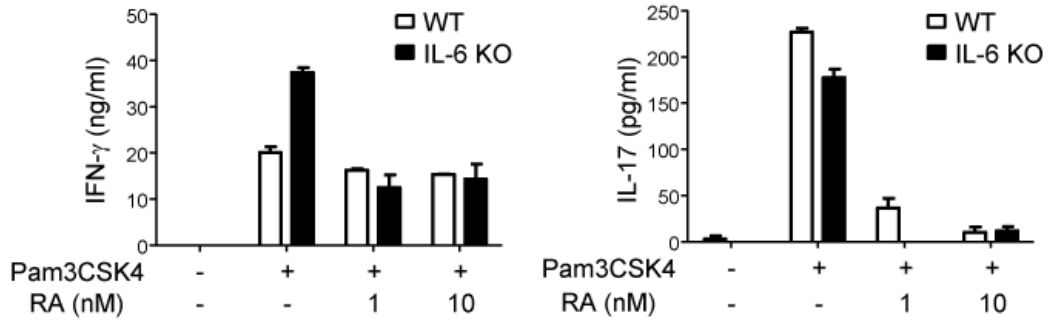

Figure 3.25. Retinoic acid inhibits Th17 differentiation.

Purified naïve WT $CD4^+$ T cells were cultured in the presence of purified splenic DCs from indicated strains and anti-CD3 (10ng/ml) for 5 days in the presence or absence of the TLR2 ligand Pam3CSK4 (100ng/ml). Retinoic acid was added into the culture as indicated. Five days later, culture supernatants were collected and analyzed for IFN-γ and IL-17 by ELISA. Bar graphs represent mean $\pm$ SEM. Data are representative of three independent experiments.

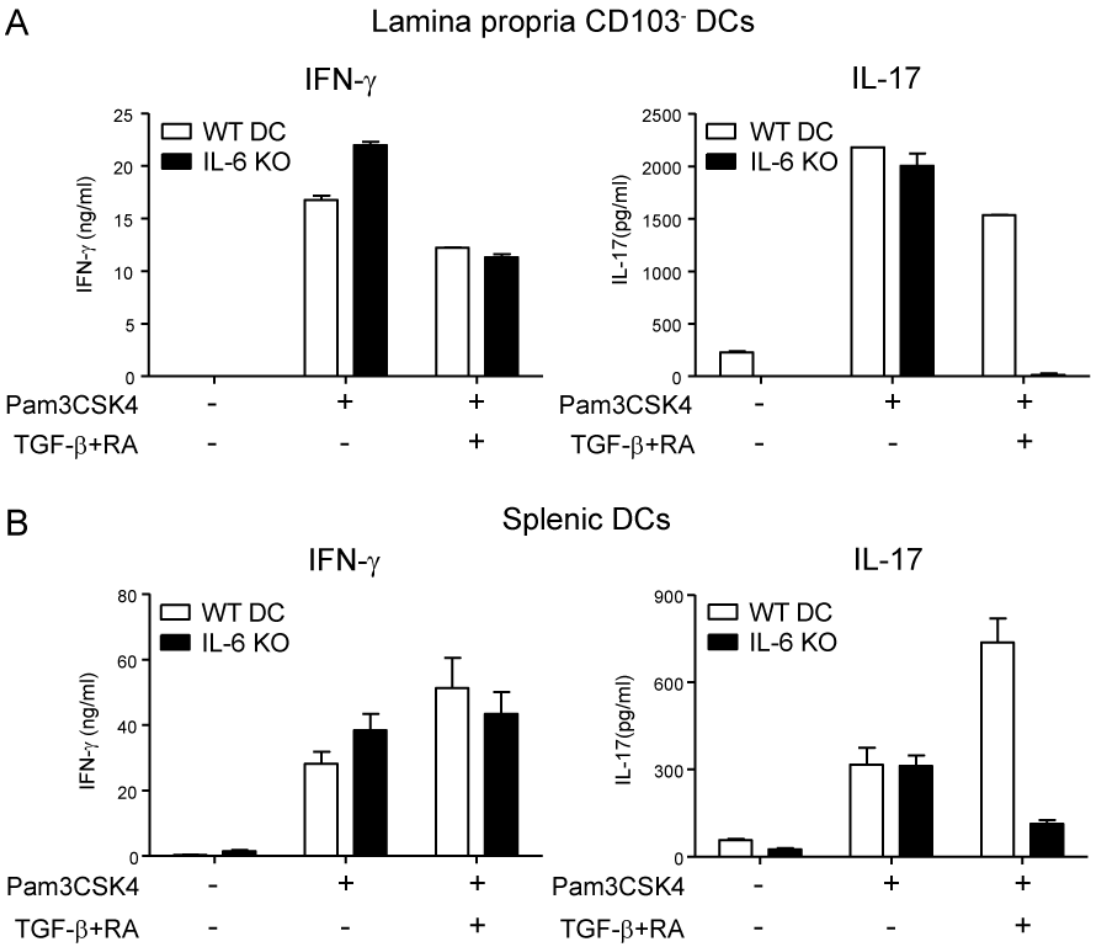

Figure 3.26. Adding TGF-β+RA blocks IL-6 independent Th17 priming.

Purified naïve WT $CD4^+$ T cells were cultured with LP $CD103^-$ DCs (A) or splenic DCs (B) from indicated strains and anti-CD3 (10ng/ml) for 5 days in the presence or absence of Pam3CSK4 (100ng/ml). TGF-β (0.3ng/ml) and RA (0.1 nM) was added into the culture as indicated. Five days later, culture supernatants were collected and analyzed for IFN-γ and IL-17 by ELISA. Bar graphs represent mean ± SEM. Data are representative of three independent experiments.

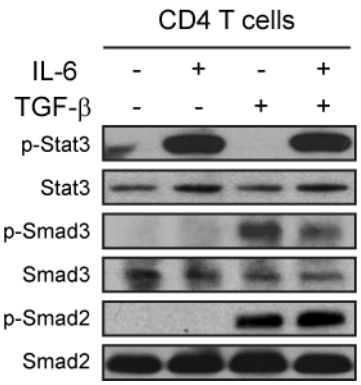

Figure 3.27. IL-6 desensitizes TGF-β signaling in CD4$^+$ T cells.

Purified CD4$^+$ T cells were cultured with 100ng/ml of IL-6 for 6 hours followed by stimulation with 0.2ng/ml of TGF-β for 1 hour. Phospho-STAT3, phospho-Smad2 and phospho-Smad3 were analyzed by western blot. Data are representative of two independent experiments.

CHAPTER FOUR
IRAK-1 Bypasses Priming and Directly Links TLRs to Rapid NLRP3 Inflammasome Activation

Work presented in this chapter represents an equal and collaborative effort between Wei Hu and Keng-Mean Lin. It is currently in press in *Proceedings of the National Academy of Sciences of the United States of America*. This work is reproduced with the permission of *Proceedings of the National Academy of Sciences of the United States of America*. Experiments were performed by Wei Hu unless otherwise indicated in the figure legends.

INTRODUCTION

Toll-like receptors (TLRs) recognize conserved molecules from pathogens, and initiate signaling that activates NF-κB, mitogen-activated protein (MAP) kinases and interferon response factor (IRF) proteins [33, 314]. This signaling induces proinflammatory cytokines, chemokines, adhesion molecules, and inflammasome components, all of which facilitate effector responses [285, 314]. A second family of receptors called NLRs (NOD like receptors) resides in the cytosol and is activated in response to either microbial ligands that gain access to the cytosol or virulence factors such as bacterial toxins [315, 316]. While conserved microbial ligands that are recognized by TLRs are expressed by both commensal and pathogenic micro-organisms, only virulent microbes express the ligands that lead to NLR activation. Activation of NLRs leads to assembly of inflammasome complex leading to

activation and cleavage of cysteine protease, caspase-1, which in turn cleaves IL-1β and IL-18 leading to their secretion [317] [183-186]. There are four inflammasome complexes that respond to different kinds of microbial challenge. The NLRP1 inflammasome is activated in response to anthrax lethal toxin [188], the NLRC4 inflammasome responds to the presence of cytosolic flagellin and type III secretion system components [189] and the AIM2 inflammasome responds to cytosolic DNA [195, 318-320]. The NLRP3 inflammasome, composed of NLRP3, ASC and procaspase-1 [321], undergoes assembly in response to stimulation by several different stimuli including ATP [197, 322], nigericin [197], maitotoxin [197], uric acid crystals [204], silica [203], asbestos [205] and pathogens such as *Staphylococcus aureus* [270, 323], *Streptococcus pyogenes* [324] and *Listeria monocytogenes* [263, 325]. The NLRP3 inflammasome also gets activated in response to the type III secretion system (SPI-1) of *Salmonella typhimurium* [326].

Several hereditary pro-inflammatory diseases such as Muckle-Wells Syndrome, Cold Induced Urticaria and Familial Mediterranean Fever are all associated with mutations in the components of the inflammasome complex and the damaging effects are caused by untimely secretion of IL-1β. Since IL-1β has such important biological effects, it is one of the most tightly regulated cytokine, both in the way it is synthesized and secreted as well as the regulatory proteins that are involved in signaling by IL-1 receptor. Current understanding of the biology of IL-1β synthesis and secretion is that the TLR signaling pathway induces activation of NF-κB and MAP kinases that leads to synthesis and accumulation of pro-IL-1β in the cytosol of the cell. When the cell receives an additional signal in the form of NLRP1, NLPR3, NLRC4 or AIM2 ligands, there is assembly of the respective inflammasome

complexes leading to cleavage of pro-IL-1β by active caspase-1. The role of TLR signaling is thus understood to be limited to synthesis of the substrates or upregulation of the levels of the components of the inflammasome complexes themselves.

Inflammasome-mediated caspase-1 activation promotes inflammation and host defense by two principal avenues: secretion of mature cytokines (IL-1β and IL-18) and activation of pyroptosis [327], a pro-inflammatory cell death pathway. IL-1 is important for the acute phase response that is defined by synthesis and secretion of proteins such as SAA (serum amyloid A) and CRP (C reactive protein) that play an important role in opsonization and clearance of the microbes. In addition IL-1 plays a critical role in activation and differentiation of Th17 lineage cells [139]. IL-18 is important for the IFN-gamma secretion by Th1 lineage cells [247]. Also, IL-1 and IL-18 are important for the ability of innate lymphoid cells to make IL-17 and IFN-γ respectively [328]. Although IL-1β and IL-18 synthesis is induced by TLR signaling, IL-1α and IL-18 can also be made constitutively by myeloid cells such as macrophages. Inflammasome activation also leads to pyroptosis where the responding cell dies and releases its pre-synthesized inflammatory contents that play an important role in amplifying the innate immune response [327]. Pyroptosis is also critical for the host's ability to eliminate the infected cell and remove the niche of intracellular microbial replication [329].

In this study, we investigated whether TLRs played a direct role in activation of the NLRP3 inflammasome and discovered that there are at least two phases of NLRP3 inflammasome activation. The early phase/acute inflammasome activation that we discovered here is independent of new protein synthesis, depends on simultaneous activation of TLRs and NLRP3 and is directly regulated by TLR signaling via IRAK-1. The late phase or priming-

dependent activation of NLRP3 inflammasome requires new protein synthesis and is independent of direct participation IRAK-1. We also find that the acute IRAK-1-dependent NLRP3 inflammasome activation pathway is critical for pyroptosis and secretion of inflammatory proteins that are already pre-synthesized by the cell. Our studies provide evidence towards a direct link between TLR signaling and NLRP3 inflammasome activation and ascribe a novel function to IRAK-1 in early innate responses following exposure of cells to virulent pathogens that activate TLRs and NLRs simultaneously.

RESULTS

Simultaneous stimulation of TLRs and NLRP3 leads to acute inflammasome activation

Inflammasome studies employ sequential stimulation in which cells are first primed using a TLR ligand and hours later a second stimulus, such as ATP or a microbial toxin (nigericin or maitotoxin) is added to trigger NLRP3 inflammasome assembly and caspase-1 activation [197, 203-205]. Host cells at the site of infection, however, are likely to be exposed to both TLR and NLR ligands simultaneously or in rapid succession. Therefore, to mimic natural infection with a pathogenic microbe, we tested the responses of bone marrow derived macrophages (BMDMs/macrophages), as well as peritoneal resident macrophages (PCMs) to simultaneous stimulation of TLR(s) and NLRP3. WT BMDMs and PCMs stimulated with a TLR ligand alone or ATP alone for 30 minutes did not activate caspase-1, but simultaneous treatment with both ligands for 30 minutes led to rapid caspase-1 activation in BMDMs (Figure 4.1). Ligands for TLRs 4, 9, 7 and 2 – LPS, CpG , R837 or Pam3CSK4 –triggered rapid caspase-1 cleavage in macrophages costimulated with ATP, but poly I:C, a TLR3 ligand, did not (Figure 4.1 A).

While WT macrophages activated caspase-1 in response to costimulation with LPS and ATP, TLR2/4 double KO (DKO) macrophages did not, though they remained responsive to R837 and ATP (Figure 4.2 A). Conversely, TLR7 KO macrophages retained responsiveness to LPS but could not activate caspase-1 when stimulated with R837 and ATP (Figure 4.2 B). As expected, rapid inflammasome activation was abolished in NLRP3-deficient macrophages,

suggesting a necessary role for both TLRs and NLRP3 (Figure 4.2 C). The LPS receptor, TLR4, signals downstream via two different adaptors, MyD88 and TRIF [16, 25-27]. Interestingly, TLR-driven rapid caspase-1 activation took place only in TRIF KO macrophages (Figure 4.3 A), and was absent in MyD88 KO macrophages (Figure 4.3 B). As previously noted, TLR3 signaling did not trigger rapid caspase-1 activation (Figure 4.1), suggesting that TRIF and its downstream components do not directly activate the NLRP3 inflammasome. Thus, rapid caspase-1 activation downstream of all TLRs depended entirely on the adapter MyD88. Previous studies have shown that TLR signaling in either MyD88- or TRIF-dependent pathways [330] leads to NF-κB-dependent upregulation of inflammasome components, particularly NLRP3 [211], suggesting a requirement for inflammasome "priming" prior to activation [211, 331]. Combined stimulation of macrophages with LPS and ATP, pretreated with cycloheximide or an NF-κB inhibitor, led to caspase-1 cleavage comparable to untreated cells, suggesting that rapid NLRP3 inflammasome activation is independent of "priming" since both NF-κB activation and new protein synthesis are not necessary (Figure 4.4). Collectively these data suggest that constitutive expression of NLRP3 is sufficient to activate caspase-1 when cells receive signals from both TLR and NLRP3 ligands simultaneously.

Rapid NLRP3 inflammasome activation is dependent on IRAK-1

To understand the mechanism of priming-independent caspase-1 activation, we focused on the role of signaling components directly downstream of MyD88, in particular the IRAK family of molecules. This was also prompted by our findings that IL-1β, but not TNFα,

induced rapid caspase-1 activation (Figure 4.5). Although MyD88-dependent signaling requires IRAK-4 [18], the functions of IRAK-1 and IRAK-2 remain less clear. IL-1R and TLR signaling seem to be only mildly affected in the absence of either IRAK-1 or IRAK-2 [19, 332, 333]. More recently, IRAK-2 has been implicated in sustaining NF-κB activation [332] as well as stabilizing mRNA of various pro-inflammatory cytokines [333]. To determine if either IRAK-1 or IRAK-2 played a role in TLR-triggered rapid NLRP3 inflammasome activation, we examined early caspase-1 activation in macrophages from WT, IRAK-1- and IRAK-2 KO mice. IRAK-1 KO BMDMs and peritoneal cavity resident macrophages (PCMs) exhibited a severe defect in rapid caspase-1 cleavage (Figure 4.6 A, B) while IRAK-2 KO BMDMs activated caspase-1 slightly more robustly than WT cells (Figure 4.6 A). These data suggest the existence of a TLR- and IRAK-1-dependent pathway that leads to rapid NLRP3 inflammasome assembly and caspase-1 activation. Interestingly, priming of macrophages for 4 hours with LPS abolished the requirement of IRAK-1 for caspase-1 cleavage (Figure 4.6 C). We next tested if the kinase activity of IRAK-1 was important for activation of this pathway. We found that rapid caspase-1 activation was compromised in macrophages from IRAK1 kinase dead (KD) knock in mice [278] (Figure 4.7 A). Since IRAK-1's kinase function is dependent on IRAK-4's kinase activity [334], we tested if rapid NLRP3 inflammasome activation required the kinase activity of IRAK4 and found that it is completely abolished in macrophages from IRAK-4 kinase dead knock in mice [277] (Figure 4.7 B). Priming-dependent NLRP3 activation was independent of both IRAK-1 and IRAK-4 kinase (Figure 4.7). Strikingly, TLR priming-dependent late phase caspase-1 activation was abolished when macrophages lacked both IRAK-1 and IRAK-2 (Figure 4.8). This suggests a critical role for

IRAK-1 and IRAK-2 in canonical NLRP3 activation since it has been previously shown that inflammasome activation following LPS priming is intact in the absence of MyD88 [211]. Since this pathway is independent of NF-κB activation, this suggests that the MyD88-IRAK-4-IRAK-1 signaling axis has a critical role in NLRP3 assembly that is unrelated to their roles in NF-κB activation.

We also discovered that TRAF-6 and TAK-1 were important to activate rapid NLRP3 inflammasome (Figure 4.9). Since this pathway is independent of NF-κB activation, this suggests that the MyD88-IRAK-4-IRAK-1-TRAF-6-TAK-1 signaling axis has a critical role in NLRP3 assembly that is unrelated to their roles in NF-κB activation.

The acute inflammasome activation, unlike priming-dependent inflammasome activation, is independent of reactive oxygen species (Figure 4.10). ROS functions to upregulate NLRP3 [220], whereas we are describing rapid activation that depends on constitutively expressed NLRP3 and consistent with the described role of ROS we find that it is not required for priming-independent NLRP3 activation. Among the other NLRP3 activators, nigericin induced IRAK-1-dependent rapid inflammasome activation, other stimuli such as crystals (monosodium urate and silica) [203] failed to induce rapid inflammasome activation and activated priming-dependent NLRP3 inflammasome in IRAK-1-independent manner (Figure 4.11). These data collectively suggest that TLR-mediated priming induces a mechanistically different, IRAK-1-independent caspase-1 activation pathway.

