REGULATION OF INFLAMMATION DURING *PSEUDOMONAS AERUGINOSA* LUNG INFECTION: A ROLE OF AIM2 AND RCAN1

by

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that is an important cause of infection among immunocompromised individuals. *P. aeruginosa* chronically infects cystic fibrosis patients, leading to declined pulmonary function and increased morbidity and mortality. Host innate immune system employs a variety of pattern recognition receptors (PRRs) to detect the conserved molecular patterns on invading *P. aeruginosa*. In healthy individuals, activation of these PRRs triggers a complex of downstream signalling pathways that induce production of various proinflammatory cytokines and efficiently eliminates *P. aeruginosa* from the host. However, dysregulation of these signalling pathways increases the susceptibility of the host to bacterial infection. Herein we identify that AIM2 inflammasome, an intracellular dsDNA sensor, is functionally redundant in IL-1β production and caspase-1 activation during *P. aeruginosa* lung infection. Furthermore, we reveal regulator of calcineurin-1 as a novel negative regulator of the TLR-MyD88-dependent signalling pathway in a mouse model of acute pneumonia using *P. aeruginosa* lipopolysaccharide. These findings improve our understanding of the molecular mechanisms of host defense against *P. aeruginosa* in innate immunity.

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CHAPTER 1: INTRODUCTION

1.1 Cystic fibrosis

Cystic fibrosis (CF) is a genetic disorder most common in the Caucasian population, approximately 1 in 2500 individuals. This disease affects a variety of organs including the lung, pancreas, liver, and intestine, and is characterized by a mutation in the cystic fibrosis transmembrane conductance receptor (CFTR) gene (1). The most frequent mutation in CFTR is a deletion of ΔF508, which causes defective transportation of chloride. Dysfunctional CFTR results in abnormally high levels of sodium ions in epithelial cells and dehydration of the mucus layer on the cell surface (2). The dehydrated mucus in the airway surface is thick and sticky, providing a perfect habitat for colonization and propagation of bacteria, such as *Staphylococcus aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa*. Among these bacteria, *P. aeruginosa* is the major pathogen in CF patients (3). Furthermore, the thick sticky mucus on the epithelium cell surface leads to formation of bacterial biofilm that increases antibiotic resistance of bacteria and reduces the rate of phagocytosis by immune cells (2, 4).

1.2 *Pseudomonas aeruginosa*

P. aeruginosa, an opportunistic Gram-negative bacterium, is an important cause of infection among immunocompromised individuals (5). *P. aeruginosa* chronically infects CF patients, leading to declined pulmonary function and increased morbidity and mortality (2). Airway infection with *P. aeruginosa* in healthy individuals triggers strong

inflammatory responses including production of various cytokines and chemokines, which leads to neutrophil recruitment and effective clearance of the bacteria (6).

1.2.1 *P. aeruginosa* colonization and biofilm formation

P. aeruginosa is normally found in environmental sources, such as water and soil. It typically infects airways, the urinary tract, bloodstream, skin, eyes and ears. Chronic infection with *P. aeruginosa* associates with declined pulmonary function in CF patients, and the treatment of this infection is challenging due to poor prognosis and antibiotic resistance (2). *P. aeruginosa* employs a variety of strategies that promote chronic colonization in the lungs, including biofilm formation, evasion of the host immune system, and inhibition of phagocytosis and induction of immune cell apoptosis (6). *P. aeruginosa* pili and flagella are two major bacterial adhesins that ensure efficient mobility and adhesion in the CF airways. Moreover, both of them are required for the biofilm formation. Biofilm is defined as communities of microbes embedded within an extracellular polymeric substrate (EPS) composed of nucleic acids, proteins, polysaccharides and lipids, and it greatly increases the difficulty of *P. aeruginosa* antibiotic treatment (4).

The strains of *P. aeruginosa* isolated from chronically infected CF lungs display a mucoid phenotype, which produces a large amount of alginate exopolysaccharide that protects *P. aeruginosa* from host factor killing and promotes the persistence of infection (7, 8). In addition, the mucoid strains of *P. aeruginosa* lack mobility and express modified lipopolysaccharide (LPS). These two characteristics enable *P. aeruginosa* to evade host immune system detection. For example, down-regulated expression of

flagellin, a component of flagella, in mucoid *P. aeruginosa* reduces the Toll like receptor 5 (TLR5)-mediated inflammatory responses, and the modified LPS structure is poorly recognized by TLR4 (6).

1.2.2 *P. aeruginosa* exotoxins

Like many other pathogenic Gram-negative bacteria, *P. aeruginosa* uses a type III secretion system (T3SS) which has a needle structure to directly inject cytotoxic effector proteins into the cytoplasm of host cells (9). The T3SS is a major virulence determinant of *P. aeruginosa* and has been implicated in the pathogenesis of acute infections. Four T3SS effectors, exoenzyme U (ExoU), ExoS, ExoT, and ExoY, have been identified in *P. aeruginosa*. *P. aeruginosa* ExoU is a potent phospholipase A_2 that causes a rapid cytolytic cell death and inhibits inflammasome activation (10, 11). ExoS and ExoT are bifunctional enzymes that possess a GTPase activating protein (GAP) activity targeting on Rho GTPases on the N-terminal domain and an ADP-ribosyltransferase activity on the C-terminal domain (12). Both ExoS and ExoT are able to inhibit phagocytosis of *P. aeruginosa* during pulmonary infection and induce apoptosis of immune cells (13-15). ExoY, an adenylate cyclase, impairs endothelial cell proliferation and repair following lung injury (16).

1.3 Pattern recognition receptors initiate host immune responses to *P. aeruginosa*

The innate immune system is the first line of defense against invading microorganisms. The early host defence against *P. aeruginosa* is predominately mediated by resident immune cells such as macrophages, mast cells and dendritic cells (17-19). The

immune cells utilize pattern-recognition receptors (PRRs) to sense the conserved molecular patterns on pathogens, initiating immune responses (20). Two major PRRs, Toll-like receptors (TLRs) and the nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs), have been identified to recognize *P. aeruginosa* in the past decades (11, 21, 22).

1.3.1 Toll-like receptors

The TLRs play an essential role in activation of innate immunity by binding to the pathogen-associated molecular patterns (PAMPs) (23). They are glycoprotein receptors comprised of an extracellular domain for ligand binding, a transmembrane domain and an intracellular domain for signalling (24). TLRs are located on cell membrane or intracellular vesicle membranes, and a total of 12 functional TLRs have been identified in animals (23, 25). TLR2 is involved in recognition of a broad range of microbial cell-wall components including peptidoglycan, lipoprotein and lipoteichoic acid (26, 27). The ligand specific recognition by TLR2 requires formation of a heterodimer with TLR1 or TLR6 (28). TLR3 functions as a dsRNA sensor important for detection of various viruses (29). TLR4 is able to recognize LPS, an outer membrane protein of Gram-negative bacteria (30). TLR5 is activated by flagellin, a conserved protein that forms bacterial flagella (31). TLR7 and TLR8 are structurally related, and both of them can recognize guanosine or uridine-rich single-stranded RNA (ssRNA) from viruses (32, 33). TLR9 is responsible for detecting unmethylated CpG motifs in bacterial DNA. The CpGs are highly methylated in mammalian genome, and methylation of a cytosine residue within the bacterial CpG motif would abrogate the immunostimulatory activity (34). TLR11 is

capable of binding to a profilin-like protein identified from *Toxoplasma gondii* and uropathogenic *Escherichia coli* (35). TLR1, -2, -4, -5, -6 and -9 have been found to be involved in the recognition of *P. aeruginosa in vivo* and *in vitro* (36-39). Activation of TLRs triggers two distinct downstream signaling pathways, the myeloid differentiation primary-response protein 88 (MyD88) pathway and the Toll/IL-1R domain-containing adaptor protein inducing interferon-β (TRIF) pathway (23). The MyD88 pathway mediates NF-κB activation, leading to proinflammatory gene expression, including IL-1β, IL-6, TNF-α. The TRIF pathway induces IFN regulatory factor (IRF) 3 and IRF7 activation, leading to IFN-α and -β expression (40).