IRAK-1 associates with inflammasome components and regulates NLRP3 inflammasome assembly

Previous results show TLR activation leads to IRAK-1 phosphorylation and degradation [334]. However, simultaneous exposure of macrophages to LPS and ATP delayed IRAK-1 degradation, as compared to LPS stimulation alone (Figure 4.12). Interestingly, IκBα phosphorylation/degradation were also delayed (Figure 4.12). These results suggest that combined activation of TLR and NLRP3 favours IRAK-1's participation in inflammasome activation thus causing a delay in NF-κB activation.

This prompted us to further examine the possible interactions between IRAK-1, ASC and NLRP3. We observed that in WT macrophages, LPS or ATP treatment for 15 minutes led to re-localization of ASC from the nucleus into the cytosol (Figure 4.13) as has been previously reported [335]. In IRAK-1 KO macrophages, LPS stimulation failed to induce nuclear-cytoplasm translocation of ASC while ATP treatment still induced this re-localization (Figure 4.13). Upon inflammasome activation, oligomerized ASC forms specks in the cells, representing assembled inflammasome complexes [335]. We observed speck formation by ASC only when cells were exposed to both LPS and ATP and this was significantly more pronounced in WT macrophages than in IRAK-1 KO macrophages (Figures 4.13 and 4.14). LPS and ATP treatment induced more cells with specks in WT macrophages when compared to IRAK-1 KO macrophages (Figure 4.14 C), and any specks formed in IRAK-1 KO macrophages were of much smaller size and lower intensity (Figure 4.13). More importantly, we found that IRAK-1, together with MyD88 and IRAK-4, co-localized in the specks of inflammasome complexes that contain both ASC and NLRP3 (Figures 4.14 and 4.15). However, IRAK-2 was not part of the specks during rapid NLRP3 activation (Figure 4.15).

The speck formation and co-localization of IRAK-1 with NLRP3 were abolished in the presence of a kinase inhibitor of IRAK-4 and IRAK-1 (Figure 4.16), or in IRAK-1 kinase dead knock in macrophages (Figure 4.15). Together, these data strongly suggest that IRAK-1 and its kinase activity are critical for TLR-dependent inflammasome assembly.

Rapid IRAK-1-dependent NLRP3 inflammasome activation is important for secretion of pre-synthesized IL-18

Next, we investigated the physiological relevance of the rapid NLRP3 inflammasome activation pathway. Although they do not express pro-IL-1β, BMDMs are known to express pro-IL-18 without any TLR stimulation. In addition, caspase-1 activation triggers a proinflammatory cell death program called pyroptosis, which could play a major role in host defence by eliminating infected cells and releasing inflammatory cellular contents [327]. We hypothesized that early inflammasome activation perhaps allows the cells to secrete pre-synthesized caspase-1 substrates and undergo pyroptosis, and these events would be compromised in the absence of IRAK-1 due to defective caspase-1 activation. Confirming this model, WT BMDMs secreted IL-18 whereas IRAK-1 KO BMDMs did not, despite similar pro-IL-18 expression between the two. Importantly, WT BMDMs failed to secrete IL-18 when stimulated only with LPS or ATP, suggesting the importance of simultaneous activation of TLR4 and NLRP3 (Figure 4.17 A). IRAK-1 deficiency did not hamper the ability of TLR-primed BMDMs to secrete either IL-1β or IL-18, consistent with their ability to cleave caspase-1 when primed by TLR ligands (Figure 4.17 B). Small intestine lamina propria macrophages

constitutively express IL-1β [336], which could also be cleaved and secreted upon caspase-1 activation in response to simultaneous activation of TLR and NLRP3 (Figure 4.17 C).

IRAK-1 is important for pyroptosis induced by acute inflammasome activation

We also found that WT BMDMs underwent rapid pyroptosis, as indicated by propidium iodide (PI) uptake, whereas caspase-1 KO and MyD88 KO macrophages did not undergo pyroptosis (Figure 4.18 A). IRAK-1 deficiency severely compromised the ability of cells to undergo pyroptosis (Figure 4.18 A), suggesting that IRAK-1 is critical for triggering pyroptotic cell death through regulation of caspase-1 activation. We were able to detect cleaved caspase-1 and the pro-inflammatory mediator HMGB-1, a hallmark of pyroptosis [337, 338], in the supernatants of WT macrophages (Figure 4.18 B). These proteins were considerably reduced or absent in the supernatants of IRAK-1 KO, caspase-1 KO and MyD88 KO macrophages (Figure 4.18 B). Additionally, cell lysis, as measured by LDH release, was reduced in the absence of IRAK-1, caspase-1 and MyD88 (Figure 4.18 C). Consistent with the caspase-1 activation data, pyroptosis in IRAK-2 KO macrophages remained unaffected, as measured by both PI incorporation as well as HMGB-1 release (Figure 4.19).

***Listeria monocytogenes* induces rapid IRAK-1-dependent NLRP3 inflammasome activation and pyroptosis**

Since many pathogens express both TLR and NLR ligands, we hypothesized that infection with a live pathogen would activate TLRs and NLRs simultaneously or in rapid sequence. The pathogen *Listeria monocytogenes* has been implicated in activating several inflammasome complexes, including the NLRP3 inflammasome [197, 263, 325]. When exposed to *L. monocytogenes* for 1 h (without prior priming by TLR ligands), WT macrophages underwent pyroptosis as evident by PI uptake, caspase-1 cleavage, HMGB-1 release and LDH release, all of which were absent NLRP3 KO macrophages (Figure 4.20). Consistent with our earlier findings, caspase-1 activation, pyroptosis and HMGB-1 release were absent in MyD88 KO and caspase-1 KO macrophages and severely compromised in IRAK-1 KO cells infected with *L. monocytogenes* (Figure 4.21). Together, these data suggest that acute *L. monocytogenes* infection causes NLRP3-dependent and TLR-MyD88-IRAK-1-dependent activation of caspase-1 and pyroptosis.

IRAK-1 promotes early host responses to *Listeria monocytogenes* infection

We reasoned that the early inflammasome activation pathway promotes host defence to infections. Indeed, when *L. monocytogenes* was injected into the peritoneal cavity of mice, the percentage of resident macrophages rapidly reduced in WT mice but were unaffected in both caspase-1- and IRAK-1 KO mice (Figure 4.22). Since there was a higher proportion of WT macrophages that incorporated PI, we reasoned that the peritoneal macrophages from WT mice were depleted because of pyroptotic cell death (Figure 4.22). These data suggest that pyroptosis resulting from acute inflammasome activation may help reduce the growth of

intracellular pathogens, a host defence mechanism previously observed in *Salmonella* infection [329]. The reduction of the proportion of resident macrophages could also result from their migration. However, it is less likely as the reduction occurred shortly after *Listeria* infection (20 minutes).

IFN-γ has been shown to play important roles in host defence against *L. monocytogenes* infection [339]. The innate source of IFN-γ is an important part of host defence during the early stage of infection [340]. Previous studies have shown that memory $CD8^+$ T cells can secrete IFN-γ in response to IL-18 and IL-12 in the absence of cognate antigen, serving as an important source of innate IFN-γ [252]. When we examined *in vivo* IFN-γ production 12 hours following *L. monocytogenes* infection, we found that memory $CD8^+$ T cells from IRAK-1- and Caspase-1-deficient mice were severely compromised in IFN-γ production (Figure 4.23 A). Consistent with previous reports [252], IL-18 receptor deficiency abrogates the ability of memory $CD8^+$ T cells to produce IFN-γ (Figure 4.23 A). These data suggest that IRAK-1/Capsase-1-dependent IL-18 production through acute inflammasome activation is a critical inducer of early innate IFN-γ. IL-18 is also known to be important for innate induction of IFN-γ from NK cells following infection [341]. Indeed, we observed reduced IFN-γ production by NK cells from IRAK-1, Caspase-1, and IL-18R-deficient mice following infection (Figure 4.23 B). Serum IFN-γ levels in both IRAK-1 and caspase-1 KO mice were also severely compromised (Figure 4.23 C). Interestingly, IL-18R KO mice were slightly more defective compared to both IRAK-1 and caspase-1 KO mice (Figure 4.23), suggesting that some IL-18 could be made independent of caspase-1.

To uncouple the effects of IRAK-1 in regulating IL-18R mediated induction of IFN-γ, we generated antigen specific memory T cells by transferring OVA specific WT OT-I T cells into either WT or IRAK-1-deficient mice. Mice were exposed to OVA-expressing vesicular stomatitis virus, followed by 45 days of rest and were rechallenged with non-OVA expressing *Listeria* to measure IFN-γ production by OVA specific memory T cells (Figure 4.24 A). We confirmed that there was no difference in expansion and survival of OT-I T cells between WT and IRAK-1 KO hosts (Figure 4.24 B). Importantly, although there was no difference in the ability of IRAK-1 KO recipients to make IL-12 or IL-6 (Figure 4.24 C), there was a significant defect in the OT-I T cells transferred into IRAK-1 KO mice to make IFN-γ (Figure 4.24 D). These data argue that IRAK-1 plays a significant role in the ability of infected cells to sense *L. monocytogenes*, induce NLRP3 inflammasome activation, trigger pyroptosis, and release IL-18 *in vivo,* which may influence the subsequent inflammatory responses such as IFN-γ production by memory T cells.

IRAK-1 does not regulate NLRC4, AIM, or non-canonical inflammasome

We finally tested whether IRAK-1's involvement in the activation of inflammasomes other than the NLRP3 inflammasome. *Salmonella typhimurium* and flagellin [342] mediated caspase-1 activation and subsequent pyroptosis were comparable between WT and IRAK-1-deficient macrophages, suggesting that IRAK1 plays no role in NLRC4 inflammasome activation (Figure 4.25). Cytosolic delivery of poly dA:dT also led to equivalent caspase-1

activation in WT and IRAK-1-deficient macrophages, suggesting that the AIM2 inflammasome [195] can also be activated independent of IRAK-1 (Figure 4.26).

Pathogens such as *Citrobacter rodentium* and *Escherichia coli* have also been shown to induce NLRP3-dependent caspase-1 cleavage and this pathway of activation is dependent on type I interferon-induced upregulation of caspase-11[241]. We found that these pathogens did not induce rapid inflammasome activation and the late phase inflammasome activation induced by them was independent of IRAK-1 (Figure 4.27). It will be important to further identify and characterize other pathogens and their virulence factors that induce TLR-IRAK-1 dependent NLRP3 inflammasome activation.

DISCUSSION

Our studies reveal a critical early cellular response pathway in macrophages induced by simultaneous engagement of TLRs and NLRP3. This previously uncharacterized rapid pathway of NLRP3 inflammasome activation is distinct from the commonly studied late NLRP3 inflammasome activation pathway, where TLR and NLRP3 are engaged in a sequential manner. Traditionally, TLRs have only been implicated in the sensitization of NLRP3 inflammasome activation, through a priming phase involving upregulation of inflammasome components such as NLRP3, as a result of NF-κB activation and ROS generation. TLR signaling *per se* was not thought to be directly involved in delivering signals to trigger the assembly of the inflammasome complex. Our findings have expanded the role of TLR signaling beyond that of inflammatory gene induction through NF-κB. Our data showed that during early NLRP3 inflammasome activation, MyD88-dependent TLRs plays a direct role via the MyD88-IRAK1 signaling axis, and dual signals from TLRs and NLRP3 synergistically provoke inflammasome activation.

The early inflammasome activation pathway appears to operate mechanistically different from the late or the priming-dependent pathway. We discovered that the TLR signaling molecule IRAK-1 plays a unique role in rapid inflammasome activation and seems to regulate NLRP3 inflammasome assembly and activation at several different levels. Since the kinase activity of IRAK-4 is important for activation of IRAK-1, we find that abolishing the kinase function of IRAK-4 completely abrogates the rapid inflammasome activation pathway. On the other hand, abrogation of kinase activity of IRAK-1 does not completely

prevent rapid inflammasome activation suggesting that IRAK-1 might have additional kinase-independent functions that regulate NLRP3 inflammasome activation. For example, we find that following LPS stimulation, IRAK-1 seems to be important for redistribution of ASC from nucleus into the cytoplasm. Moreover, IRAK-1 itself seems to be part of the inflammasome complex during early activation, as evident by specks formed by IRAK-1 that overlap with those formed by ASC and NLRP3. Interestingly, when we compared the kinetics of IRAK-1 disappearance and NF-κB activation, we found that IRAK-1's disappearance was delayed when TLR and NLRP3 are both activated, as compared to TLR activation alone, suggesting that IRAK-1 is being redistributed between the NF-κB and inflammasome pathways. IκBα phosphorylation and degradation are also delayed and reduced in magnitude when TLR and inflammasome are both activated, compared TLR activation alone, indicating that usage of IRAK-1 by the inflammasome pathway reduced the availability of IRAK-1 for activating NF-κB. Thus, when encountering TLR and inflammasome activators at the same time as might happen in the case of encountering a virulent pathogen, IRAK-1 serves as a controlling node for the cells to make a decision between triggering the activation of the pro-survival NF-κB pathway that will lead to new gene synthesis that takes time or the pro-death inflammasome pathway that immediately eliminates the niche for survival and replication of pathogens as well as secretes pro-inflammatory contents of the cell. Our data also reveal that, although rapid NLRP3 inflammasome activation is defective in IRAK-1-deficient macrophages. Priming of macrophages with LPS, however, abrogates the requirement for IRAK-1 in inducing inflammasome activation. It is possible that elevated NLRP3 protein level bypasses additional regulators of inflammasome activation. It is important to note that IRAK-1 and IRAK-2

combined are still necessary for late inflammasome activation. Whether this is due to failure of NLRP3 up-regulation or lack of other signals transduced by these two IRAKs remains to be investigated.

The early inflammasome pathway induces rapid caspase-1 cleavage, which renders the macrophages to undergo rapid pyroptosis and processing and secretion of pre-synthesized IL-1 family members, such as IL-18. The rapidity of caspase-1 cleavage and pyroptosis suggests that this pathway contributes to detection and limitation of early infection by depriving pathogens such as *Listeria* of an intracellular sanctuary for survival and replication, and by initiating local inflammation through release of pre-synthesized IL-18 and other pro-inflammatory mediators such as HMGB-1. These rapid events are likely to be critical for lowering early pathogen burden [329] and do not depend on new protein synthesis that could be targeted by virulence factors [343]. The differential requirement for IRAK-1 in early and late pathways allowed us to investigate the importance of rapid NLRP3 inflammasome pathway *in vivo* using IRAK-1-deficient animals. Particularly, we found that IL-18 secretion resulting from early inflammasome activation plays an important role in inducing IFN-γ production by memory $CD8^+$ T cells, which happens early on during the infection (12 hours). The late inflammasome pathway induced following priming of cells by TLR ligands however, leads to *de novo* synthesis of IL-1 family members, such as pro-IL-1β, and its subsequent processing and secretion, which could be important for a powerful systemic inflammatory response.

Out findings also highlight the non-redundant roles of the IRAK family members downstream of TLRs. Among the four IRAK family members (IRAK-1, IRAK-2, IRAK-

3/IRAK-M and IRAK-4), IRAK-4 has been shown to mediate recruitment of IRAK-1, IRAK-2 and IRAK-M to the signaling complex nucleated by MyD88, and is therefore indispensable for both NF-κB and inflammasome activation. It has been shown that IRAK-1, IRAK-2 and IRAK-M play redundant roles in NF-κB activation. While IRAK-1 plays a unique role in early inflammasome activation we find that IRAK-1 and IRAK-2 together are important for late inflammasome activation. These divergent roles of IRAKs extend our understanding of the IRAK family members beyond gene transcription and link them to signaling events not involving new gene synthesis.

In summary, these results reveal a critical early cellular response pathway in macrophages induced by simultaneous engagement of TLRs and NLRP3. We demonstrate that TLRs, through the kinase activities of IRAK-4 and IRAK-1, participate in acute NLRP3 activation leading to rapid caspase-1 cleavage, pyroptosis and release of pre-synthesized inflammatory molecules. The rapid NLRP3 inflammasome pathway we describe here could enable the host to mount a "true innate" response immediately upon pathogen invasion, before the induction of inflammatory cytokines and chemokines. This has a direct impact on regulating rapid innate responses such as IFN-γ secretion by antigen nonspecific memory $CD8^+$ T cells. The rapidity of caspase-1 cleavage and pyroptosis suggests that this pathway contributes to detection and limitation of early infection by depriving pathogens such as *Listeria* of an intracellular sanctuary for survival and replication, and by initiating local inflammation through release of presynthesized IL-18 and other pro-inflammatory mediators such as HMGB-1. These rapid events are likely to be critical for control of early pathogen burden [329] and do not depend on new protein synthesis that could be targeted by virulence

factors [343]. Our findings also expand the intracellular role of TLR signaling beyond that of inflammatory gene expression through NF-κB. Priming of cells by TLR ligands however, bypasses the continued requirement of TLR signaling (Table 4.1), as demonstrated in previous studies [211, 331], and leads to secretion of higher quantities of IL-1 and therefore could be important for a powerful systemic inflammatory response. Finally, although the necessity of IRAK-1 in NF-κB activation downstream of TLRs and MyD88 is not completely clear, our study identifies a unique function for IRAK-1 and its kinase activity in regulation of rapid NLRP3 inflammasome activation and a critical initial host response to infection.

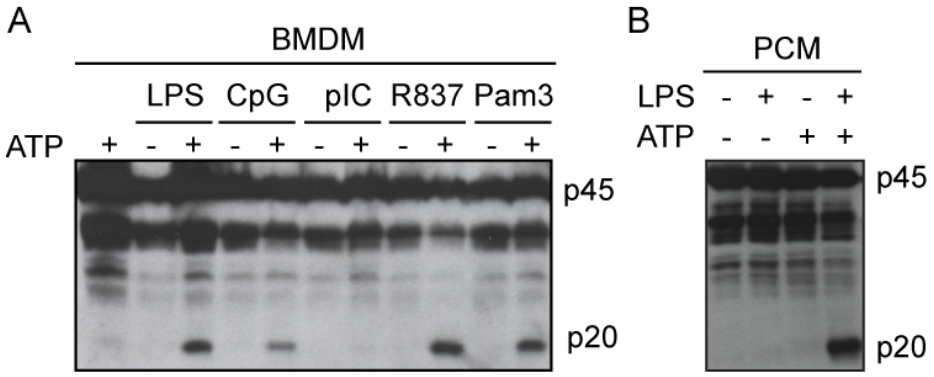

Figure 4.1. Simultaneous stimulation of TLR and NLRP3 induces rapid inflammasome activation.