1.3.1.1 Recognition of *P. aeruginosa* by TLRs

1.3.1.1.1 TLR2

The *P. aeruginosa* lipoproteins and cytotoxic protein ExoS have been reported to be the TLR2 ligands (21). TLR2 forms a heterodimer with either TLR1 or TLR6 to ensure specific recognition of ligands (28). Overproduction of alginate in the mucoid phenotype of *P. aeruginosa* is associated with increased expression of lipoproteins, which are potent agonists of TLR2 signaling (41). Moreover, *P. aeruginosa* infection results in upregulation of TLR2 expression (42). During the *P. aeruginosa* respiratory infection, TLR2 was found to contribute to activation of p38 mitogen-activated protein kinase (MAPK), an important inflammatory mediator, in addition to TLR5 (43). A study showed that *P. aeruginosa* lipoprotein I (OprI) can bind to both TLR2 and TLR4 of dendritic cells and subsequently enhance T cell activation and IFN-γ production (44). TLR2 is also able to recognize *P. aeruginosa* slime-glycolipoprotein (slime-GLP) an extracellular glycolipid

component produced by mucoid and nonmucoid strains. Activation of TLR2 by *P. aeruginosa* slime-GLP triggers TNF-α production and NF-κB activation in human monocytes (45). The *P. aeruginosa* virulence factor, ExoS, has been found to induce proinflammatory cytokine production, and activation of TLR2 is mediated by its C-terminal domain (46). However, in a murine model of acute *P. aeruginosa* pulmonary infection, TLR2-deficient ($TLR2^{-/-}$) mice had partially reduced cytokine production but the bacterial clearance and neutrophil recruitment in lung were not impaired and the mortality was not affected (38, 47, 48). It implies that TLR2 may be redundant in the host immune response to *P. aeruginosa* lung infection.

1.3.1.1.2 TLR4

LPS, a major outer membrane component of Gram-negative bacterium, triggers potent inflammatory responses through activation of TLR4 (30, 49). LPS consists of an O-antigen, a core oligosaccharide and a lipid A. *P. aeruginosa* expresses many types of LPS due to the different variety of lipid A, and the specific recognition of LPS by TLR4 depends on its structure of lipid A (49). A 82-amino acid hypervariable region in the extracellular domain is responsible for LPS recognition. The environmental strains of *P. aeruginosa* synthesize a penta-acylated lipid A, whereas the strains isolated from CF patients produce a hexa-acylated lipid A. Moreover, the hexa-acylated lipid A is more readily to be recognized by human TLR4, but not murine TLR4, and triggers stronger proinflammatory responses than penta-acylated lipid A. (50, 51).

Recognition of LPS by TLR4 requires accessory proteins, LPS binding protein (LBP) and CD14 (28). LPS is first bound to the serum protein LBP, and then transferred to

CD14. CD14 can be expressed as a glycosyl-phosphatidylinositol (GPI)-linked receptor on the surface of myelomonocytic cells or a soluble protein in serum. After binding of LPS by CD14, LPS is presented to a TLR4–MD-2 complex (52, 53). MD-2 is a cell surface protein associated with TLR4 to form a heterodimer that mediates LPS recognition. MD-2 deficiency leads to impaired TLR4 activation by LPS and reduced cytokine production *in vitro* (54).

TLR4 has been shown to play an essential role in host defense against *P. aeruginosa* pulmonary infection (48, 55). TLR4-deficient ($TLR4^{-/-}$) mice display reduced cytokine production and neutrophil recruitment but unaffected bacterial clearance in lung during wild-type *P. aeruginosa* infection. Additionally, the $TLR4^{-/-}$ mice infected with the flagellin mutant strains manifest more severe impairment in cytokine production (48). Consistent with this finding, another study showed that mice with a deletion of both TLR2 and -4 are more susceptible to challenge with the *P. aeruginosa* strain lacking of flagellin compared with the wild-type strain infection (47, 55). These findings suggest that TLR4 is required to function together with TLR5 to effectively clear *P. aeruginosa* from the host.

1.3.1.1.3 TLR5

Flagellin, a major component of bacterial flagella, has been implicated in acute *P. aeruginosa* pneumonia (56, 57). Flagella facilitate *P. aeruginosa* mobility and attachment on host cells. The environmental strain of *P. aeruginosa* is flagellated, whereas the mucoid strains of *P. aeruginosa* isolated form CF patients usually lack flagella, which allows *P. aeruginosa* to evade immune system detection and resist

phagocytosis (6). *P. aeruginosa* flagellin is able to activate the TLR signaling in alveolar macrophages and airway epithelial cells, and the recognition of flagellin is mediated by TLR5 (31, 37, 58). A 10-amino-acid region (aa 88 to 97) in the N-terminal domain of *P. aeruginosa* flagellin is critical for binding to TLR5, and mutations within this region disrupt the TLR5-flagellin interaction, as well as inflammatory responses and bacterial mobility (59).

As mentioned previously, a study shows that the cytokine production and neutrophil recruitment in TLR4$^{-/-}$ or TLR2/4$^{-/-}$ mice were more severely impaired when infected with flagellin-deficient *P. aeruginosa* than that when infected with wild-type *P. aeruginosa*. This indirectly suggests that TLR5 contributes to the MyD88-dependent responses during *P. aeruginosa* pulmonary infection (48). Another study used a TLR5-deficient (TLR5$^{-/-}$) mice model to directly investigate the role of TLR5 in *P. aeruginosa* pneumonia. Following airway infection with an environmental strain of *P. aeruginosa* PAK, the TLR5$^{-/-}$ mice showed diminished early cytokine secretion and delayed neutrophil recruitment. Moreover, the role of TLR5 in clearance of *P. aeruginosa* from the lung was found to be inoculum-dependent. Bacterial clearance is impaired at low-inoculum but not high-inoculum of *P. aeruginosa* (57). In addition, TLR5 is also involved in phagocytosis and destruction of *P. aeruginosa* by alveolar macrophages. Non-flagellated *P. aeruginosa* or loss of TLR5 reduces phagocytosis by alveolar macrophages. TLR5 is able to mediate IL-1β production during wild-type *P. aeruginosa* infection, which is important for endosomal acidification and bacterial killing (60).

1.3.1.1.4 TLR9

TLR9 is localized on intracellular vesicle membranes, and functions intracellularly to detect unmethylated CpG motifs in bacterial DNA (34). The unmethylated CpG motif has a potent immunostimulatory activity. In mammalian genomes, CpG motif is highly methylated, leading to complete abrogation of the immunostimulatory activity (61). Unlike other TLRs, TLR9 plays an adverse role in host defense against pulmonary infection. A paper by Benmohamed et al. shows that TLR9-deficient ($TLR9^{-/-}$) mice significantly resist lethal lung infection by *P. aeruginosa* compared to wild-type mice, and the TLR9 deficiency is associated with increased proinflammatory cytokine and nitric oxide production in alveolar macrophages (62). Production of IL-1β and nitric oxide are critical for clearance of *P. aeruginosa* from the lung. Furthermore, they identified that TLR9 activation down-regulates LPS-induced NF-κB activation, suggesting that TLR9 activation may suppresses TLR4 signaling. However, the molecular mechanisms involved in this down-regulation are still under investigation.

1.3.1.2 TLR signaling

After binding to specific ligands, TLRs trigger diverse signal transduction pathways that induce expression of various genes (Figure 1.3.1). The first signalling pathway is MyD88-denpendent. All TLRs except TLR3 use MyD88 as an adaptor protein. TLR5 can directly recruit MyD88 but TLR 2, -4 and -9 require a second adapter protein toll-interleukin 1 receptor (TIR) adapter protein (TIRAP) for MyD88 recruitment. MyD88 recruits IL-1 receptor-associated kinase 1 (IRAK1) and IRAK4 to TLR. IRAK1 is activated through phosphorylation, and then associates with TNF receptor-associated

factor 6 (TRAF6), leading to activation of two distinct signaling pathways. One pathway activates a downstream protein complex consisting of TGF-β-activated kinase 1 (TAK1), TAB1 (TAK1-binding protein 1), and TAB2. TAK1 couples to the IκB kinase (IKK) complex, leading to phosphorylation and degradation of the inhibitory protein IκB. This phosphorylation results in the dissociation of IκB from NF-κB. The liberated NF-κB then translocates into the nucleus and activates the NF-κB–dependent proinflammatory gene expression. Another pathway is the activation of mitogen activated protein kinases (MAPK) family, including p38, ERK, and JNK. The MAPKs migrate into the nucleus and activate a variety of transcriptional factors, mediating cell growth, apoptosis and inflammation (21). The MyD88-dependent signaling pathway is essential for early host defense against *P. aeruginosa* lung infection. In the early stage of *P. aeruginosa* infection, MyD88-deficient mice display impaired neutrophil recruitment, proinflammatory cytokine production and bacterial clearance (38). However, the MyD88-deficient mice are able to eventually clear off *P. aeruginosa* from the lung by producing significant amounts of cytokines and recruiting sufficient neutrophils (63). Activation of TLR4 also triggers a MyD88-independent signaling pathway, which recruits the adapter molecule TRIF to TLR-4 with the assistance of a second adaptor molecule TRIF related adapter molecule (TRAM). TRIF is capable of activating TRAF6 and inducing a delayed NF-κB activation. Most importantly, TRIF-dependent signaling can activate interferon regulatory factors IRF-3 and IRF-7, which regulate IFN-α and -β expression (21). TRIF deficiency leads to markedly diminished clearance of *P. aeruginosa* from lung associated with delayed neutrophil recruitment and partly impaired production of proinflammatory cytokines, including RANTES, KC, and TNF (64).

1.3.2 Inflammasome

Another inflammatory pathway is the cleavage and activation of caspase-1, which is initially produced as an inactive form that requires proteolytic cleavage to become active. This process is controlled by a multiprotein complex termed inflammasome (65). Inflammasome can be formed by members of NLR family or the pyrin and HIN domain-containing protein (PYHIN) family absent in melanoma (AIM2). NLRs and AIM2 are cytoplasmic pattern recognition receptors (PRRs) that detect microbial molecules and endogenous danger signals as intracellular sensors (66). NLR family proteins share a tripartite structure consisting of a C-terminal leucine-rich repeats (LRRs) that are responsible for sensing pathogen molecules, a central NACHT domain that mediates self-oligomerization, and a N-terminal effector domain composed of a caspase activation and recruitment domain (CARD) or a Pyrin domain (PYD) or a baculovirus inhibitor of apoptosis protein repeat (BIR) domain (67, 68). Based on the different structure of N-terminal effector domain, NLRs are divided into subfamilies. The NLR family CARD domain-containing protein 4 (NLRC4) uses its N-terminal CARD to directly recruit pro-caspase-1 through CARD-CARD interaction, whereas the NLR subfamily with N-terminal PYD domain (NLRP) proteins and AIM2 employ a PYD domain at the N-terminal to interact with the PYD domain of an adapter protein ASC (Apoptosis associate speck-like containing a CARD domain) that recruits pro-caspase-1 via CARD-CARD interactions (67). The NAIP subfamily members contain BIR at their N-terminal. Although lacking PYD or CARD domains, NAIP can indirectly activate caspase-1 by interacting with NLRC4 to form an oligomeric protein complex (69). Proteolysis of pro-

caspase-1 results in the generation of two characterized subunits, P20 and P10. The activated caspase-1 is responsible for cleaving two proinflammatory cytokines, interleukin (IL)-1β and IL-18, which are also synthesized as inactive pro forms upon TLR pathway activation in response to inflammatory stimuli (70) (Figure 1.3.2). IL-1β is a key proinflammatory cytokine that plays a critical role in mediation of innate and adaptive immune responses upon pathogen infection by promoting inflammatory cell recruitment, antibody production and Th17 cell generation (70, 71). Correspondingly, IL-1β deficiency leads to reduced acute-phase response, increased susceptibility to pathogen infection and impaired neutrophil recruitment (72-74). IL-18 together with IL-12 induces production of interferon-γ (IFN-γ) in Th1, natural killer (NK) and cytotoxic T cells (70, 71, 75). Moreover, the levels of IL-1β and IL-18 are significantly increased in the bronchoalveolar lavage fluid and sputum of *P. aeruginosa* lung infected cystic fibrosis patients, and murine models of acute *P. aeruginosa* lung infection (22, 76, 77).

1.3.2.1 Inflammasome induces pyroptosis

Cell death is an effective strategy to limit intracellular pathogen replication (78). Inflammasome has been shown to induce cell pyroptosis, a form of programmed cell death that is distinct from apoptosis. Pyroptosis is caspase-1-dependent. Activation of caspase-1 leads to rapid formation of pores on plasma membrane. Those pores disrupt cellular osmotic homeostasis, thus causing water influx, cell swelling and lysis ultimately (79). Pyroptosis results in not only elimination of cellular niches for bacterial replication, but also release of proinflammatory cytokines and other intracellular contents such as ATP and IL-1α to extracellular space to induce inflammation. In contrast to pyroptosis,

cell membrane integrity is normally maintained during apoptosis. Moreover, the apoptosis depends on caspase-3, caspase-6 and caspase-7, and it does not induce inflammatory consequence (78, 80, 81). In addition, to measure the level of pyroptosis induced cell death, lactate dehydrogenase (LDH) is considered to be a good indicator, which is normally maintained in cell cytosol and released from cell during pyroptotic cell death (82). Pyroptosis has been identified to play an essential role in defending against intracellular bacteria, such as *Salmonella typhimurium*, *Burkholderia pseudomalle* and *Burkholderia thailandensis* (80, 83, 84). A study by Miao and colleagues found that clearance of *S. typhimurium* does not require IL-1β and IL-18. Instead, it associates with pyroptotic cell death, which release *S. typhimurium* from macrophages to extracellular environment and then the bacteria was taken up and killed by neutrophils through reactive oxygen species (ROS) (80). However, excessive cell death, especially those mediated by inflammasome, may cause tissue damage and interfere with bacterial clearance. A study by Taylor and his colleagues demonstrate that activation of inflammasome signalling in alveolar macrophages causes impaired *P. aeruginosa* clearance and increased lung injury in the acute pulmonary infected mouse model, leading to elevated mortality. Authors applied multiple strategies to limit inflammasome activation, including using flagellin-deficient *P. aeruginosa*, deletion of NLRC4, reduction of IL-1β and IL-18, inhibition of caspase-1, all of which resulted in enhanced bacterial clearance, reduced mortality and less lung damage (22). It suggests that inflammasome activation needs to be tightly controlled, and inhibition of this excess activation is a valid way to limit the pathological consequences of acute *P. aeruginosa* lung infection.

1.3.2.2 Inflammasome in host defense against pathogen infection

Inflammasomes play an essential role for host defense against a variety of microbial infections or danger molecules by mediating IL-1β and IL-18 production. Each inflammasome recognizes specific intracellular PAMPs and serve as a platform for caspase-1 activation. NLRP1 inflammasome is required for caspase-1 activation in response to anthrax lethal toxin (LeTx), a major virulence factor of *Bacillus anthracis* (85). NLRP3 inflammasome is the most well characterized inflammasome family member due to its ability of recognizing a broad range of stimuli, including both microbial and non-microbial ligands (66). However, the molecular mechanism that triggers NLRP3 inflammasome activation remains poorly understood. NLRP6 is a new characterized member of the NLR family that is highly expressed in the intestinal tissue. It has been shown to be a negative regulator of NF-kB and MAPK signaling, and NLRP6 deficiency enhances host defense against several bacterial pathogens, including *Listeria monocytogenes, Salmonella typhimurium* and *Escherichia coli* (86). NLRP12 is a novel protein important for regulation of IL-1β and IL-18 production during *Yersinia pestis* infection (87). Moreover, NLRP12 is recently found to have similar function as NLRP6, which maintains intestinal homeostasis and prevent colorectal tumorigenesis (88). NLRC4 inflammasome is activated by bacterial flagellin or T3SS rod proteins. Activation of NLRC4 inflammasome has been identified upon several bacterial infections, including *Salmonella typhimurium, Legionella pneumophila, Klebsiella pneumoniae* and *P. aeruginosa* (11, 89-91). AIM2 is a cytosolic double-stranded DNA (dsDNA) sensor essential for innate immune responses against DNA viruses and bacteria (66).