(A) BMDMs were stimulated with LPS, CpG, Poly I:C (pIC), R837 or Pam3CSK4 together with ATP for 30 min. (B) PCMs were stimulated with LPS together with ATP for 30 min. Cell lysates were analyzed for caspase-1 cleavage by western blot. Data are representative of four independent experiments.

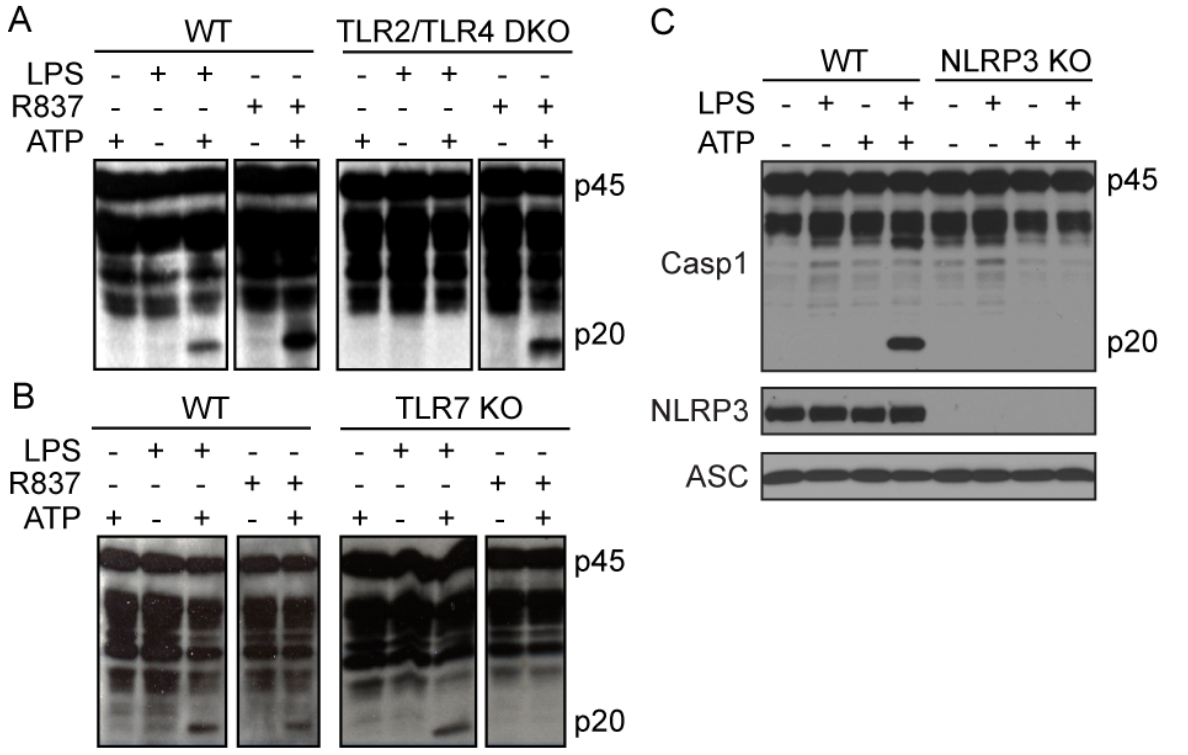

Figure 4.2. Rapid Caspase-1 cleavage is abrogated in macrophages lacking TLR signaling and NLRP3.

BMDMs from mice of indicated genotypes were stimulated with TLR4 ligand, LPS, and ATP or TLR 7 ligand, R837, and ATP for 30 minutes and cell lysates were analyzed for caspase-1 cleavage by western blot analysis. Bone marrow derived macrophages express constitutive NLRP3 protein that is sufficient for rapid caspase-1 cleavage but requires signals from both TLRs and NLRP3. Data are representative of two or three independent experiments.

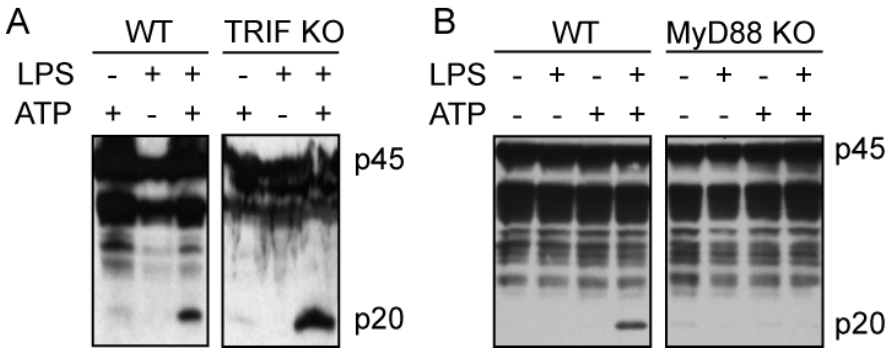

Figure 4.3. MyD88 but not TRIF is required for rapid NLRP3 inflammasome activation.

BMDMs of indicated genotypes stimulated with LPS and ATP for 30 min were analyzed for caspase-1 cleavage by western blot. Data are representative of three independent experiments.

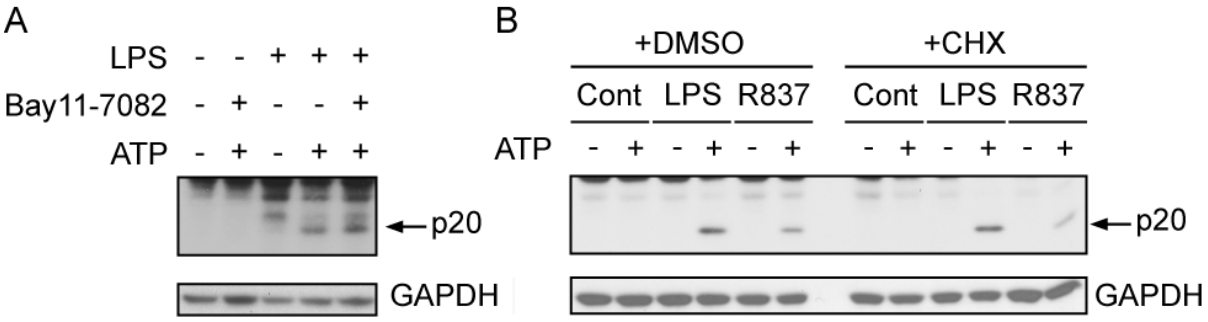

Figure 4.4. Rapid NLRP3 inflammasome activation is independent of NF-κB and new protein synthesis.

BMDMs were treated with cycloheximide (CHX, 50 ng/ml) (d) or Bay11-7082 (10 μM) (e) for 60 min prior to stimulation with LPS or R837 together with ATP for 30 min, and then analyzed for caspase-1 activation by western blot. Data are representative of two independent experiments. This work was performed by Keng-Mean Lin.

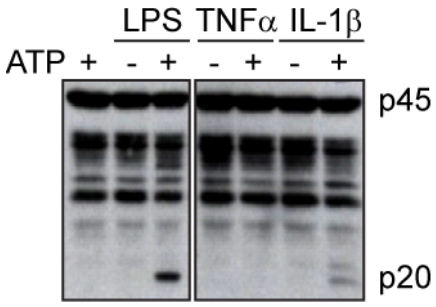

Figure 4.5. IL-1β but not TNF-α can induce rapid NLRP3 inflammasome activation.

BMDMs from WT mice were stimulated with LPS, TNF-α and IL-1β with and without ATP for 30 minutes. Only LPS or IL-1β were able to induce caspase-1 cleavage. Macrophages express very low levels of IL-1R and this could explain the lower level of caspase-1 cleavage induced by IL-1 when compared to LPS. Data are representative of three independent experiments.

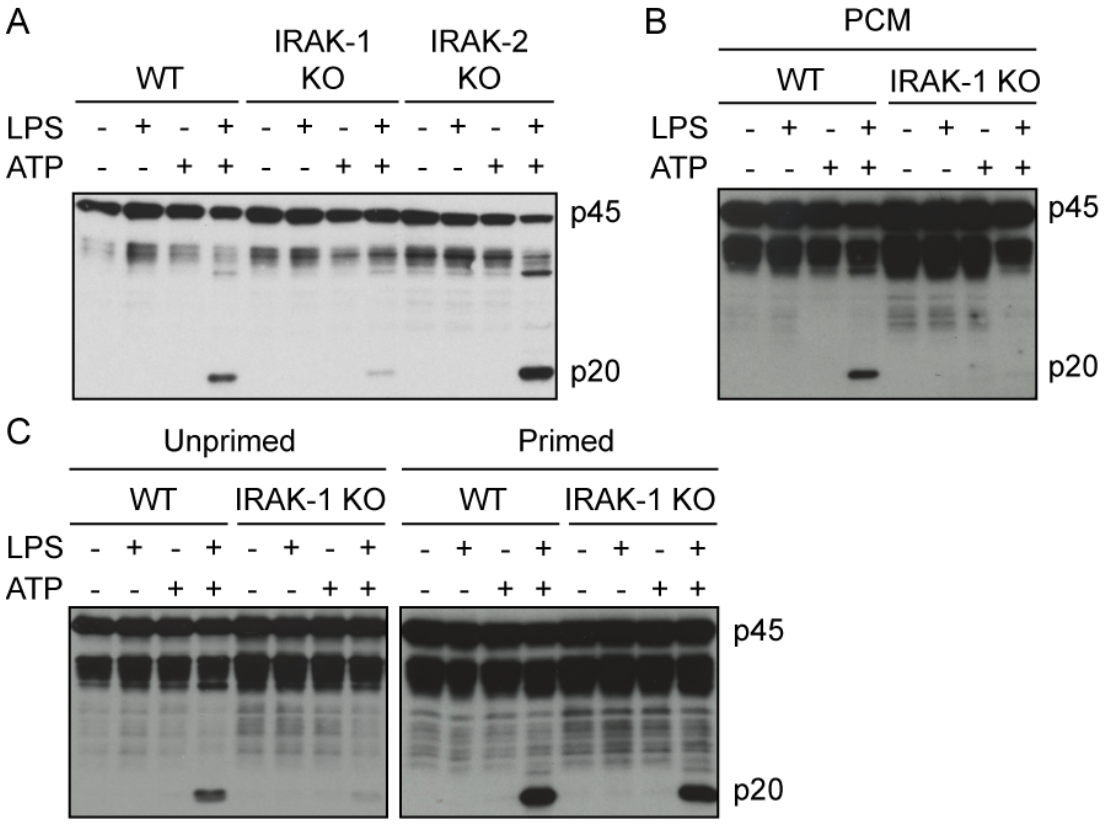

Figure 4.6. IRAK-1 is required for rapid but not priming dependent NLRP3 inflammasome activation.

(A) BMDMs from WT, IRAK-1 and IRAK-2 KO mice were stimulated with LPS together with ATP for 30 min and analyzed for caspase-1 activation by western blot. (B) PCMs from WT and IRAK-1 KO mice were stimulated with LPS together with ATP for 30 min and analyzed for caspase-1 activation by western blot. (C) BMDMs from WT or IRAK-1 KO mice were stimulated with LPS together with ATP for 30 min (unprimed) or primed with LPS for 4 hours followed by stimulation with ATP for 30 min (primed). Lysates were probed for caspase-1 activation by western blot. Data are representative of two to five independent experiments.

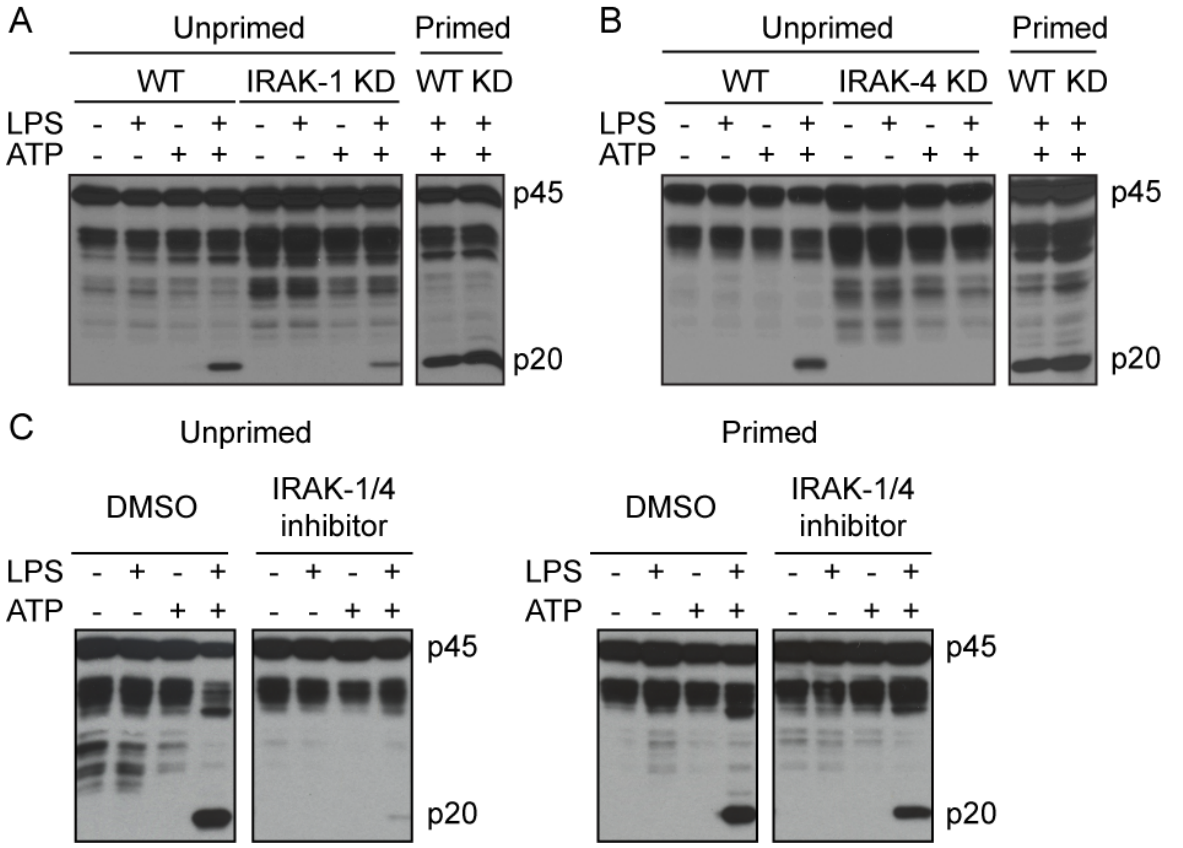

Figure 4.7. Rapid NLRP3 inflammmasome activation depends on kinase activity of IRAKs, but priming dependent NLRP3 inflammasome activation does not require kinase activity of IRAKs.

(A-B) BMDMs from indicated genotypes of mice were stimulated with LPS together with ATP for 30 min (unprimed) or primed with LPS for 4 hours followed by stimulation with ATP for 30 min (primed). Lysates were probed for caspase-1 activation by western blot. (C) BMDMs from WT mice were pretreated with DMSO or an IRAK 1 and 4 kinase inhibitor (CAS 509093-47-4, Millipore) for 1hour, followed by stimulation with LPS together with ATP for 30 minutes (unprimed), or were first stimulated with LPS for 4 hours before addition of ATP for an additional 30 minutes (primed). Cell lystes were subjected to western blot analysis to detect caspase-1 cleavage. Data are representative of three or four independent experiments.

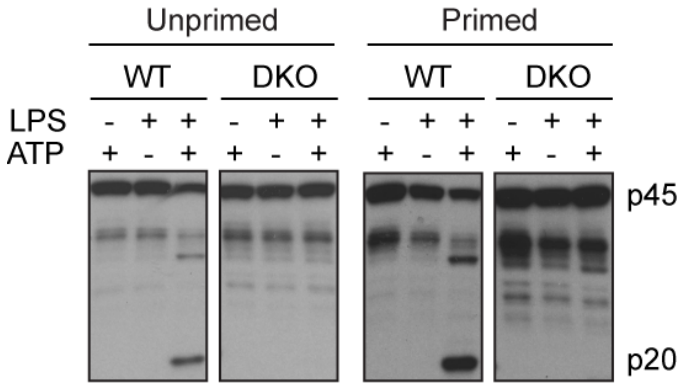

Figure 4.8. IRAK-1/2 double dificiency abrogates both rapid and priming dependent NLRP3 inflammasome activation.

BMDMs from WT or IRAK-1/2 double KO (DKO) mice were stimulated with LPS together with ATP for 30 min (unprimed) or primed with LPS for 4 hours followed by stimulation with ATP for 30 min (primed). Lysates were probed for caspase-1 activation by western blot. Data are representative of four independent experiments.

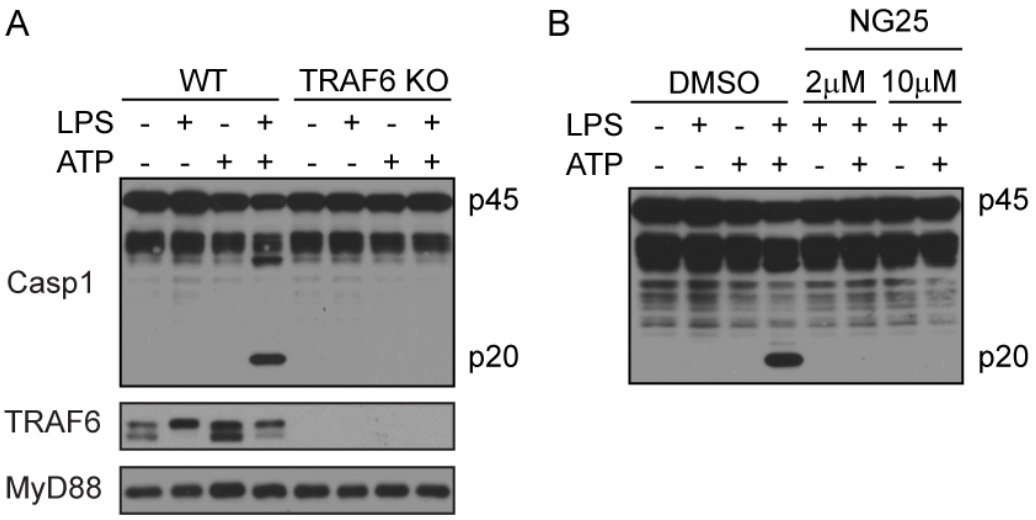

Figure 4.9. Rapid NLRP3 inflammasome activation is dependent on TRAF6 and TAK-1.