1.3.2.3 NLRC4 inflammasome

NLRC4, also known as interleukin-converting enzyme protease-activating factor (IPAF), contains an N-terminal CARD for direct recruitment of pro-caspase-1 through CARD-CARD interaction, a central NACHT domain, and a C-terminal LRR domain (66). Although NLRC4 can recruit pro-caspase-1 directly, it requires the adapter protein ASC to facilitate caspase-1 activation in response to some bacteria, such as *S. typhimurium* and *Legionell pneumophila* (89, 92). ASC has been shown to greatly enhance the NLRC4-mediated cytokine production. However, the cell death induced by NLRC4 inflammasome activation is independent of ASC (93). Additionally, a study by Yan et al. identified that phosphorylation of an evolutionarily conserved residue, Ser 533, on NLRC4 is critical for NLRC4 activation following *S. typhimurium* infection. Phosphorylation-defective NLRC4 was found failed to activate caspase-1 and induce pyroptosis (94). The NLRC4 inflammasome activation largely depends on bacterial flagellin and T3SS rod proteins (95-97). Flagellin-deficient strains of *S. typhimurium* and *L. pneumophila* abrogates the NLRC4-mediated caspase-1 activation and bacterial replication (90, 98). *P. aeruginosa* induced activation of NLRC4 inflammasome requires an intact T3SS but the induction of NLRC4 inflammasome-mediated caspase-1 activation is in a flagellin-independent manner (11). The bacterial T3SS rod proteins are also important ligands for NLRC4 inflammasome. The rod proteins of T3SS from *S. typhimurium* (PrgJ), *Burkholderia pseudomallei* (BsaK), *Escherichia coli* (EprJ and EscI), *Shigella flexneri* (MxiI), and *Pseudomonas aeruginosa* (PscI) has been identified to be detected by NLRC4, and a carboxyl terminal amino acid sequence shared by these T3SS

rod proteins is critical for the recognition (97). Bacteria utilize T3SS to deliver virulence factors into host cytosol. NLRC4 activation is T3SS-dependent in response to some bacterial infections, suggesting that flagellin is transported into host cytosol via T3SS (11, 99, 100).

1.3.2.3.1 Mechanism of NLRC4 inflammasome activation

Some studies indicate that NLRC4 is not able to interact with flagellin directly and the interaction requires NAIP proteins (96, 101). A study by Lightfield et al. identified that NAIP5 is required for inflammasome activation following *L. pneumophila* infection. NAIP5-deficient macrophages manifest diminished *L. pneumophila*-induced caspase-1 activation, IL-1β secretion and pyroptotic cell death (101). Another study by Zhao et al. uses *L. pneumophila* flagellin to directly stimulate the mouse macrophages with knockdown of *NAIP5*, which failed to trigger caspase-1 activation and pyroptosis. Moreover, the *Legionella* flagellin was found to interact with NAIP5 directly but not NLRC4 using yeast two-hybrid assay. They also mentioned that NAIP6 functions similarly to NAIP5 that interacts with flagellin, but the effect of NAIP6 involved in NLRC4 activation is limited due to lower expression compared with NAIP5 (96). Furthermore, a study by Halff et al. characterized that flagellin-bound NAIP5 recruits NLRC4 to form a hetero-oligomeric complex, the leucine residues near the flagellin C-terminus is critical for the complex formation. The flagellin-bound NAIP5 then facilitates NLRC4 inflammasome activation (102). NAIP proteins also facilitate T3SS-induced NLRC4 activation. Zhao and colleagues found another NAIP protein, NAIP2, which functioned analogously to NAIP5, recognizing the conserved T3SS rod proteins such as

Salmonella PrgJ and *Burkholderia* BsaK. Caspase-1 activation is impaired in *NAIP2* knockdown mouse macrophages upon PrgJ and BsaK stimulation (96). In contrast to mouse macrophages, only one type of NAIP exists in human macrophages, and it does not associate with flagellin and T3SS rod proteins, but specifically recognizes the needle proteins of T3SS to induce NLRC4 inflammasome activation. The ortholog of human NAIP (hNAIP) in mouse has been identified to be NAIP1, which is also responsible for recognition of cytosolic T3SS needle proteins (103). Altogether, NLRC4 requires association with NAIP proteins to recognize bacterial flagellin and T3SS.

1.3.2.3.2 *P. aeruginosa* recognition by NLRC4 inflammasome

To date, the NLRC4 inflammasome is the only type of inflammasome identified to mediated immune responses to *P. aeruginosa*. *P. aeruginosa*-induced caspase-1 activation requires NLRC4 (11, 104). NLRC4-deficient macrophages display reduced IL-1β production and delayed cell death following *P. aeruginosa* infection. The *P. aeruginosa*-induced NLRC4 inflammasome activation is flagellin-independent but it requires an intact T3SS. Additionally, the *P. aeruginosa* effector molecule ExoU can inhibit caspase-1 activation (11). Of note, a recent study identified a novel inflammasome activator pilin, the main protein constituent of bacterial type IV pili. *P. aeruginosa* pilin stimulated NLRC4 or NLRP3-deficient macrophages from mouse produced similar levels of IL-1β as the wild-type cells did (105). Thus, additional members of inflammasome may also contribute to *P. aeruginosa*-mediated IL-1β production.

1.3.2.4 AIM2 inflammasome

AIM2 is a cytosolic dsDNA sensor essential for innate immune responses against DNA viruses and bacteria. It consists of an N-terminal PYD and a C-terminal haematopoietic interferon-inducible nuclear protein (HIN) domain. The HIN domain is responsible for binding to cytosolic dsDNA. Because of absence of CARD, AIM2 also requires the adapter protein ASC to recruit caspase-1 through a PYD-PYD interaction (66). AIM2 PYD has a six-helix bundle structure and the highly conserved lysine residue at the α2 helix may indirectly facilitate the PYD-ASC PYD interaction. Although AIM2 lacks a central NACHT domain, which mediates self-oligomerization, dsDNA as the activator provides the oligomerization platform for the assembly of AIM2 inflammasome. In the absence of the dsDNA ligand, the AIM2 PYD and HIN domains interact with each other to form an intramolecular complex, thus leading to an autoinhibited state of AIM2. Moreover, the acidic residues at the α2 helix were found to be essential for the PYD-HIN interaction (106). By looking at the crystal structures of HIN domains in complex with dsDNA derived from the vaccinia virus genomic sequences, the positively charged HIN domains were observed to interact with the negatively charged dsDNA sugar-phosphate backbone in a concave surface of the protein. This interaction is non-sequence-specific, and it refers to electrostatic attraction. The DNA binding leads to the release of PYD from HIN, and then the liberated PYD domain facilitates binding the adaptor ASC and activation of caspase-1. The length of dsDNA needs to be more than 80bp in order to sufficiently activate AIM2 inflammasome (107).

AIM2 inflammasome has been found to mediate IL-1β production and caspase-1 activation in response to bacterial pathogens including *Francisella tularensis*, *Listeria monocytogenes*, *Porphyromonas gingivalis* and *Mycobacterium bovis* as well as DNA viruses such as vaccinia virus, murine cytomegalovirus and Human papillomaviruses (HPV) (108-113). These microbial invaders described above can get into host cytosol through phagocytosis, and release DNA to trigger AIM2 inflammsome activation. However, the mechanism involved in release of bacterial dsDNA into host cytosol is quite mysterious. From our previous knowledge, immune cells internalize bacteria by phagocytosis, and the fusion between the phagosome and lysosome can kill the bacteria directly (114). Evasion from phagosomes is a critical step allows invading microorganisms to replicate and grow inside of host cytosol. Many intracellular pathogens have evolved mechanisms to counter these responses by inhibiting lysosome fusion and escaping from the phagosome. *L. monocytogenes* is a Gram-positive intracellular bacterium that employs a pore forming cytolysin listeriolysin O (LLO) to escape from phagosome and undergoes bacteriolysis to release DNA into host cell cytosol, thus leading to AIM2 inflammasome activation and pyroptosis (115). Additionally, in order to evade the host immune detection by AIM2 inflammasome, bacteria tends to keep their cellular integrity in host cytocol to avoid release of DNA.

1.3.2.5 Regulation of inflammasome

Inflammasome activity needs to be tightly controlled by host immune system to avoid overproduction of cytokines and excessive pyroptotic cell death, which cause tissue damage in the host. Dysregulation of inflammasome activity associates with many

autoinflammatory disorders such as familial cold autoinflammatory syndrome (FCAS) and cryopyrin-associated periodic syndromes (CAPS) (116). Many regulatory mechanisms have been identified to be employed by host to limit tissue damage by attenuating inflammasome signaling.

1.3.2.5.1 Autophagy

Autophagy is an evolutionarily conserved process that degrades and recycles cellular organelles and long-lived proteins in eukaryotic cells. It is essential for maintaining cellular homeostasis, and stimulated under both extracellular and intracellular stress conditions, including nutrient deprivation, hypoxia, high temperature, microbial infection and accumulation of dysfunctional cytoplasmic components (117). During autophagy, the cytoplasmic components are engulfed by autophagosomes and then delivered to lysosomes for degradation (118).

Autophagy has been found to negatively regulate inflammasome activity. An early study showed that autophagy-deficient macrophages display significantly enhanced IL-1β production and TRIF-dependent caspase-1 activation in response to LPS stimulation. Moreover, the autophagy-deficient mice were found to suffer more severe dextran sulphate sodium-induced acute colitis due to high level of inflammation. Interestingly, after injection of neutralizing antibodies for IL-1β and IL-18, the mice recovered rapidly and the survival rate was increased (119). It suggests that autophagy is involved in inhibition of the inflammasome-mediated proinflammatory cytokine production. A recent study found that activation of AIM2 or NLRP3 inflammasomes triggers autophagosome

formation and the increased autophagy activity leads to destruction of inflammasomes through ubiquitination (120).

1.3.2.5.2 Type I interferon

Type I interferon (IFNs) can inhibit IL-1β production through two mechanisms. Type I IFNs diminish the level of intracellular IL-1β by inducing the production of anti-inflammatory cytokine IL-10, which inhibits the synthesis of pro-IL-1α and pro-IL-1β via the signal transducer and activator of transcription 3 (STAT3) signaling pathway. In addition, Type I IFNs inhibits the activity of the NLRP1 and NLRP3 inflammasomes with a STAT1-dependent manner, leading to suppression of the caspase-1-dependent IL-1β maturation (121). In consistence with this finding, a study showed that *M. tuberculosis* induces production of Type I IFN in human macrophages, and Type I IFN is able to down-regulate the *M. tuberculosis*-induced IL-1β secretion (122). Interestingly, type I IFN also functions as an activator that is required for activation of the AIM2 inflammasome in response to *Francisella tularensis*. Type I IFN-deficient macrophages showed reduced activation of the AIM2 inflammasome compared to wild-type macrophages (123).

1.3.2.5.3 T cell

Effector and memory $CD4^+$ T cells are capable of inhibiting the activation of NLRP1 and NLRP3 inflammasomes in macrophages in a contact-dependent manner, leading to impaired caspase-1 activation and IL-1β release. This inhibitory event may require the stimulation with the ligands of the tumor necrosis factor family, such as CD40L (124). In

addition, T cell-derived type II interferon IFNγ has been found to inhibit the NLRP3 inflammasome activity. This effect relies on nitric oxide (NO) production that disrupts the assembly of NLRP3 inflammasome via S-nitrosylation (125).

1.3.2.5.4 MicroRNA

MicroRNA (miRNA) is a small, single-stranded, non-protein coding RNA molecule that plays an important role in controlling gene expression at post-transcriptional level (126). A study reported that miR-223 is able to suppress NLRP3 inflammasome activity by binding to the 3' untranslated region of *NLRP3* mRNA, thus leading to reduced NLRP3 protein expression (127). In addition, another study found that miRNA can also positively regulate inflammasome activation. MicroRNA-133a-1 promotes NLRP3 inflammasome activation by targeting the mitochondrial uncoupling protein 2 (UCP2), which plays a role in ATP synthesis and ROS production. Overexpression of microRNA-133a-1 results in increased cleavage of caspase-1 and IL-1β (128).

1.3.2.5.5 Pyrin-only and CARD-only proteins

Regulation of the inflammasomes can be achieved by interfering with the PYD-PYD or CARD–CARD interaction in the inflammasomes. Pyrin-only (POPs) and CARD-only proteins (COPs) are two important negative regulators of the inflammasome in humans. The POPs include POP1 and POP2. POP1 has high degree of similarity with the PYD domain of ASC so that it can interact with the ASC and sequester it from binding to the PYD domain of NLRs (129). However, POP2 is highly similar to the PYD domain of NLRs, which directly inhibits the inflammasome activation (130). In contrast to POPs,

the COPs have high similarity to the CARD of caspase-1. It inhibits the CARD–CARD interaction between adaptor ASC and caspase-1 by binding to the CARD of caspase-1, thereby leading to impaired caspase-1 activation (131).

1.3.2.5.6 Special regulatory mechanisms of AIM2 inflammasome

The AIM2 inflammasome is tightly regulated by some specific regulatory mechanisms. A recent study reported that mouse p202, another member of PYHIN family, can inhibit AIM2-dependent caspase-1 activation (132). Unlike AIM2, p202 lacks a PYD but consists of two HIN domains. It binds rapidly to the dsDNA introduced into the cytosol without triggering downstream signalling (133). Moreover, it can also bind to AIM2 directly, which results in interfere of DNA binding by AIM2 and a spatial separation of the AIM2 pyrin domains, preventing ASC recruitment and AIM2 inflammasome activation (132). Therefore, p202 negatively regulates AIM2 inflammasome activation by competing for DNA binding or directly disrupting the DNA binding ability of AIM2. Another study reported that the synthetic oligodeoxynucleotides (ODNs) A151 comprised of the immunosuppressive motif TTAGGG blocks cytosolic DNA-driven inflammatory cytokine production. The DNA sequences containing TTAGGG repeats are commonly found in mammalian telomeric DNA and they function as suppressors of immune activation. In this study, Kaminski and colleagues generated synthetic ODNs that contain the TTAGGG motif, which competes with cytosolic DNA for binding to AIM2, resulting in interference of AIM2 inflammasome activation (134).

1.4 The NFAT/Calcineurin pathway and regulator of calcineurin 1

After elimination of the invading microorganisms from host, the proinflammatory responses must return to a homeostatic state. Unrestrained production of proinflammatory mediators may be harmful to host, leading to development of autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (135). Therefore, TLR signalling pathways must be tightly regulated. Recently, a novel negative regulator of proinflammatory responses, regulator of calcineurin-1 (RCAN1), was identified (136-138). The human *RCAN1* gene is located on chromosome 21 within the Down syndrome critical region and is highly expressed in brain, heart, muscle, liver, kidney, lung and testis (139-141). It suppresses the nuclear factor of activated T cells (NFAT)-mediated gene expression through inhibition of calcineurin (142).

The NFAT pathway is activated by increase of intracellular calcium (Ca^{2+}) in response to cellular stimuli, such as oxidative stress. The increased Ca^{2+} leads to activation of the calmodulin, which associates with phosphatase calcineurin. The activated calcineurin is able to dephosphorylate cytosolic NFAT leading to nuclear translocation of NFAT (143). NFAT activation has been implicated in production of a variety of proinflammatory cytokines, including IL-2, IL-3, IL-4, GM-CSF and TNF-α (144-148). Calcineurin is a heterodimeric protein, consisting of catalytic subunit calcineurin A and a regulatory subunit calcineurin B for calcium binding (149). RCAN1 interacts with calcineurin A and inhibits the calcineurin-dependent phosphatase activity, preventing NFAT activation. Moreover, the interaction region is located in the linker region between calcineurin A and B (142).

RCAN1 is also able to negatively regulate NF-κB signaling pathway through enhancing the stability of NF-κB inhibitor IκBα (138). A previous study in our lab has identified that RCAN1 functions as a negative regulator of inflammation during *P. aeruginosa* acute lung infection. RCAN1 deficiency elevates NF-κB and NFAT activation, as well as a variety of proinflammatory cytokine production (137). However, the molecular mechanisms involved in the regulation of *P. aeruginosa*-induced TLR signaling pathway by RCAN1 have not yet been elucidated.