(A) TRAF6$^{fl/fl}$(WT) and TRAF6$^{fl/fl}$x Mx-Cre Tg mice (TRAF6 KO) were injected with Poly I:C as described in the methods and BMDMs were prepared and stimulated with LPS and ATP for 30 minutes. Cell lystates were examined for caspase-1 cleavage by western blot. (B) WT BMDMs were pretreated with NG25, an inhibitor of TAK-1, prior to stimulation with LPS and ATP for 30 minutes. Cell lystates were examined for caspase-1 cleavage by western blot. Data are representative of two independent experiments.

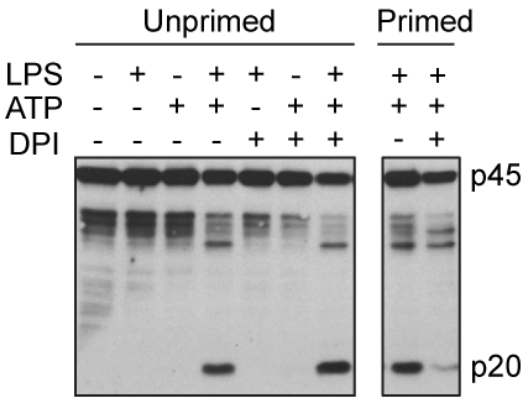

Figure 4.10. Acute inflammasome activation occurs independent of reactive oxygen species (ROS).

WT BMDMs were pretreated for 1h with DMSO or 5 μM diphenyleneiodonium chloride (DPI, an inhibitor of ROS generation) before stimulation. Cells were then stimulated with LPS together with ATP for 30 minutes (unprimed) or LPS for 4 hours followed by ATP for 30 minutes (primed). Cell lysates were analyzed by western blot for caspase-1 cleavage. Data are representative of two independent experiments.

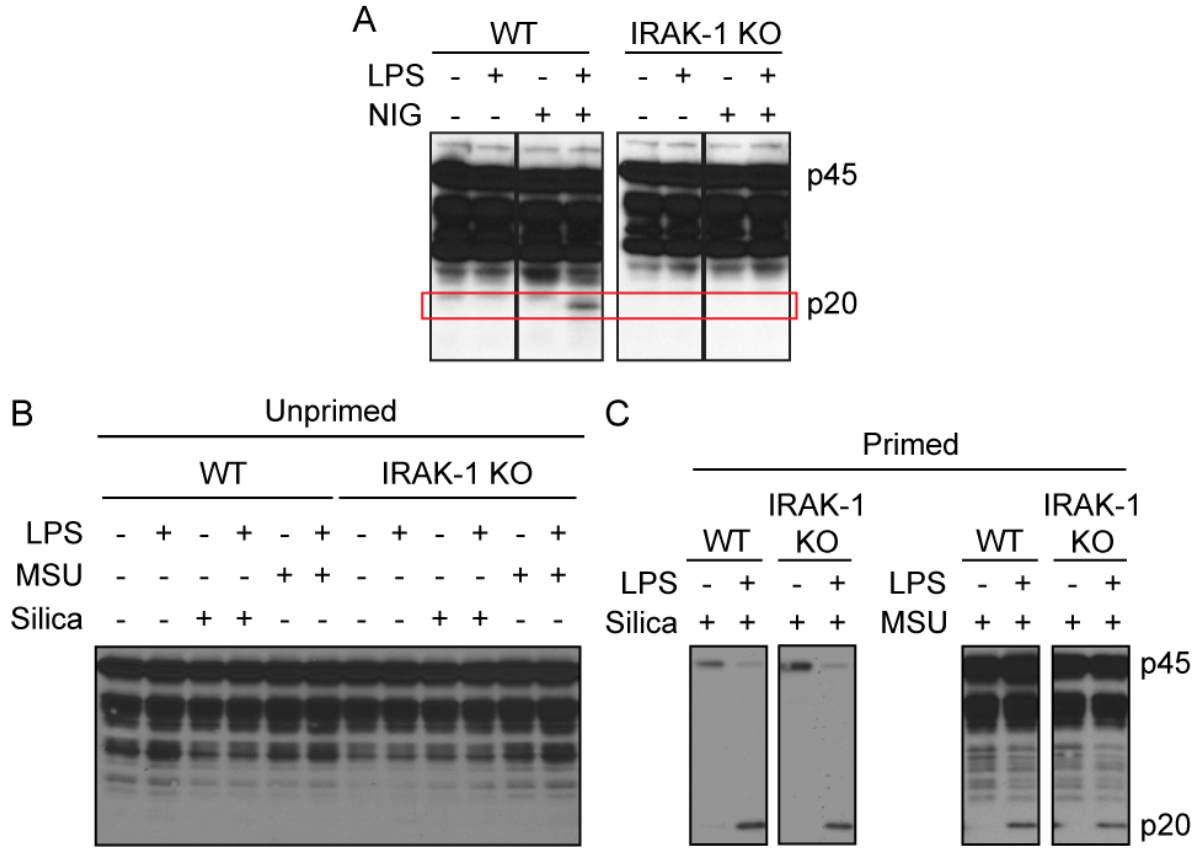

Figure 4.11. Differential ability of NLRP3 ligands to induce rapid caspase-1 activation.

(A) WT or IRAK-1 KO BMDMs were stimulated with LPS plus nigericin for 30 minutes. (B) WT or IRAK-1 KO BMDMs were stimulated with LPS plus monosodium urate (MSU), or LPS plus silica crystals for 30 minutes. (C) WT or IRAK-1 KO BMDMs were primed using LPS for 4 hours followed by stimulation with MSU and silica crystals for an additional 6 hours. Cell lystates were examined for caspase-1 cleavage by western blot analysis. Data are representative of two or three independent experiments. Work in (A) was performed by Keng-Mean Lin.

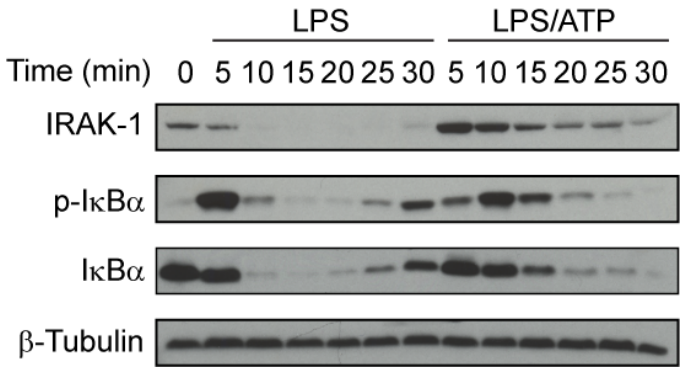

Figure 4.12. Simultanous stimulation of TLR and NLRP3 delays IRAK-1 degradation and NF-κB activation.

Cell lysates from WT BMDMs stimulated with LPS alone or LPS together with ATP for the indicated periods of time were immunoblotted for the indicated proteins. Data are representative of three independent experiments.

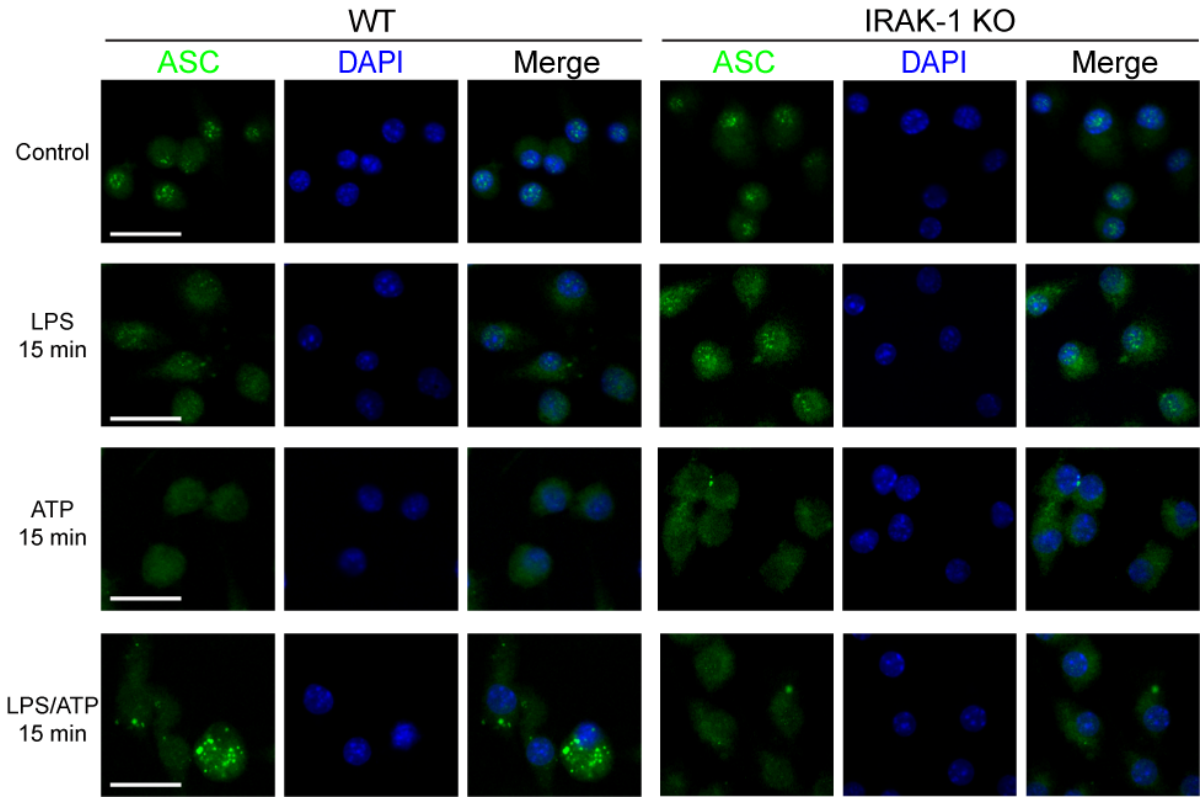

Figure 4.13. Relocalization and speck formation by ASC following TLR and NLRP3 stimulation.

WT (left panels) or IRAK-1 KO (right panels) BMDMs underwent nuclear staining with DAPI and immunofluorescence of endogenous ASC after stimulation with LPS alone, ATP alone, or LPS and ATP simultaneously. Scale bar, 20 μm. Data are representative of three independent experiments. This work was performed by Keng-Mean Lin.

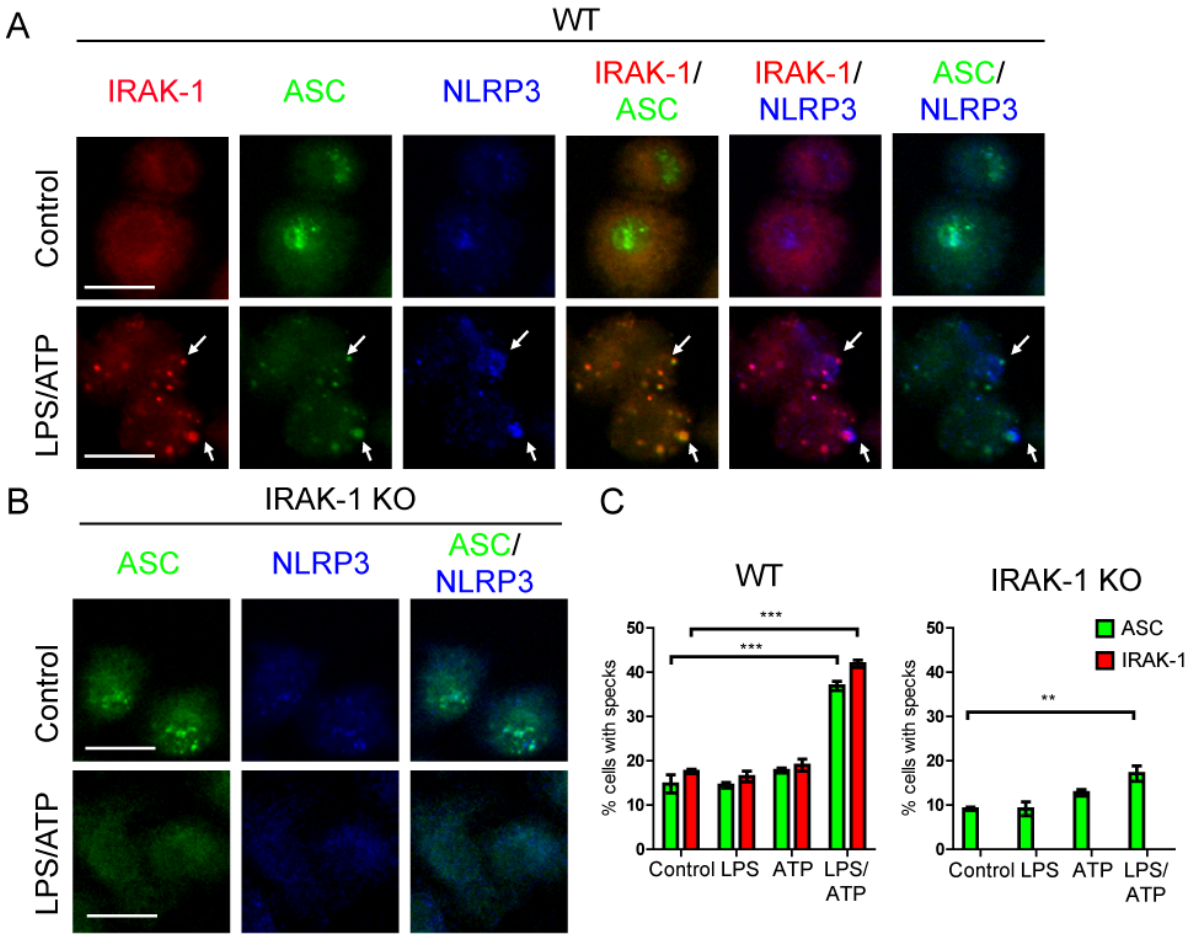

Figure 4.14. IRAK-1 colocalizes with the NLRP3 inflammasome complex.

(A-B) Immunostaining of endogenous ASC, IRAK-1 and NLRP3 in WT (A) and IRAK-1 KO (B) BMDMs stimulated with LPS and ATP for 15 minutes. Arrows show specks formed by the indicated proteins. Scale bars, 20 μm. (C) Quantification of percentages of cells containing ASC or IRAK-1 specks in WT and IRAK-1 KO macrophages. Bar graphs represent mean ± SEM from three experiments and at least 100 cells were counted in every condition. $**p < 0.01$. $***p < 0.005$, one-way ANOVA. This work was performed by Keng-Mean Lin.

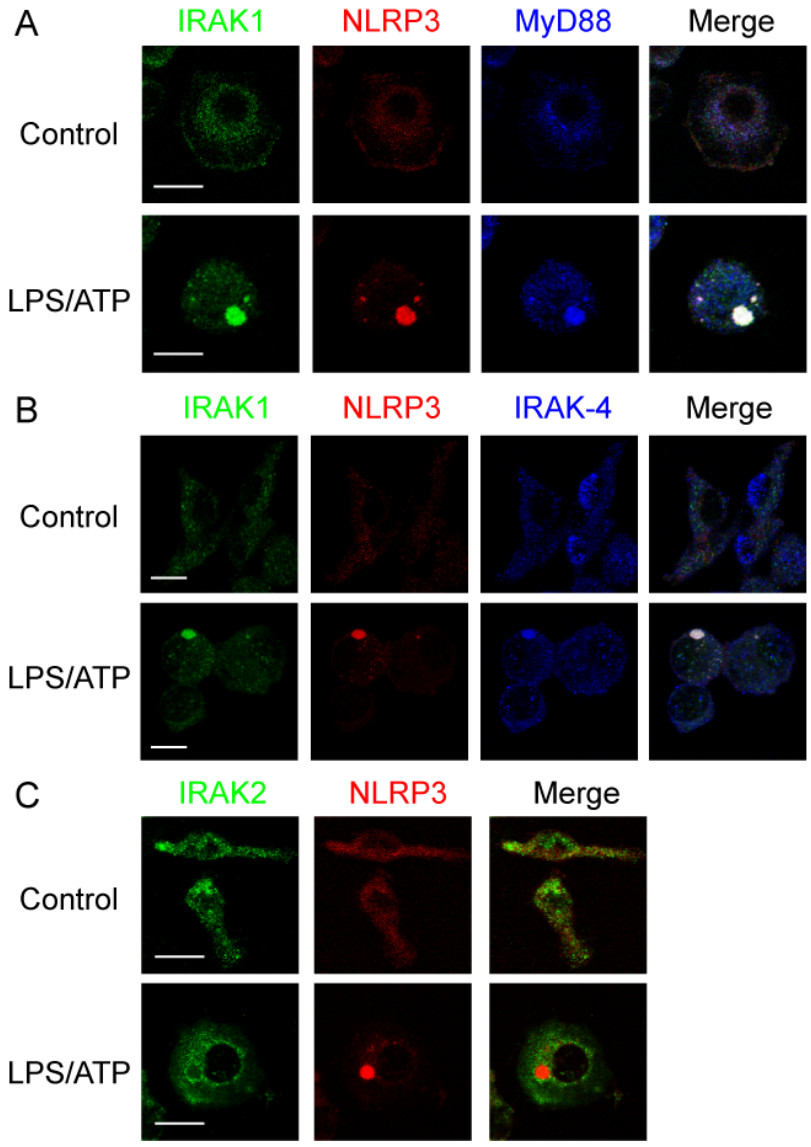

Figure 4.15. MyD88, IRAK-4 and IRAK-1, but not IRAK-2, colocalize with the inflammasome complex.