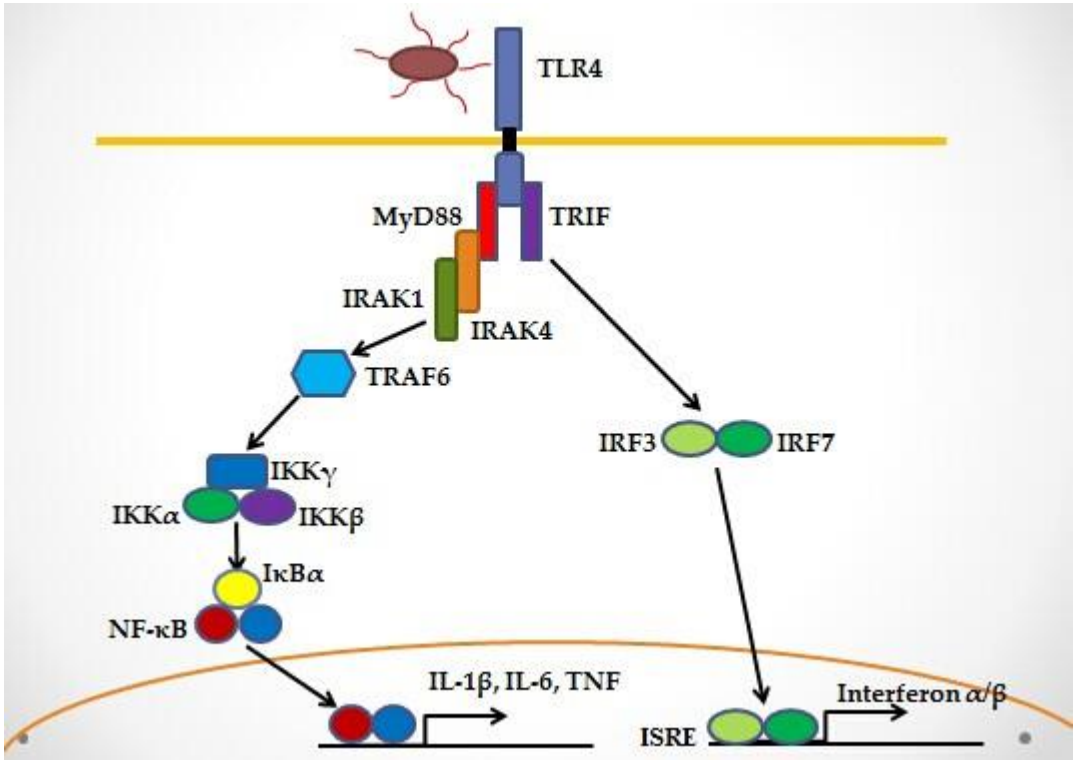

Figure 1.3.1: TLR-mediated MyD88- and TRIF-dependent signaling pathways. After binding to specific ligands, TLRs trigger two distinct signaling pathways, MyD88- and TRIF-dependent pathways. The MyD88-dependent pathway leads to activation of TRAF6 and IKK complex which phosphorylates IκB and frees NF-κB. The liberated NF-κB then translocates into nucleus and mediates gene expression of various proinflammatory cytokines, including IL-1β, IL-6, TNF-α. The TRIF-dependent pathway induces IRF3 and IRF7 activation, leading to IFN-α and -β expression.

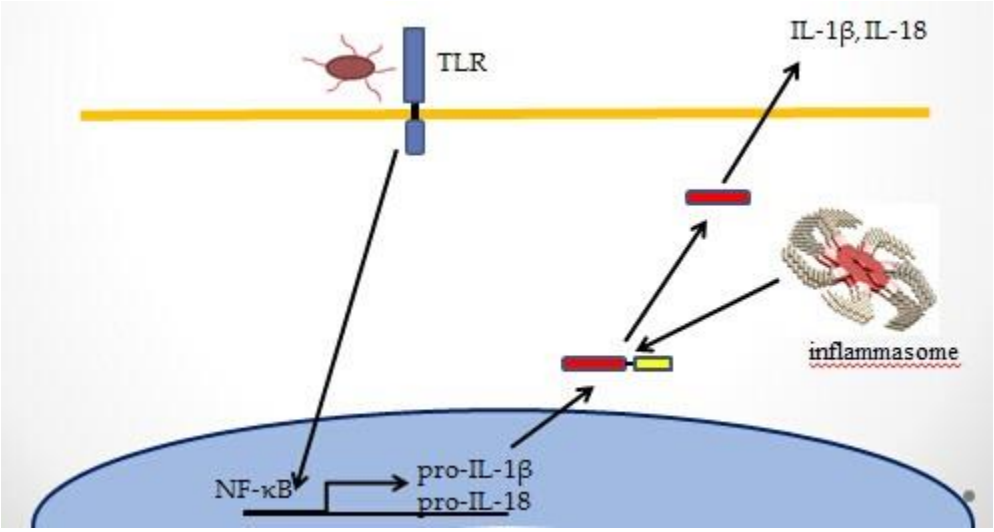

Figure 1.3.2: Mechanisms resulting production of IL-1β and IL-18. Transcription of the inactive pro forms of IL-1β and IL-18 is induced by TLR signaling. Cytoplasmic stimuli can activate NLRs or AIM2, leading to caspase-1 recruitment and inflammasome assembly. The activated caspase-1 is able to process pro-IL-1β and pro-IL-18 into their active and secretable form.

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CHAPTER 2: AIM2 INFLAMMASOME IS DISPENSABLE FOR HOST DEFENSE AGAINST *PSEUDIMONAS AERUGINOSA* INFECTION

2.1 Abstract

Respiratory tract infection with *Pseudomonas aeruginosa* is a major cause of hospital-acquired pneumonia in immune-compromised individuals. Lung infection with *P. aeruginosa* is often associated with production of various inflammatory cytokines including IL-1β. Production of IL-1β requires proteolytic cleavage by a multiprotein complex termed inflammasome. AIM2 inflammasome recognizes foreign cytosolic double stranded DNA. A role of AIM2 in *P. aeruginosa* infection has not been reported previously. In this study, we found that *P. aeruginosa* infection induced degradation of AIM2 protein in macrophages and induction of AIM2 mRNA expression in macrophages and in the lungs of mice. Interestingly, *P. aeruginosa* infection induced a similar level of IL-1β, IL-6 and TNF production in wild-type and AIM2-deficient mice. Similarly, no significant differences in bacterial clearance, neutrophil infiltration and NF-κB activation were observed between wild-type and AIM2-deficient mice following *P. aeruginosa* lung infection. Our data suggest that AIM2 inflammasome is dispensable for the host defense against *P. aeruginosa* infection.

2.2 Introduction

Pseudomonas aeruginosa, an opportunistic Gram-negative bacterium, is an important cause of infection among immunocompromised individuals (1). *P. aeruginosa*

chronically infects cystic fibrosis (CF) patients, leading to declined pulmonary function and increased morbidity and mortality (2). *P. aeruginosa* infection in the airway triggers excessive production of various cytokines and chemokines including IL-1β (3). The levels of IL-1β are significantly increased in the bronchoalveolar lavage fluid and sputum of cystic fibrosis patients, and in mouse lung following *P. aeruginosa* infection (4, 5).

Production of mature form of IL-1β requires proteolytic cleavage of its inactive precursor (6). This proteolytic cleavage is tightly controlled by caspase-1, a cysteine protease in cytosol. Activation of caspase-1 is regulated by multiprotein complexes termed inflammasomes, which consist of members of nucleotide binding and oligomerization domain (NOD)-like receptors (NLR) family or pyrin and HIN domain-containing protein (PYHIN) family member absent in melanoma-2 (AIM2) (6-8). NLRs and AIM2 are cytoplasmic pattern recognition receptors (PRRs) that detect microbial molecules and endogenous danger signals as intracellular sensors. Once activated they rapidly assemble into inflammasomes and recruit procaspase-1 for cleavage and activation (9). The NLR family CARD domain–containing protein 4 (NLRC4) inflammasome has been shown contributing to IL-1β production in response to *P. aeruginosa* infection (5, 10-12). However, cells from animals with homozygous deletion of NLR family members NLRC4, or NLRP3 produced similar levels of IL-1β in response to *P. aeruginosa* pilin stimulation as the wild-type cells did (13). Thus, additional members of inflammasome may also contribute to *P. aeruginosa*-mediated IL-1β production.

AIM2 is a cytosolic double-stranded DNA (dsDNA) sensor essential for innate immune responses against DNA viruses and bacteria (14-20). It is composed of an N-terminal pyrin domain (PYD) associated with adaptor protein ASC for caspase-1 recruitment and a C-terminal haematopoietic interferon-inducible nuclear protein 200 (HIN200) domain, which is responsible for binding to cytosolic dsDNA (6). Upon binding to dsDNA, AIM2 releases from an auto-inhibited state and assembles into a large inflammasome complex with ASC and procaspase-1 (21). However, the role of AIM2 inflammasome in the innate immune responses during *P. aeruginosa* infection remains undefined. Given the ability of *P. aeruginosa* to reside and replicate within host cells (22), and the important role of AIM2 inflammasome in the activation of IL-1β during host defense against various intracellular bacteria (14-20), it is possible that the AIM2 inflammasome is involved in mediation of inflammatory responses during *P. aeruginosa* lung infection.

In this study, we found that *P. aeruginosa* infection mediates degradation of AIM2 protein and stimulation of AIM2 mRNA expression, suggesting a potential involvement of AIM2 in *P. aeruginosa* infection. Surprisingly, AIM2-deficient mice showed no defect in bacterial clearance, neutrophil infiltration, IL-1β production and transcription factor NF-κB activation in the lung following *P. aeruginosa* infection. These findings suggest that there may be functional redundancy in inflammasome-mediated caspase-1 activation and IL-1β production.

2.3 Material and Method

Ethic statement

Animal protocols for this study were approved by the University Committee on Laboratory Animals, Dalhousie University (permit number: 13-115), in accordance with the guidelines of the Canadian Council on Animal Care. Animals were housed in specific pathogen free facilities, and anesthetized with ketamine to minimize suffering during relevant procedures.

Animals

AIM2-deficient ($AIM2^{-/-}$) mice on the C57BL/6 background were purchased from Jackson Laboratory**.** C57BL/6 mice were purchased from Charles River Laboratories and used as wild-type controls. $AIM2^{-/-}$ mice were age- and sex- matched with C57BL/6 mice.

Antibodies

Abs to AIM2 (13095) was purchased from Cell Signaling Technology (Beverly, MA). Abs to actin (sc1616) and all HRP-linked secondary Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Abs to caspase-1 (p20) (AG-20B-0042) was purchased from AdipoGen (San Diego, CA).

Bacterial preparation and macrophage activation

P. aeruginosa strain 8821, a gift from Dr. A. Chakrabarty (University of Illinois, Chicago, IL, USA), was a mucoid strain isolated from a cystic fibrosis patient (23). *P. aeruginosa* was cultured as described previously (24). Briefly, suspension culture was grown until reaching the early stationary phase. Bacteria were washed in phosphate buffer

solution (PBS) and resuspended in saline for *in vivo* studies or PBS for *in vitro* macrophage activation experiments.

Bone marrow-derived macrophages (BMMs) were used for *in vitro* study. Bone marrow cells were flushed from C57BL/6 or AIM2$^{-/-}$ mice femurs and tibias and cultured in complete medium (DMEM medium containing 10% FBS, 1% Penicillin/Strep and M-CSF from supernatant of L929). Cells were cultured for 7 days until they were mature, and the medium was replaced with antibiotic-free DMEM medium with 10% FBS. Cells were infected with *P. aeruginosa* strain 8821 at the multiplicity of infection (MOI) of 10 or 20 bacteria per macrophage for various time points. After incubation, cell-free supernatants were collected for measuring cytokines by ELISA. Cell pellets were lysed for Western blotting or real-time quantitative PCR.

Cytokine production

The concentrations of IL-1β, IL-6 and TNF in the lungs, bronchoalveolar lavage fluid (BALF) and cell-free supernatants were determined by ELISA as described previously using Ab pairs from R&D System (Minneapolis, MN), and developed with an amplification system (Invitrogen) (25).

Western blotting

Cells were lysed in RIPA buffer supplemented with a cocktail of protease and phosphatase inhibitors. Cells lysates (36 – 42 μg) were subjected to electrophoresis in 12% SDS-polyacrylamide gels. Gels were transferred to polyvinylidene difluoride (PVDF) membrane, blocked with 5% non-fat milk powder, blotted with primary and secondary

antibodies, and detected by an ECL detection system (Western Lightning Plus-ECL; PerkinElmer). For detection of caspase-1 p20 in cell supernatants, proteins were precipitated by the addition of an equal volume of methanol and 0.25 volumes of chloroform as described previously (26).

Real-time quantitative PCR

1-2 X 10^6 cells or 100 mg of mouse lung tissues were collected and processed in TRIzol (Invitrogen). The total mRNA was purified using RNeasy kit (Qiagen) and cDNA was synthesized using a reverse transcription system (Clontech). AIM2 primer sequences, Forward 5'- AGCTGAAAACTGCTCTGCTGC -3' and Reverse 5'- AGCACCGTGACAACAAGTGG- 3', were designed by Primer-BLAST (NCBI). According to manufacturer's instruction, RT-PCR arrays were conducted in triplicate and AIM2 mRNA was quantified using syber green method on a sequence detection system (ABI Prism 7000; Applied Biosystems). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as an endogenous reference. Data were analyzed using relative standard curve method according to the manufacturer's protocol.

Lung infection with *P. aeruginosa*, collection of lung and BALF

Mice were intranasally infected with 1 X 10^7 CFU of *P. aeruginosa* strain 8821 for 4 h or 1 X 10^9 CFU for 24 h. After 4 h or 24 h infection, BALF was obtained by lavaging the lung with 3 X 1 ml of PBS containing soybean trypsin inhibitor (100 μg/ml). The lung tissues were obtained for detection of cytokines, myeloperoxidase (MPO), bacterial CFU counting and histology.

Lung tissues were homogenized at maximum speed for 10s in 50 mM HEPES buffer (4 µl/mg lung) containing soybean trypsin inhibitor (100 µg/ml). For bacterial CFU counting, 10 µl of homogenate was plated on an agar dish and incubated for 24 h at 37^{o}C. For cytokines and MPO assays, the homogenate was centrifuged at 4^{o}C for 30 min at 18000 × g. The supernatant was stored at -80^{o}C for later cytokine analysis. The pellets were resuspended and homogenized in 0.5% cetyltrimethylammonium chloride (CTAC) (4 µl/mg lung) and centrifuged again at 18000 × g for 30 min. The clear extracts were collected for MPO assay.

BALF (10 µl) was plated on an agar dish and incubated for 24 h at 37^{o}C for CFU counting. For detection of cytokines and MPO assay, BALF was centrifuged at 480 × g for 5 min at 4^{o}C. The supernatants were used for cytokine analysis. The cell pellets were resuspended in 1 ml of NH_4Cl buffer to lyse erythrocytes and centrifuged. The supernatants were discarded. The pellets were resuspended in 0.5% CTAC (250 µl/mouse) and centrifuged again. The clear extracts were collected for MPO assay.

MPO assay

The MPO assay was used to determine the infiltration of neutrophils into the lungs of the mice as described previously (27). Briefly, samples in duplicate (75 µl) were mixed with equal volumes of the substrate (3,3′,5,5′-tetramethyl-benzidine dihydrochloride, 3 mM; Resorcinol, 120 µM; and H_2O_2, 2.2 mM) for 2 minutes. The reaction was stopped by adding 150 µl of 2 M H_2SO_4. The optical density was measured at 450 nm.

EMSA analysis

A consensus double-stranded NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Promega) was used for EMSA. Nuclear protein extracts were obtained using a nuclear extract kit (Active Motif, Carlsbad, CA), according to the manufacturer's protocol. EMSA performed as previously described (28). Briefly, 10 µg of extracted nuclear protein was added into 10 µl of binding buffer (Promega E358A) with 1 µg of poly(deoxyinosinic-deoxycytidylic acid) (GE healthcare) and incubated for 15 min at room temperature. Double-stranded oligonucleotides were ^{32}P-labeled and added into each reaction mixture, which was incubated for 30 min at room temperature and separated by electrophoresis on a 6% polyacrylamide gel in 0.5 X Tris–boric acid–EDTA buffer. Gels were vacuum dried and subjected to autoradiography.

Histology

Mice lungs were fixed in 10% formalin overnight then in 100% ethanol for paraffin embedding and sectioning. Slides were deparaffinized with CitriSolv (Fisher Scientific), and rehydrated through decreasing concentrations of ethanol. Slides were stained with Harris H&E to illustrate lung histology.

Statistics

Data are presented as the mean ± SEM of the indicated number of experiments. Statistical significance was determined by assessing means with ANOVA and the Tukey-Kramer multiple comparison tests, or by using an unpaired *t* test. Differences were considered significant at $p < 0.05$.