Immunostaining of indicated endogenous proteins in WT BMDMs stimulated with LPS and ATP for 15 minutes. Scale bar, 10 μm. Data are representative of three independent experiments. This work was performed by Wei Hu together with Keng-Mean Lin.

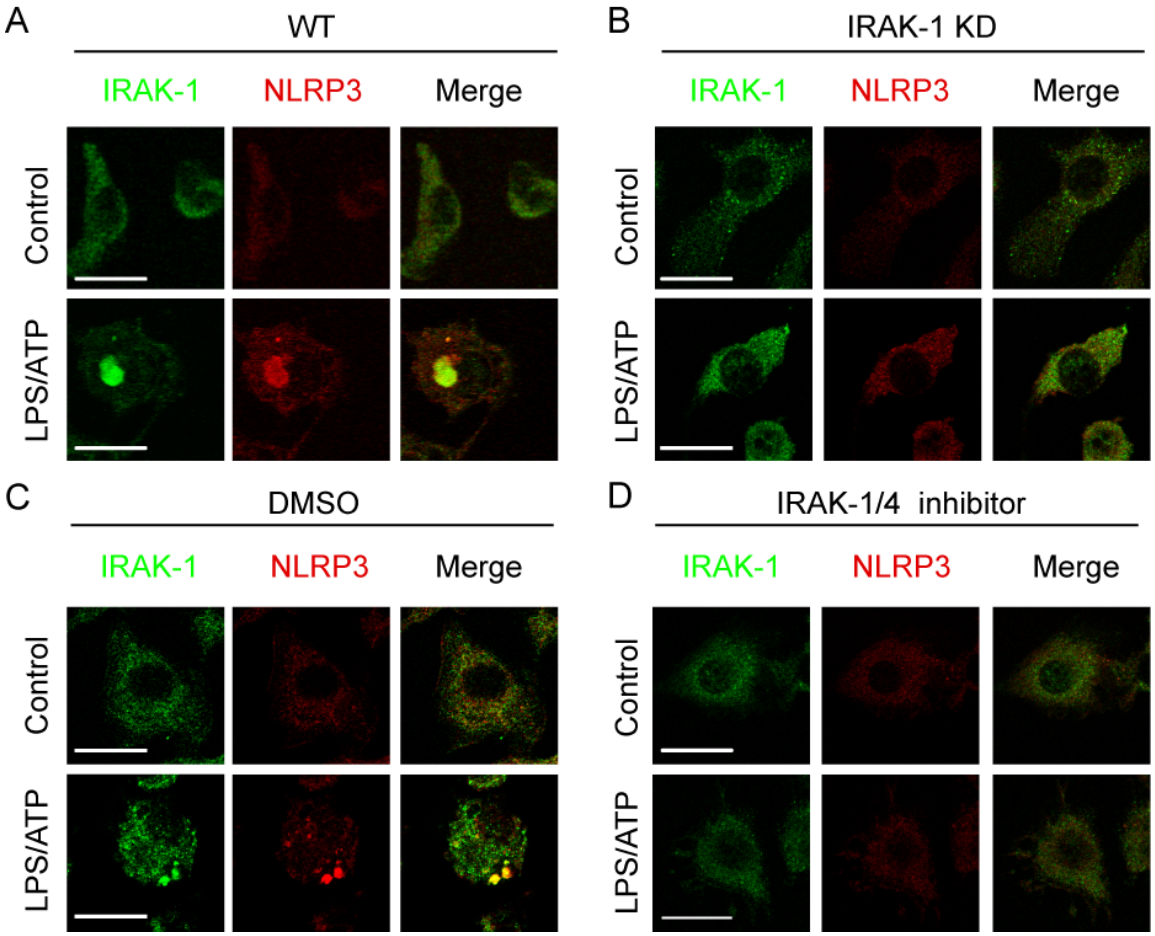

Figure 4.16. Inhibition of the kinase activity of IRAKs prevent NLRP3 Speck formation.

(A) Immunostaining of endogenous IRAK-1 and NLRP3 in IRAK-1 kinase dead (KD) knock in mouse BMDMs stimulated with LPS and ATP for 15 minutes. (B) Immunostaining of endogenous IRAK-1 and NLRP3 in WT BMDMs stimulated with LPS and ATP for 15 minutes in the presence of an IRAK-1/4 kinase inhibitor. Scale bar, 10 μm. Data are representative of two independent experiments. This work was performed by Wei Hu together with Keng-Mean Lin.

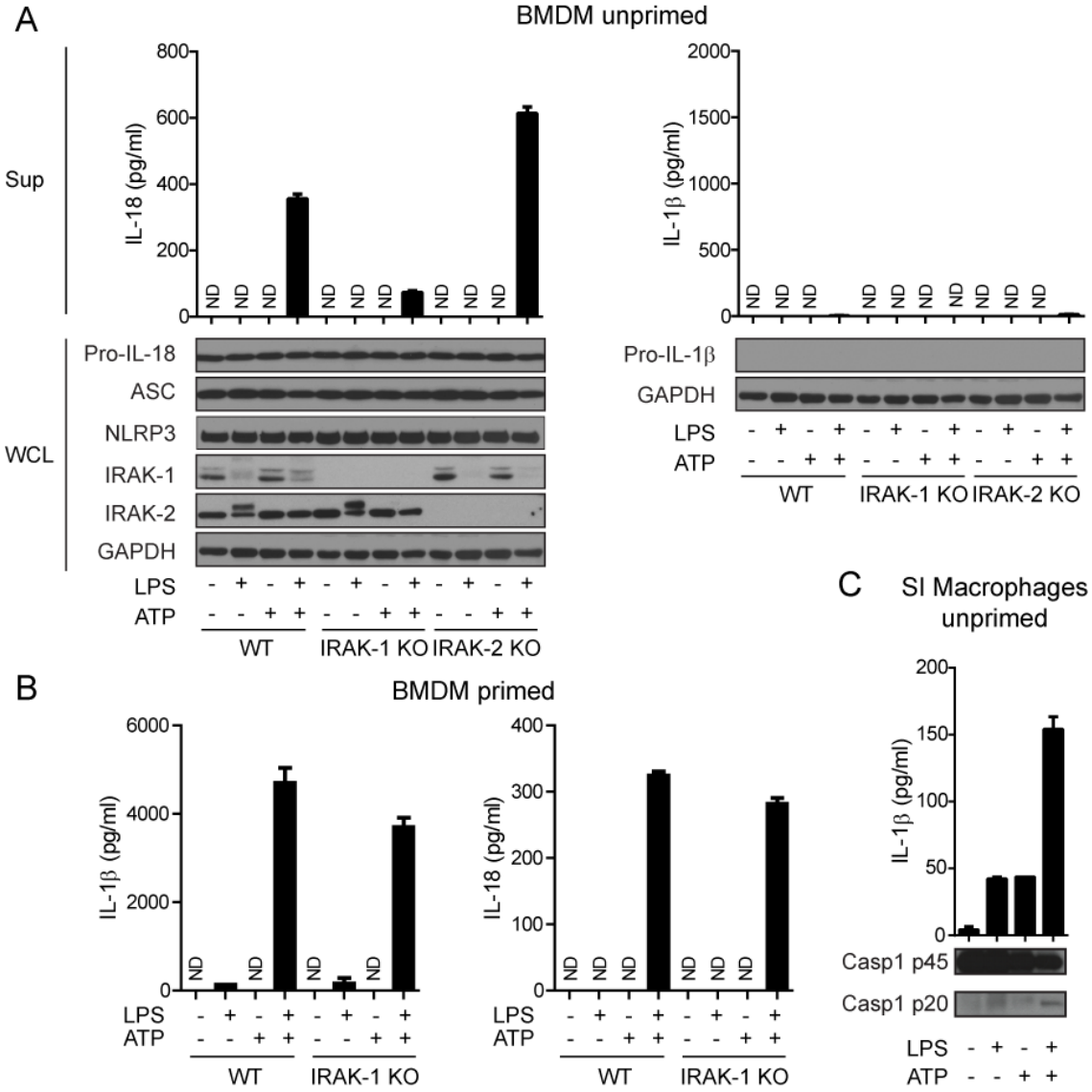

Figure 4.17. Acute NLRP3 inflammasome activation leads to processing and secretion of presynthesized IL-1 family cytokines.

(A) BMDMs of the indicated genotypes were stimulated with LPS alone, ATP alone, or both LPS and ATP for 60 min and supernatants were analyzed for IL-18 and IL-1β by ELISA. Cell lysates were subject to western blotting to detect indicated proteins. (B) BMDMs were primed for 4 hours with LPS and then were pulsed with ATP for 30 minutes and supernatants were collected after 4 hours of ATP stimulation to measure IL-1β and IL-18. (C) FACS sorted small intestine (SI) lamina propria macrophages ($CD11c^{+}MHC\text{-}II^{+}CD103^{-}F4/80^{hi}$) were stimulated with LPS alone, ATP alone, or both LPS and ATP for 60 min and supernatants were analyzed for IL-1β by ELISA. Cell lysates were analyzed for caspase-1 activation by western blot. ND, not detectable. Bar graphs represent mean ± SEM from duplicated ELISA measurements. Data are representative of two to four independent experiments.

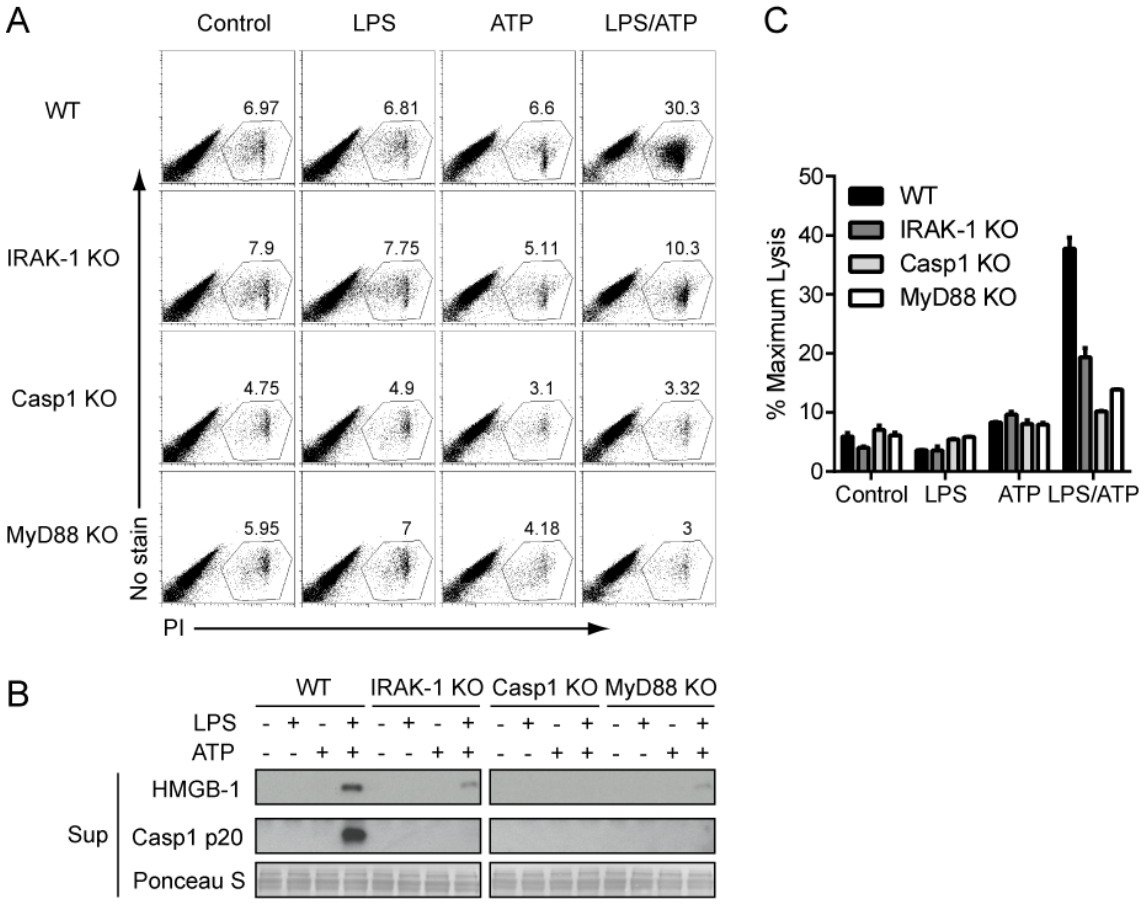

Figure 4.18. Rapid NLRP3 inflammasome activation induces pyroptosis.

BMDMs of the indicated genotypes were stimulated with LPS alone, ATP alone, or both LPS and ATP for 60 min. (A) Cells were stained with propidium iodide (PI) and analyzed by flow cytometry for pyroptosis. (B) The supernatants were blotted for activated caspase-1 and HMGB-1. (C) LDH activity in the supernatants was determined to measure cell lysis. Ponceau S staining serves as the loading control. Bar graph represents mean $\pm$ SEM of duplicated LDH measurements. Data are representative of five independent experiments.

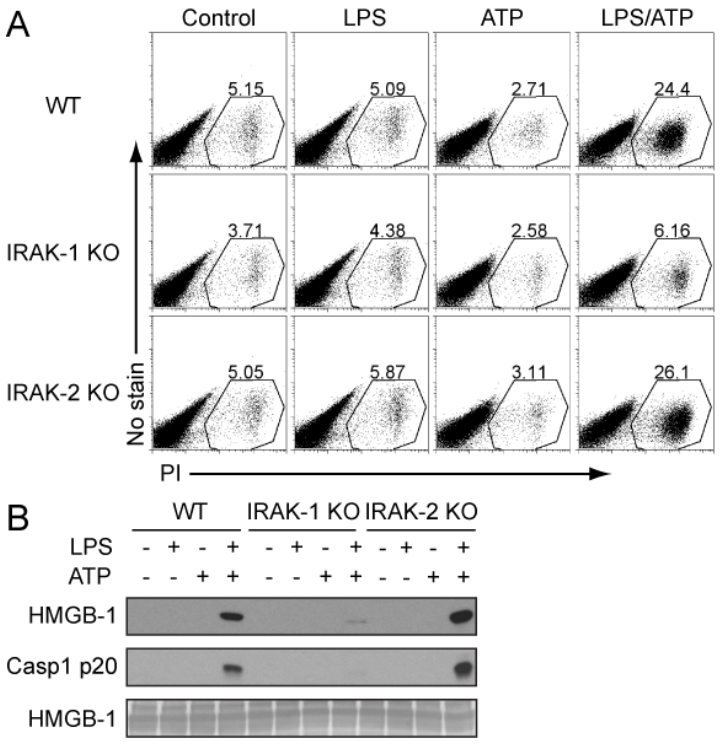

Figure 4.19. IRAK-2 deficiency does not impair pyroptosis.

BMDMs of the indicated genotypes were stimulated with LPS alone, ATP alone, or both LPS and ATP for 60 min. (A) Cells were stained with propidium iodide (PI) and analyzed by flow cytometry for pyroptosis. (B) The supernatants were blotted for activated caspase-1 and HMGB-1. Ponceau S staining serves as the loading control. Data are representative of three independent experiments.

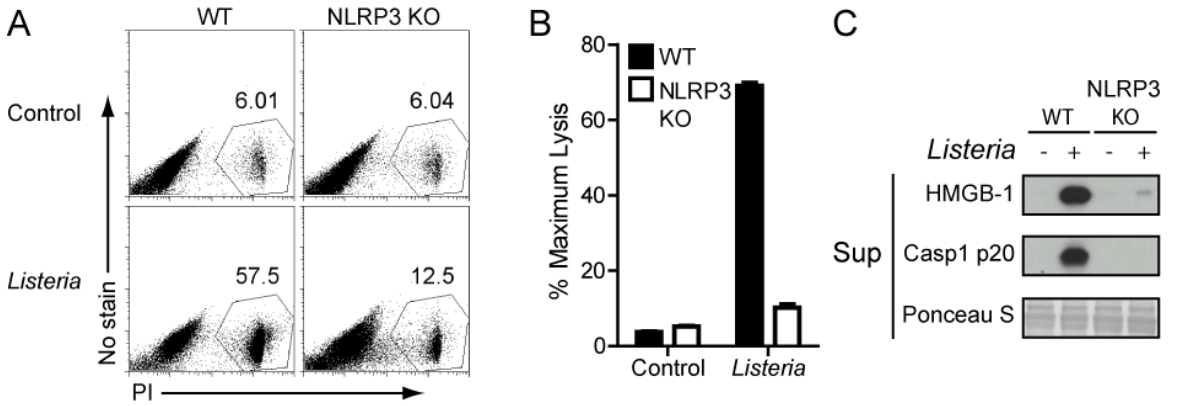

Figure 4.20. *Listeria monocytogenes* infection induces NLRP3-dependent inflammasome activation and pyroptosis.

WT or NLRP3-deficient BMDMs were infected with *L. monocytogenes* for 1 h and analyzed for pyroptosis by (A) PI incorporation, (B) LDH release and (C) cleaved caspase-1 release and HMGB-1 release. Bar graph represents mean ± SEM of duplicated LDH measurements. Data are representative of two independent experiments.

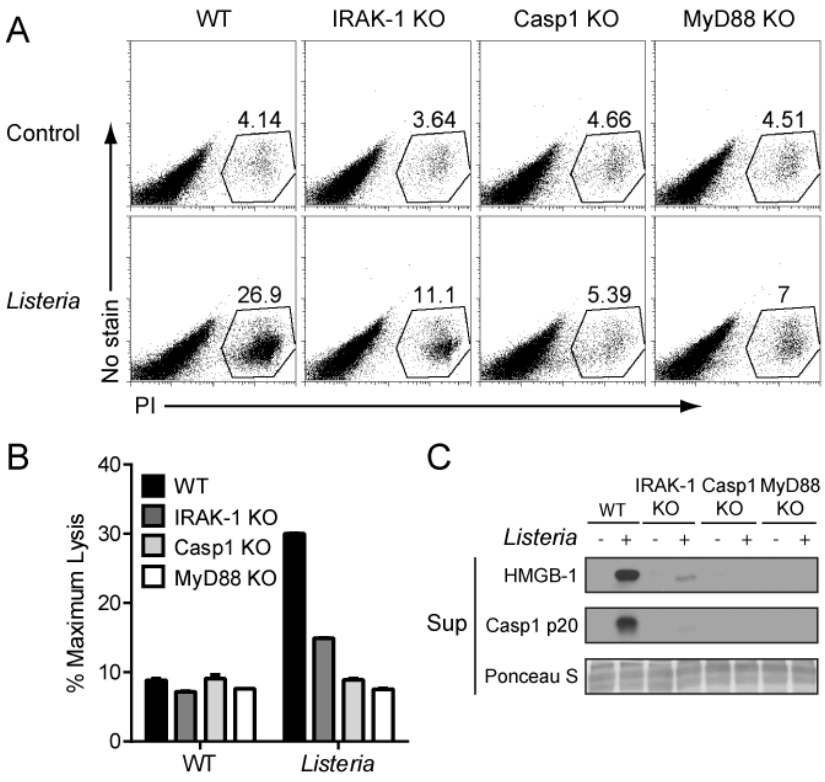

Figure 4.21. Listeria infection triggers IRAK-1 dependent rapid inflammasome activation.

BMDMs of the indicated genotypes were infected with *L. monocytogenes* for 1 h and analyzed for pyroptosis by (A) PI incorporation, (B) LDH release and (C) cleaved caspase-1 and HMGB-1 release. Bar graph represents mean ± SEM of duplicated LDH measurements. Data are representative of four independent experiments.

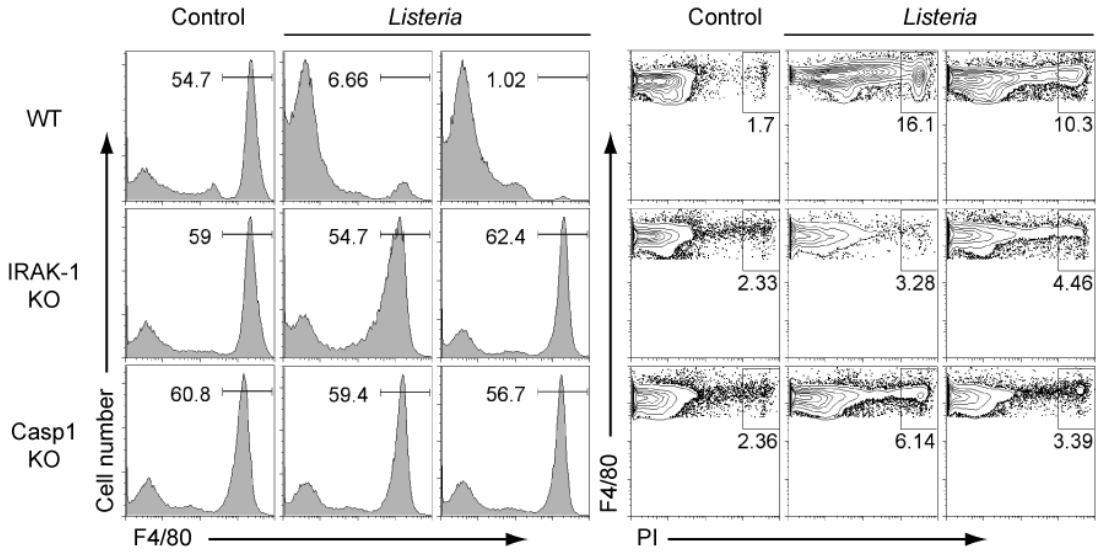

Figure 4.22. *Listeia* infection induces pyroptosis *in vivo*.

WT, IRAK-1 and caspase-1 KO mice were injected with *L. monocytogenes* and 20 min later, cells in the peritoneal cavity were stained with anti-CD45, anti-F4/80 and PI. The left panel shows the proportions of $CD45^+$ cells that stain positive for F4/80 and the right panel shows proportions of $CD45^+$ $F4/80^+$ cells that stain for PI.

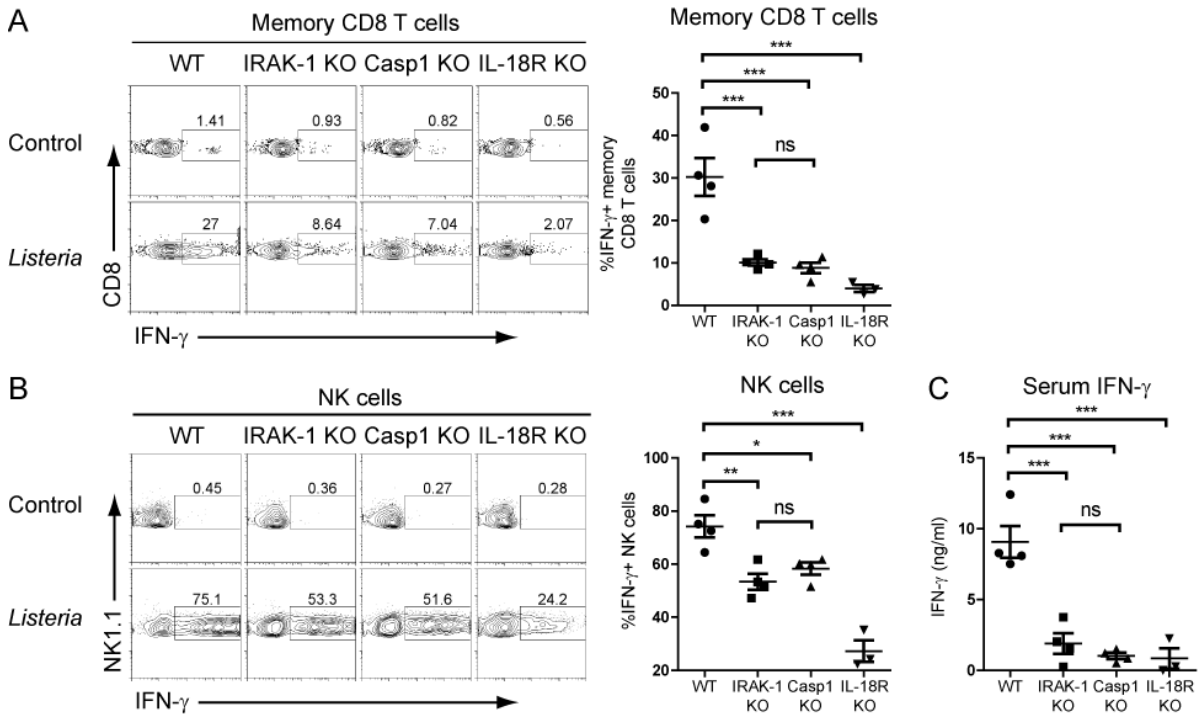

Figure 4.23. IRAK-1 dependent innate IFN-γ production during *L. monocytogenes* infection *in vivo*.

(A) Representative plots (left) and combined data (right) of IFN-γ production by polyclonal memory $CD8^+$ T cells ($CD8^+TCR\beta^+CD44^+$) from mice infected intravenously with *L. monocytogenes* for 12 hours. (B) Representative plots (left) and combined data (right) of IFN-γ production by NK cells ($NK1.1^+TCR\beta^-$) from mice infected intravenously with *L. monocytogenes* for 12 hours. (C) Serum IFN-γ concentration of mice infected intravenously with *L. monocytogenes* for 12 hours. Scatter plots show mean ±SEM (n=3-4 mice). **p*<0.05, ***p*<0.01, ****p*<0.005, ns, non-significant, one-way ANOVA test.

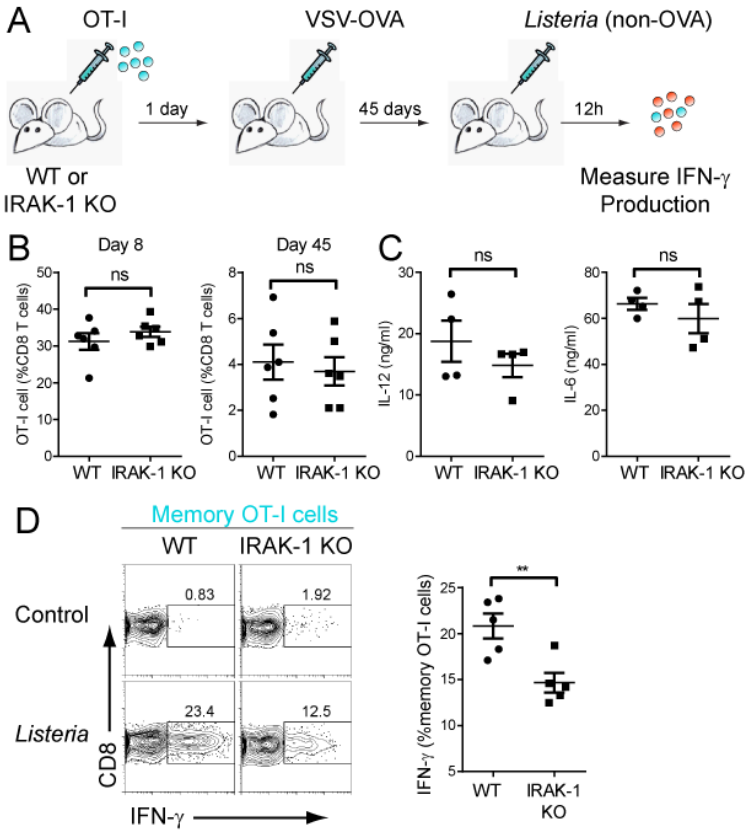

Figure 4.24. IRAK-1 in the non-T cell compartment is required for innate IFN-γ production during *L. monocytogenes* infection *in vivo*.

(A) Experimental procedure for assessing IFN-γ production by memory OT-I T cells upon *Listeria* infection. (B) OT-I T cell expansion was measured in the peripheral blood at day 8 by staining for surface CD45.1 and CD8. Similar staining was done for cells from the spleen on day 45 after challenge to measure OT-I T cell survival. (C) IL-12 and IL-6 concentrations in the serum were determined by ELISA at 12 hours following infection. (D) IFN-γ production by memory WT OT-I T cells generated in WT or IRAK-1 KO mice. Scatter plots show the mean $\pm$ SEM (n=4-5 mice). Data are representative of two independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p<0.005$, ns, non-significant, two-tailed *t* test.

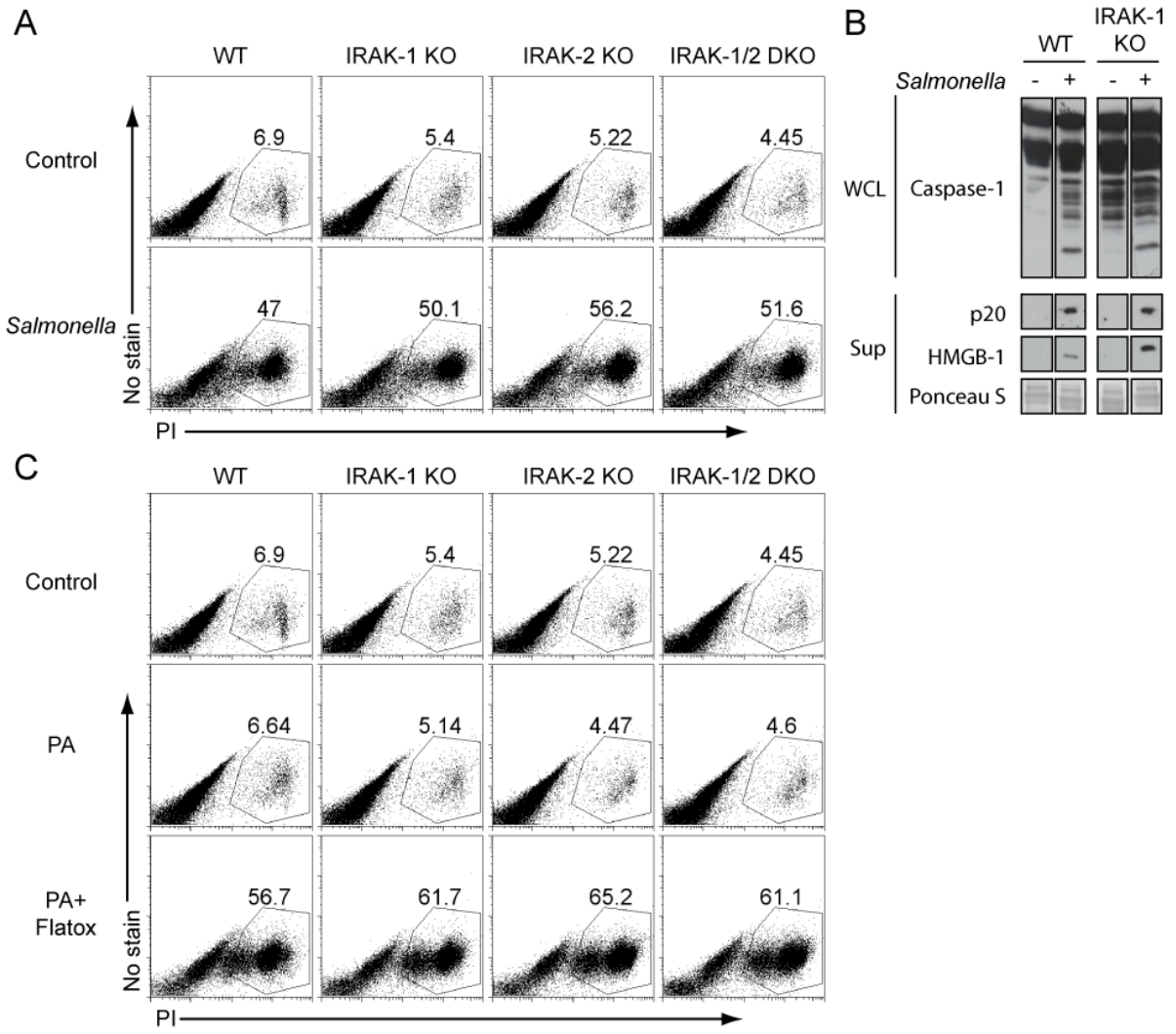

Figure 4.25. IRAK-1 does not regulate NLRC4 inflammasome activation and pyroptosis.

(A-B) BMDMs of the indicated genotypes were infected with *Salmonella typhimurium* SL1344 (MOI=2) for 1h and analyzed for pyroptosis by PI incorporation (A) and cleaved caspase-1 release and HMGB-1 release (B). Ponceau S stain of total protein serves as the loading control for the supernatant (sup). (C) BMDMs were treated with 4 μg/ml of *B. anthracis* protective antigen (PA) alone, or together with 2 μg/ml of LFn-FlaA (Flatox)[1], a fusion protein encoding the first (non-enzymatically active) 263 amino acids of the lethal factor from *Bacillus anthracis* fused to full-length flagellin (FlaA) from *L. pneumophila*, which allows cytosolic delivery of flagellin. After 1h, cells were stained with PI to measure pyroptosis. Data are representative of five independent experiments.

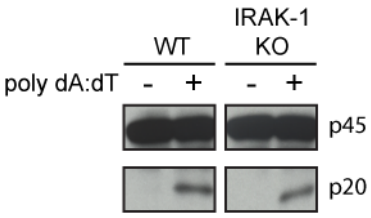

Figure 4.26. AIM-2 inflammasome activation is independent of IRAK-1.

WT and IRAK-1-deficient BMDMs were mock transfected or transfected with 5 μg/ml of poly dA:dT using lipofectamine 2000 for 1h and analyzed for caspase-1 cleavage by western blot. Data are representative of two independent experiments.

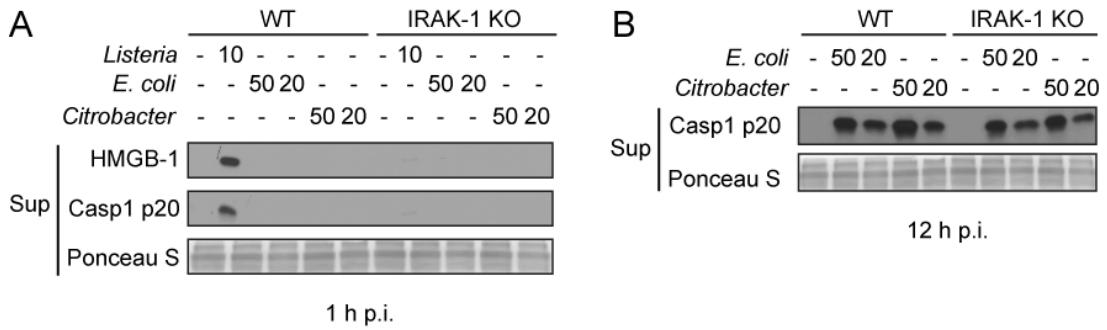

Figure 4.27. Differential ability of pathogens to induce rapid NLRP3 inflammasome activation.

(A), WT and IRAK-1-deficient BMDMs were infected with different pathogens at indicated MOI that are known to activate NLRP3 inflammasome. Culture supernatants were analyzed for HMGB-1 release and Caspase-1 cleavage by western blot. *E. coli* and *Citrobacter rodentium* fail to induce rapid caspase-1 cleavage after 1 hour of infection. (B) Culture supernatants from macrophages infected with *E. coli* or *Citrobacter* at indicated MOI for 12 hours were analyzed for Caspase-1 cleavage by western blot. Overnight infection with *E. coli* and *C. rodentium* induces IRAK-1 independent NLRP3 inflammasome activation. Ponceau S stain serves as the loading control. Data are representative of two independent experiments.

Table 4.1. Major differences between the acute and priming-dependent pathways of NLRP3 inflammasome activation.

	Acute pathway	***Priming-dependent pathway***
Activation of TLR and NLRP3	Simultaneous	Sequential
Participating TLRs	All except for TLR3	All
NF-κB activity	Not required	Required
New protein synthesis	Not required	Required
Reactive oxygen species	Not required	Required
IRAK-1	Required	Not required
IRAK-1&IRAK-2	Required	Required
IRAK-4	Required	Not tested

CHAPTER FIVE
Discussion

Part of this chapter has been published in *Journal of Leukocyte Biology*, volume 94, pages 409-421.

Overall conclusion

TLRs are microbial sensors that orchestrate the host defense program. The work I have presented here focused on two emerging new players of host defense, the Th17 cells and the inflammasome, and investigated how TLR signaling and TLR-induced proinflammatory cytokines influence these two arms of immune pathways. My work has revealed two previously unappreciated aspects of TLR-dependent mechanism of host defense. First, the TLR-induced cytokine IL-6 plays a tissue-specific role in regulating Th17 differentiation. It is specifically required in the intestine and skin, but dispensable in the spleen, for the differentiation of Th17 cells. Such differential cytokine requirements for Th17 lineage commitment are dictated by differential population of DCs resident in the priming tissue. (Figure 5.1). Second, TLR can cooperate with NLR and deliver dual signaling to activate the NLRP3 inflammasome in the absence of new protein synthesis, and the signaling downstream of TLRs leading to NF-κB/MAPK activation vs. inflammasome activation diverge at the signaling protein IRAK-1. This form of acute NLRP3 inflammasome activation contributes to early host defense against the pathogenic microbe *Listeria monocytogenes* (Figure 5.2).

TLR-induced cytokines and Th17 lineage development

Role of IL-6 in Th17 differentiation

IL-6 has been shown by multiple studies to be essential for Th17 development [122, 123]. IL-6 combined with TGF-β is sufficient to induce RORγt expression and IL-17 secretion, proving that IL-6 is a positive regulator of Th17 differentiation [140]. In support with this notion, the amount of IL-6 and IL-17 in various autoimmune diseases are often correlated [344]. However, the interpretation of these observations is complicated by the fact that IL-6 is a target gene of IL-17 in fibroblasts. Therefore, it is unclear, in these studies, whether the increased IL-6 is the cause or the consequence of elevated IL-17. IL-6-deficient mice are resistant to EAE development when immunized with MOG peptide and CFA [128]. The lack of disease is accompanied by defective MOG-specific Th17 responses in the IL-6-deficient mice [128]. However, priming of $CD4^+$ T cells also depends on IL-6, as IL-6 signaling is required by effector T cells to overcome Treg mediated suppression [60]. In fact, $CD4^+$ T cells from MOG immunized IL-6 KO mice proliferated poorly during antigen specific recall [128]. Thus, failure to develop into the Th17 lineage in IL-6 KO mice is not sufficient to prove that IL-6 is specifically required for Th17 lineage development, as opposed to general T cell activation. Depletion of Tregs prior to immunization relieves their suppressive effect and could restore antigen-specific T cell priming. In this scenario, IL-6-deficient mice are still unable to mount Th17 immunity, suggesting that IL-6 is required for antigen-specific Th17 response (Medzhitov and Schenten, personal communication).

Evidence of IL-6-independent Th17 differentiation has also been observed. In an *in vitro* differentiation system, activating naïve $CD4^+$ T cells in the presence of conditioned media from LPS-stimulated bone marrow-derived DCs results in the production of IL-17, which is not blocked by antibodies against IL-6Rα or gp130, although such blockade completely inhibited the induction of IL-7 by TGF-β plus IL-6. Congruent with this finding, conditioned media from IL-6 KO DCs also induced IL-17 production from naïve T cells [345]. These data argue that additional Th17 inducing capacity other than IL-6 are present in the DC conditioned media. Likewise, using an *in vitro* priming system employing DC-T cell culture, I have shown that the induction of IL-17 in such system does not rely on IL-6, as IL-6 KO DCs prime Th17 cells as effectively as their WT counterparts. The identity of the other factors are not yet clear. IL-21 has been suggested to synergize with TGF-β and induce Th17 differentiation in the absence of IL-6. However, DCs produce little IL-21, and T cells, unless stimulated with IL-6 or IL-21, do not secrete IL-21 either [346]. It is therefore reasonable to speculate that an unknown factor is responsible for IL-6-independent Th17 differentiation, and it would be interesting to determine the nature of this unknown activity. Since STAT3 is indispensable for initiating the Th17 differentiation program [132], it would also be informative to test if the IL-6-independent Th17 differentiation requires STAT3 activation in the T cells.

Requirement of IL-6 for Th17 differentiation in vivo is tissue specific

The *in vivo* data regarding the need for IL-6 in generating Th17 responses have been conflicting. Many Th17-dominant inflammatory diseases depend on IL-6 [347-349]. Others

have suggested that Th17 differentiation can occur in the absence IL-6. I have shown that the IL-6 dependency of Th17 differentiation is context dependent, depending on the location where the immune response is initiated. When an immune response is initiated in the skin or intestinal mucosa, such as induction of EAE via subcutaneous immunization or oral infection, the IL-6-dependent mechanism is in operation. In contrary, when an immune challenge occurs in the systemic immune system, the IL-6 impendent mechanism prevails. In summary, when discussing the cytokine requirement for Th17 lineage development, it is important to consider the route by which the immune challenge is delivered.

Compartmentalization of the immune system necessitates tissue specific control of immune responses

The immune system can be divided into three major compartments: (1) the systemic immune system, which filters the blood and detects blood-borne infections; (2) the mucosal immune system, which detects pathogens evading various mucosal surfaces including the gastrointestinal tract, the respiratory tract, the urinary tract and the uro-genital tract; (3) the cutaneous immune system, which detects pathogens entering via the skin either because of breaches or insect bites [350]. Each of the three immune compartments faces distinct challenges in generating effective anti-pathogen immune responses while maintaining homeostasis and avoiding overwhelming, self-damaging responses. As a result, immunity has to operate in a tissue-specific manner: The systemic immune system mounts immune responses to invading pathogens via the induction of robust proinflammatory and bactericidal programs

that may have damaging consequences to the host. This is understandable because the danger from the pathogen is very high when it gains access to the circulation. However, in the case of mucosal and cutaneous immune systems, maintaining organ integrity is the number one priority. Therefore, under steady state, immunity has to be tightly controlled to allow peaceful coexistence with, rather than eradication, of the symbionts. The inability to maintain homeostasis results in immunopathology and tissue destruction, as seen in emphysema (uncontrolled activation of alveolar macrophages) and colitis (uncontrolled activation of T cells against commensals). Since the same type of immune cells residing in different tissues originate from the same precursors prior to entering the tissue, it would be expected that organ-specific factors instruct regional immune responses. Consequently, each organ would have its own threshold for activating the immune system, which is inversely correlated with the relative sterility of the organ. My data have shown that the requirement for IL-6 in Th17 differentiation is specific to the mucosal and cutaneous immune systems, suggest that IL-6 may serve as an additional inflammatory signal that elevates the activation threshold for Th17 immunity in the commensal associated immune systems to avoid deleterious inflammation against the symbionts.

Priming Microenvironments and routes of infection dictate the outcome of immune response

I have shown that the route of infection may determine the outcome of an immune response. This is consistent with increasing evidence showing that the phenotype, differentiation status, longevity, turnover rate, functions, and regulatory mechanisms of

immune cells are drastically different and significantly impacted by the priming microenvironment.

It has long been noticed that the efficacy of vaccination is greatly impacted by the route of immunization. This phenomenon has been seen in vaccination against *Mycobacterium*, where intranasal immunization with BCG leads to far superior protection to airborne challenge with *Mycobacterium tuberculosis* compared to subcutaneous or intravenous route [351, 352]. Similar observations have been made with *Listeria pneumonitis*, where mice vaccinated through the airborne route, but not intravenously or in the footpad, acquire more robust and prolonged resistant to airborne rechallenge [353].

A number of studies have analyzed the type, the longevity and the differentiation mechanisms of the effectors generated via different routes of infection. When mice are infected with *Listeria monocytogenes* through either the intravenous or the intranasal route, intravenous infection elicit antigen-specific Th1 cells, while intranasal infection elicit antigen-specific Th17 cells instead [354]. Moreover, intravenous infection-elicited Th1 cells persist following the contraction phase, whereas the intranasal infection-elicited Th17 cells progressively decay. This short-lived phenotype of intranasal infection-elicited Th17 cells correlate with diminished CD27 expression, reduced homeostatic proliferation, and reduced IL-15R and Bcl2 (anti-apoptotic factor) expression [354]. Therefore, the route of infection seems to imprint fundamental differences in the resulting effector cells, shaping their differentiation pathway and survival potential. Similarly, intranasal and intravenous/subcutaneous inoculations of group A *Streptococcus pyogenes* induce antigen-specific Th17 and Th1 cells, respectively [355]. It has been demonstrated that IL-23 is produced in larger quantities by intestinal APCs,

whereas IL-12 is more highly expressed by APCs from the periphery, such as the spleen [356]. The enrichment of Th17 cells in the mucosal associated lymphoid organs, compared to other tissues, is likely, at least in part, determined by the type of pro-inflammatory cytokines resident APCs make. In the case of *Francisella trularensis* infection, the intranasal and intradermal routes favor Th17 and Th1 responses, respectively. The skewed responses correlates with enhanced prostaglandin E_2 production during intranasal infection, and upon blockade of its production, the Th1 response is partially restored [357]. The route of infection is also critical for determining which pathways provide protective immunity. Using *Yersinia enterocolitica*, DePaolo *et al.* have shown that oral infection promotes Th17 immunity, whereas systemic infection promotes Th1 immunity. Interestingly, TLR1 is specifically involved in inducing Th17-mediated protective immunity during oral infection, resulting from the TLR2/TLR1-induced IL-6/IL-23 and the presence of TGF-β in the gut environment. TLR2/TLR1 is however not required for Th1 immune responses during systemic infection [358].

The concept of tissue specific immune regulation has important implication for human health, especially vaccine design. It is important to gain deeper insights into how the route of immunization affects the immune response, and ultimately long-term protection. The rational design of a vaccine should consider the differentiation pathways the vaccine engages, and the desired type, longevity, homing pattern, and immune effectors. More knowledge on tissue specific immune regulation would lead to better selection the vaccination route and improve the efficacy of vaccination.

Role of IL-1 in Th17 differentiation and effector function

The role of IL-1 in Th17 differentiation has been less clear. Some studies suggest that IL-1 is not a differentiation factor, but enhances IL-17 production [359]. Others have argued that IL-1 indeed contributes to the differentiation of Th17 cells [79]. *In vitro*, IL-1 combined with TGF-β is not sufficient to drive Th17 differentiation, although it can enhance Th17 differentiation when a STAT3 activator – IL-6, IL-21 or IL-23 – is present along with TGF-β [124]. In addition, I have found that IL-6 in combination with TGF-β can lead to comparable Th17 polarization in IL-1R1 KO T cells and WT T cells (data not shown). Neither is Th17 polarization blocked by recombinant IL-1 receptor antagonist (data not shown). These data suggest that at least *in vitro*, IL-1 merely enhances, but is dispensable for, Th17 differentiation. However, IL-1 seems necessary *in vivo* for generating Th17 cells, as IL-1R-deficient mice develop defective Th17 response and are resistant to EAE [138]. Moreover, another reported has found that microbiota-induced IL-1β is critical for the development of steady-state Th17 cells in the intestine [336]. When analyzing the requirement of IL-1 and IL-1R signaling for Th17 differentiation, I have obtained contradicting results between ELISA and intracellular flow cytometry staining. Specifically, when ELISA is performed following TCR restimulation, IL-1R KO or MyD88 KO T cells fail to secrete IL-17. However, when intracellular staining is utilized following PMA/ionomycin treatment, which by passes TCR engagement and directly targets downstream TCR signaling effectors PKC and Ca^{2+}, IL-1R KO and MyD88 KO T cells contain Th17 populations comparable to WT T cells (Data not shown. Similar results have been observed in [282, 336, 360, 361]). Furthermore, data from our lab show that RORγt+ expression by memory $CD4^+$ T cells from IL-1R KO and MyD88 KO mice is unaltered.

Collectively, these data suggest that IL-1 is not required for the differentiation of Th17 cells, but is indispensable for the execution of their effector functions during reactivation through the TCR. When PMA/ionomycin is used to activate the cells for intracellular staining, the need for IL-1 is bypassed. Therefore, one has to be careful when assessing the contribution of IL-1 to Th17 development and effector function, as the assay used may impact the interpretation of the results.

TLR signaling-mediated acute NLRP3 inflammasome activation and its role in early host defense

Unique role of IRAK-1 in nucleating the inflammasome complex

Previous studies on the NLRP3 inflammasome have been designed based on the "two signal" model where TLRs and NLRP3 function in a stepwise fashion. First, TLRs "prime" the inflammasome pathway by inducing the expression of the inflammasome component NLRP3 and the caspase-1 substrate IL-1β and IL-18. This priming phase requires NF-κB activity as well as the generation of ROS [211, 220, 331]. Once NLRP3 and IL-1β/IL-18 are upregulated, a second signal – ATP, toxins, crystals, etc. – is sufficient to activate NLRP3, and TLR signaling no longer plays a role at stage. My data, however, suggests a direct role of the TLR signaling pathway in activating NLRP3. When TLRs and NLRP3 are simultaneously activated, a unique form of inflammasome activation is triggered. This acute form of inflammasome activation differs from the traditional inflammasome activation pathway, where

TLRs and NLRP3 are engaged sequentially. It functions independent of new protein synthesis, consistent with the fact that it is independent of NF-κB activity and ROS generation. Further dissection of the TLR pathway using genetic deficient and knock-in mice reveals that the TLR signal transducer IRAK-1, as well as its kinase activity, are specifically required for the acute inflammasome pathway.

The precise mechanism by which IRAK-1 regulates inflammasome still remains elusive. Previous studies have shown that *Helicobacter pylori* LPS primed macrophages contain an activity capable of phosphorylating caspase-1 at a particular serine residue [362]. Most recently, NLRC4 inflammasome has been shown to depend on a specific serine phosphorylation event of the NLRC4 molecule for its activation [232]. PKR, a serine/threonine kinase known to be involved in anti-viral response, has also shown to be required during inflammasome activation and its kinase activity is critical for inflammasome activation [230]. ASC phosphorylation mediated by Syk and JNK has been demonstrated to be essential for activation of the NLRP3 and AIM2 inflammasomes [363]. These results suggest that serine/threonine phosphorylation of inflammasome components may be a key step for inflammasome activation.

IRAK-1 and IRAK-4 differs from IRAK-2 in their kinase activity (Figure 5.3). Disrupting IRAK-1's kinase activity, using IRAK-1 kinase inactive knock-in alleles, reduces acute inflammasome activation. It would be of great interest to identify the substrate for IRAK-1 necessary for inflammasome activation. On the other hand, disrupting IRAK-4's kinase activity using IRAK-4 kinase inactive knock-in alleles, which simultaneously prevents the gain of kinase activity of IRAK-1, completely blocks acute inflammasome activation, indicating

that IRAK-1 and IRAK-4 share similar substrates, and play redundant roles in mediating the phosphorylation of downstream targets.

The defect in acute inflammasome activation in IRAK-1 KO macrophages is more severe than that in IRAK-1 KD macrophages, suggesting IRAK-1 has kinase-independent functions in facilitating inflammasome activation. IRAK-1 is the longest member of the IRAK-1 family. Comparison of the domain structure between IRAK-1, IRAK-2 and IRAK-4 shows that IRAK-1 possesses a unique C-terminus, which contains a number of interaction motifs that mediate the interaction between IRAK-1 and downstream molecules in the TLR signaling pathway, such as TRAF6 (Figure 5.3). A previous study has shown that IRAK-1 has the ability to interact with the pyrin domain of NLRP12 [364]. It is possible that the C-terminus of IRAK-1 also contains critical motifs that mediate the interaction between IRAK-1 and NLRP3 inflammasome components.

Role of TRAF6 and TAK-1 in acute inflammasome activation

TRAF6 and TAK-1 have been well documented for their role in activating NF-κB and inducing NF-κB-dependent genes [365]. However, how they contribute to acute inflammasome activation remains to be elucidated. In addition to NF-κB activation, TAK-1 is also responsible for activating MAPKs [23]. Since the MAPK JNK has been shown to mediate ASC phosphorylation and priming-dependent inflammasome activation, it would be worthwhile to test whether JNK and ASC are involved in a similar fashion for acute inflammasome activation. TRAF6 is an E3 ubiquitin ligase necessary for the activation of

TAK1. Given that TAK1 is required for acute inflammasome activation, it is not surprising that TRAF6 is required, too. However, TRAF6 also has TAK1-independent functions, which could also be important in inflammasome activation. A recent study has shown that K63 linked polyubiquitination of caspas-1 is essential for priming-dependent inflammasome activation [366]. It is possible that similar K63-polyubiquitination takes place in acute inflammasome activation as well and it would be interesting to test if TRAF6 in involved in synthesizing the K63 polyubiquitin chain on caspase-1.

Acute inflammasome activation is particularly important for early host protection at the pathogen entry sites

Although multiple TLR signaling molecules are required for acute inflammasome activation, including MyD88, IRAK-4, IRAK-1, TRAF6 and TAK-1, IRAK-1 has a unique advantage for probing the *in vivo* consequence of the acute inflammasome pathway. IRAK-1 plays largely redundant roles with IRAK-2 in TLR and IL-1R/IL-18R signaling pathways, in which IRAK-1 deficiency only leads to a mild defect [367]. This allows me to specifically test the physiological function of the acute inflammasome pathway *in vivo*, using whole body IRAK-1 KO mice or transfer of IRAK-1 KO T cells, without significantly compromising the TLR/IL-1R/IL-18R pathways. The priming-dependent model of NLRP3 inflammasome activation can be useful to explain the late events during pathogen encounter, when macrophages have been primed by the microbial ligands in the pathogen through TLRs. However, during the initial phase of an infection prior to new gene induction, or when new

protein synthesis is hijacked by the pathogen, whether the host is able to mount protective immunity is unclear. My data have shown that simultaneous activation of TLRs and NLRP3 early on during infection is sufficient to trigger a unique set of host defense programs that would lower the pathogen burden and promote pathogen clearance.

Pyroptosis as a mechanism for host defense has been implicated in several infection models, such as *Escherichia coli*, *Legionella pneumophila, Burkholderia thailandensis*, and *S. typhimurium* [329, 368]. The intracellular niche within the macrophage permits the intracellular bacteria to continue a replicative cycle, and eventually being released in greater numbers. When the intracellular pathogens are detected by the inflammasome, resulting in caspase-1 activation and pyroptosis, the pathogen can be released from the macrophage prior to replication. Released bacteria are subsequently exposed to additional clearance mechanisms, such as phagocytosis and destruction by neutrophils [369]. My data clearly show that *Listeria monocytogenes* can trigger IRAK-1-dependent acute inflammasome activation and pyroptosis *in vivo*. However, the contribution of pyroptosis to host defense mechanism against *Listeria* has not been directly analyzed, which could be subjected to future investigation.

Caspase-1 activation *per se* can happen without the induction of new protein. This has been widely accepted for the NLRP1 and NLRC4 inflammasomes. I have presented evidence here that activation of the NLRP3 inflammasome can also occur in the absence of new protein synthesis, as is seen in acute inflammasome activation regulated by TLR signaling via IRAK-1. Many macrophages at the barrier sites of the body contain presynthesized IL-1 family members, which are ready to be cleaved by caspase-1 and secreted. For instance, red pulp macrophages and lung resident macrophages constitutively express IL-18, whereas intestine

resident macrophages constitutively express IL-1β (Figure 5.4). Acute inflammasome activation in these cells would allow their respective IL-1 family cytokine deposition to be immediately processed and released, thereby allowing rapid response to invading pathogens at their primary entry sites. I have shown that IL-18 secretion triggered by blood borne *Listeria*, likely from red pulp macrophages, induces innate IFN-γ production from $CD8^+$ T cells and NK cells. I have also shown that small intestine macrophages are capable of triggering the acute inflammasome activation pathway, and release presynthesized IL-1β. IL-1β, together with IL-23, can promote IL-17 production from NKT cells [370], γδT cells [371], and type 3 innate lymphoid cells [328]. Whether this is essential for host defense against oral pathogens remains to be investigated.

Concluding remarks and future perspectives

Traditionally, we have gained most of our knowledge about the immune system by analyzing cells from the spleen and LNs, as a result of the convenience to obtain large number of cells. The work I have presented here, together with works from other labs, however, have highlighted the importance of tissue-specific microenvironments in the regulation of immune responses. Tissue-specific factors play unique roles in generating different types of immune responses, recruiting different immune cells, as well as dictating different immune regulatory pathways. Studies that introduce pathogens directly into the circulation to cause systemic infection may overlook the complexity imposed by the local microenvironment, and could miss valuable information regarding how the immune responses are shaped by non-immune

cells in various tissues and commensal-derived factors. A better knowledge of these information would be particularly important for understanding host defense against cutaneous and mucosal pathogens, which could lead to rational design of better vaccines and therapies. The tissue specific regulation of Th17 development I have presented here is only an example of many tissue specific immune regulation pathways. Future studies will likely reveal more tissue-specific modes of immune regulation, ranging from innate immune responses to effector T cell development and memory formation.

Human pathogens such as *Listeria monocytogenes*, *Salmonella typhi*, *Staphylococcus aureus* and *Streptococcus pneumonia* can be detected by the NLRP3 inflammasome, which by mouse studies have been shown to be essential for host defense. The acute NLRP3 inflammasome pathway we discovered is particularly important for early host response to invading pathogens. Understanding how it impacts innate and adaptive immunity will likely provide new therapies aiming for enhanced host defense against these pathogens. Additionally, increasing evidence have suggested an important role of inflammasome and inflammasome derived cytokines in driving autoimmune and inflammatory diseases. Anakinra (IL-1 receptor antagonist) has been widely used in the treatment of these diseases. On the other hand, polymorphisms in the IRAK-1 gene has been shown to associate with disease susceptibility to SLE [372], rheumatoid arthritis [373, 374], and sepsis [375, 376]. If IRAK-1 mediates similar acute inflammasome activation in humans as it does in mice, IRAK-1 inhibition could provide additional strategies for treating these diseases. This will be particularly important for patients suffering from unresponsiveness and side effects of other currently available treatments.

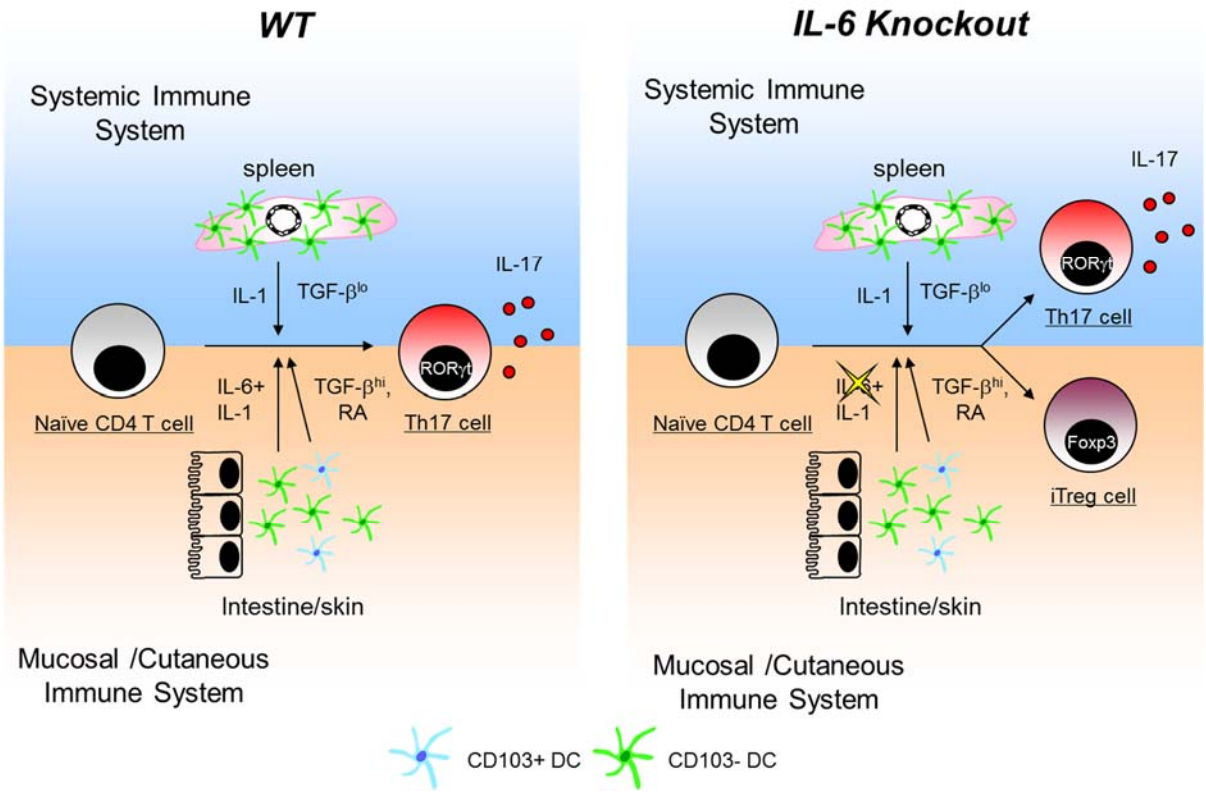

Figure 5.1. Tissue-specific role of IL-6 in Th17 differentiation.

In the systemic immune system (upper), which lacks the $CD103^+$ DCs, IL-6 is not required but IL-1 is important for generating Th17 cells. In the mucosal and cutaneous immune systems (lower), the $CD103^+$ DCs produce TGF-β and retinoic acid (RA) to inhibit Th17 differentiation and favor immune regulation. IL-6 is necessary to overcome the inhibitory effect of TGF-β and RA, and is therefore required for Th17 differentiation in the mucosal and cutaneous immune systems. In the absence of IL-6 (right), the systemic immune system can still generate Th17 cells, but the mucosal/cutaneous immune systems fail to do so but instead generate iTreg cells as a result of TGF-β and RA signals from $CD103^+$ DCs.

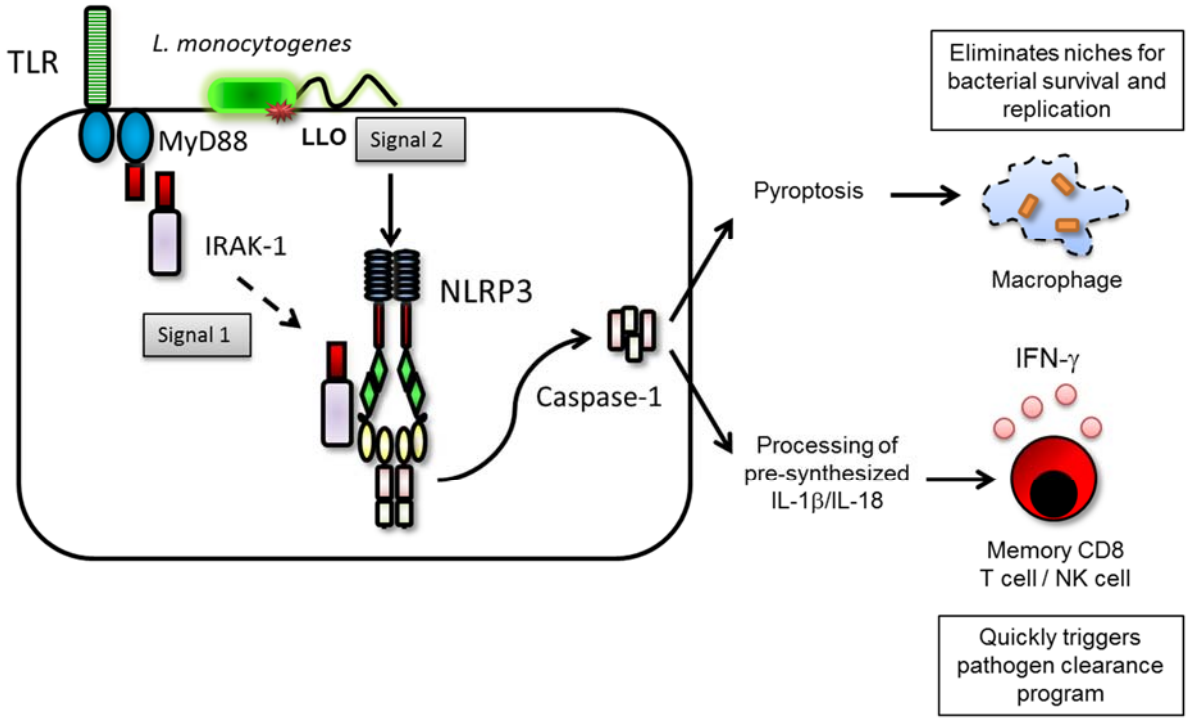

Figure 5.2. IRAK-1 dependent acute NLRP3 inflammasome activation promotes host defense to *L. monocytogenes*.

Upon *L. monocytogenes* infection, TLRs and NLRP3 are simultaneously engaged by microbial ligands and bacterial toxin (Listeriolysin O, LLO), respectively. This combined recognition leads to association of the TLR signaling protein IRAK-1 with the NLRP3 inflammasome complex and induces its rapid activation, which renders activation of caspase-1 and subsequent host defense mechanisms: pyroptosis eliminates the niches for bacterial survival and replication; processing/secretion of pre-synthesized IL-1β/IL-18 quickly triggers pathogen clearance program in neighboring immune cells. These responses are critical for lowering the pathogen burden and promote host defense during the early phase of infection.

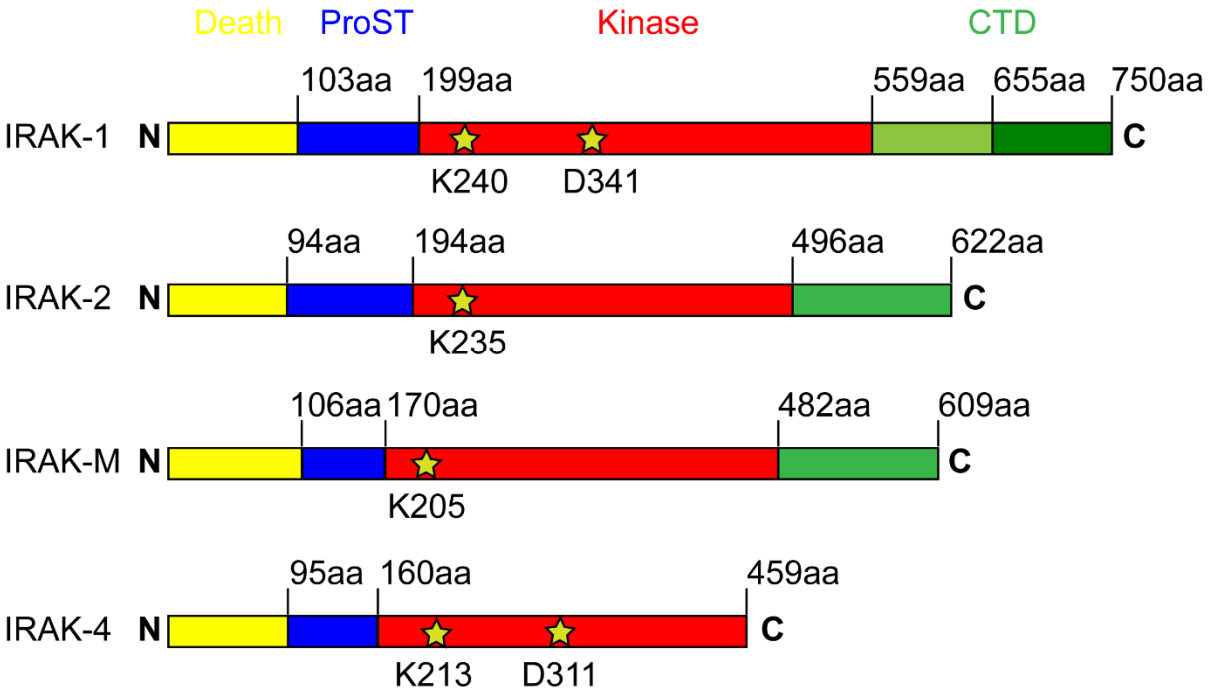

Figure 5.3. Domain structure of mouse IRAK family members.

Each member has a death domain, a proST domain, a conserved kinase domain, and, with the exception of IRAK-4, a C terminal domain. The proST domain has been shown to be vital for autophosphorylation. The invariant lysine and aspartate residues within the kinase domain (marked by stars) are critical for IRAK kinase function. Due to the lack of the critical aspartate residue, IRAK-2 is believed not to have kinase activity.

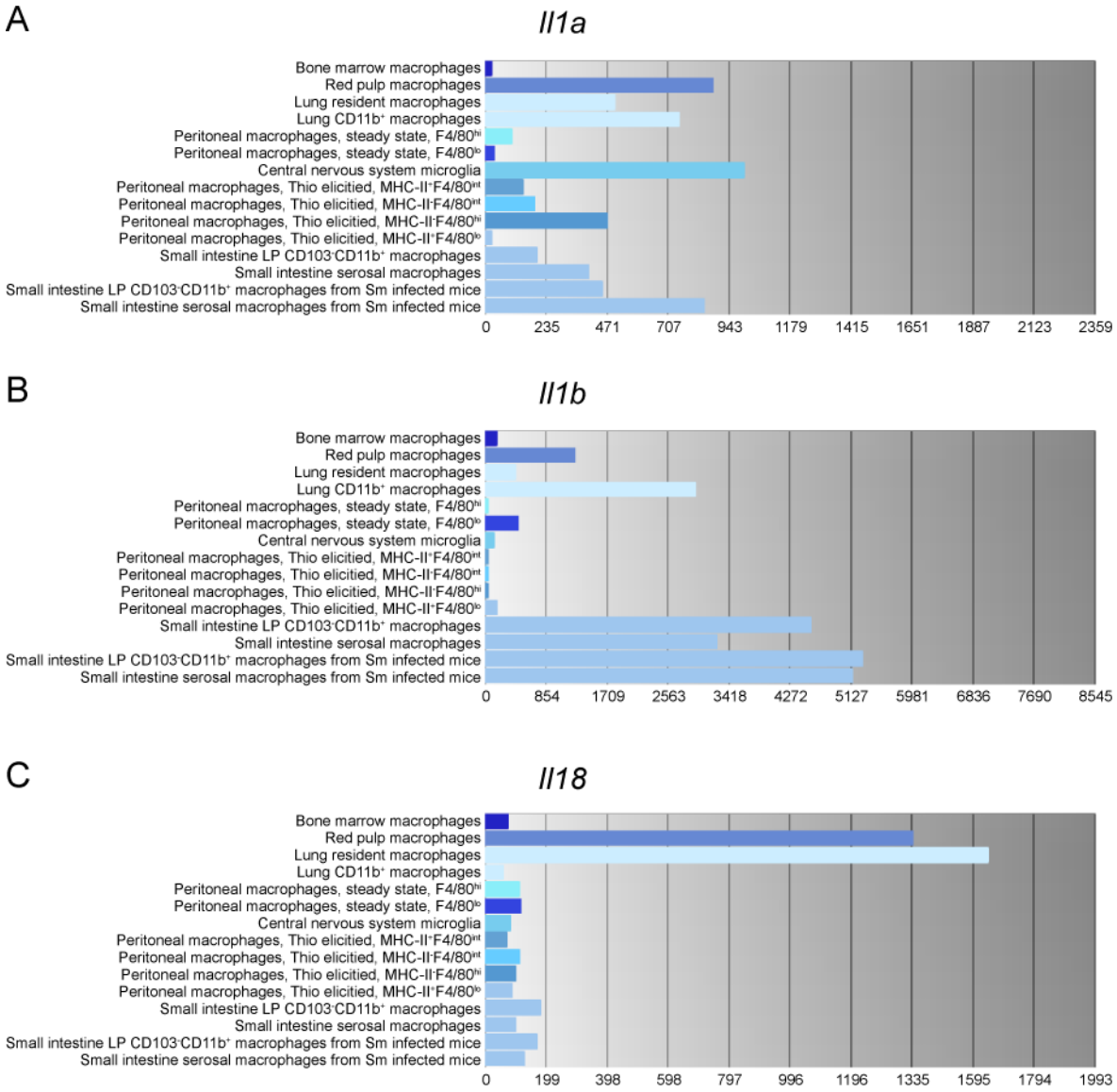

Figure 5.4. Expression level of IL-1 family cytokines in different macrophage populations.

Data from the Immunological Genome Project (http://www.immgen.org). The x axes represent normalized expression values obtained using hybridization to the Affymetrix 1.0 ST microarray platform.

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