2.4 Results

***P. aeruginosa* infection induces AIM2 protein degradation and stimulates AIM2 mRNA expression.** The early host responses to pathogen in airway are mainly mediated by local resident cells. Macrophages have been identified to play an important role in the first line of defense against *P. aeruginosa* in the respiratory tract (29, 30). To determine the AIM2 gene expression in macrophages during *P. aeruginosa* infection, macrophages cultured from murine bone marrow were infected with *P. aeruginosa* strain 8821 at a MOI of 1:10 for various times. We first examined the AIM2 mRNA expression in macrophages at 2, 4 or 6 h time points by real-time quantitative PCR. The AIM2 mRNA expression levels was found to be increased and reached their highest levels around 4 h after *P. aeruginosa* infection (Figure 2.1A). Similarly, the AIM2 mRNA expression was enhanced in the lung *in vivo* after *P. aeruginosa* lung infection for 4 h (Figure 2.1B).

We next assessed the AIM2 protein level in macrophages. Mouse bone marrow derived macrophages were treated with *P. aeruginosa* (MOI of 1: 10) for 1, 2, 4 or 6 h. Cell lysates were subjected to Western blot analysis for AIM2. Surprisingly, the level of AIM2 protein was significantly reduced over the infection time, and it was mostly degraded at 6 h (Figure 2.1C and 2.1D).

AIM2 deficiency does not impair IL-1β production in macrophages following *P. aeruginosa* infection in vitro. To examine whether AIM2 plays a role in IL-1β production during *P. aeruginosa* infection, AIM2-decificent and wild-type macrophages were treated with *P. aeruginosa* at a MOI of 1:10 for various times up to 24 h. Cell free supernatants were collected for detection of IL-1β production by ELISA. The level of IL-

1β started to increase at 3 h. There is no significant difference of IL-1β production between wild-type and AIM2-decificent cells (Figure 2.2A). Moreover, we tested additional two cytokines, TNF (Figure 2.2B) and IL-6 (Figure 2.2C), which are two inflammatory cytokines independent of the inflammasome-mediated activation pathway. Similarly, no significant difference of TNF and IL-6 production was found between wild-type and AIM2-deficient macrophages.

AIM2 deficiency does not impair caspase-1 activation. Assembly of inflammasome complexes leads to activation of caspase-1 by cleavage of procaspase-1 into two subunits, p20 and p10 (31). To examine whether AIM2 inflammasome is required for *P. aeruginosa*-induced caspase-1 activation *in vitro*, we set out to detect the activated form of caspase-1 p20 in both wild-type and AIM2-deficient cell lysates and supernatants, as well as its inactive form procaspase-1, by Western blotting. Wild-type and AIM2-deficient macrophages were infected with *P. aeruginosa* stain 8821 at a MOI of 1:20 for 2, 4 or 6 h. The activated caspase-1 p20 was detected in both wild type and AIM2-decificent cell lysates and supernatants in response to *P. aeruginosa* infection. The active caspase-1 subunits have been previously shown to be released into extracellular environment (32-34). The p20 cleavage in cell lysates was found to be induced at 2 h and then decreased up to 6 h, which is in agreement of the increase of p20 in culture supernatants along the infection time (Fig. 2.2D). This suggests that caspase-1 activation is not impaired in AIM2-deficient macrophages upon *P. aeruginosa* infection.

AIM2-deficiency does not impair the clearance of *P. aeruginosa* and neutrophil infiltration in vivo. To determine whether AIM2 inflammasome plays a role in the

clearance of *P. aeruginosa* and neutrophil infiltration in the airways, wild-type and AIM2-deficient mice were infected intranasally with 1×10^7 or 1×10^9 CFU/mouse for 4 h or 24 h. Lungs and BALF were collected for detection of bacterial burden by CFU counting (24 h) and neutrophil infiltration by MPO assay (4 and 24 h). The bacterial burden in the lungs and BALF of AIM2-deficient mice was not significantly different to that in wild-type mice at 24 h (Figure 2.3A and 2.3B).

Upon pathogen infection, neutrophils are recruited into the infected site (35, 36). We further characterized the neutrophil infiltration into the respiratory tract by measuring the activity of the neutrophil specific MPO. The MPO activity was not significantly impaired in the lungs and BALF of AIM2-deficient mice compared to that in wild type mice at 4 h and 24 h (Figure 2.3C and 2.3D). Moreover, lung histology showed similar level of inflammatory cell infiltration in the lung between AIM2-deficient and wild-type mice (Figure 2.4). These results suggest that AIM2 deficiency has no effect on bacterial clearance and neutrophil recruitment in the airways following *P. aeruginosa* lung infection *in vivo*.

AIM2 deficiency does not impair IL-1β production and NF-κB activation in response to *P. aeruginosa* lung infection in vivo. Synthesis of pro-IL-1β is primarily regulated by the transcription factor NF-κB (37). To determine a role for AIM2 inflammasome in cytokine production following *P. aeruginosa* lung infection *in vivo*, the lung and BALF supernatants were collected for determination of IL-1β, TNF and IL-6 by ELISA after 4 or 24 h infection. IL-1β (Figure 2.5A and 2.5B), TNF (Figure 2.5C and 2.5D) and IL-6 (Figure 2.5E and 2.5F) in lungs and BALF were induced as early as 4 h

and significantly increased at 24 h. However, no statistically significant difference was found in these cytokines between wild-type and AIM2-deficient mice. Consistent with this finding, *P. aeruginosa*-induced activation of NF-κB in the lungs of AIM2-deficient mice was not impaired compared to that in wild-type mice as measured by EMSA (Figure 2.6A and 2.6B). These findings suggest that AIM2 deficiency has no significant effect on transcription factor NF-κB activation and its downstream cytokine production following *P. aeruginosa* lung infection.

2.5 Discussion

P. aeruginosa infection impairs lung function which often is associated with excessive production of cytokines and chemokines including IL-1β in immunocompromised individuals and CF patients (1, 2). Previous studies have revealed that host immune system employs two types of pattern recognition receptors, the TLRs and NLRs, to recognize the invading *P. aeruginosa* (5, 10-12, 38). We and others have demonstrated the involvement of several cell membrane-associated TLRs including TLR2, 4, 5, 6 in the recognition of the extracellular *P. aeruginosa* (24, 38-40). The NLRs are cytoplasmic receptors that are responsible for detecting the *P. aeruginosa* inside cells (9, 38). Activation of the TLRs or NLRs triggers downstream signaling pathways leading to the production of cytokines and chemokines. Specifically, the expression of inactive pro-form of the proinflammatory cytokine IL-1β is regulated by TLR pathways, and the pro-IL-1β was subsequently cleaved by inflammasome, which consists of activated NLRs (41, 42). NLRC4 inflammasome has been found to recognize *P. aeruginosa* and mediates IL-1β production (10). However, a recent report showed that *P. aeruginosa* pillin was able to

trigger the inflammasome-mediated immune responses in a NLRC4-independent manner (13), suggesting that additional mechanisms may be involved in the recognition of *P. aeruginosa*. In this study, we examined the role of AIM2 inflammasome in host response to *P. aeruginosa* infection *in vitro* and *in vivo*. Unexpectedly, our data suggest that AIM2 is not required for *P. aeruginosa*-mediated IL-1β production.

AIM2 mRNA expression was increased following *P. aeruginosa* infection. In contrast, the AIM2 protein level was reduced in macrophages, suggesting the involvement of AIM2 in *P. aeruginosa* infection. However, how and why AIM2 is degraded remains to be determined. It has been well recognized that inflammasome activity needs to be tightly controlled by host immune system to avoid overproduction of cytokines and excessive pyroptotic cell death (43, 44). To achieve this, host regulatory mechanisms down-regulate the inflammasome activity by inhibition or degradation of inflammasome (45-51). A host regulatory process termed autophagy may be involved in AIM2 degradation following *P. aeruginosa* infection. Autophagy is an evolutionarily conserved process that degrades and recycles cellular organelles and long-lived proteins in eukaryotic cells (52, 53). A study has shown that induction of AIM2 inflammasome triggers autophagosome formation and increased autophagy activity leading to destruction of AIM2 through ubiquitination (51). We recently showed that autophagy is induced by *P. aeruginosa* infection (54). It is possible that autophagy accompanies inflammasome activation to temper inflammation by eliminating active inflammasomes.

AIM2 inflammasome senses the dsDNA released from microbes in host cytosol (6). AIM2 has been found to be activated in dendritic cells and macrophages upon sensing

several intracellular pathogens, including cytosolic bacterial pathogen *Francisella tularensis*, *Listeria monocytogenes*, *Porphyromonas gingivalis*, *Mycobacterium bovis* and human papillomaviruses (HPV) (16-20). AIM2 deficiency results in impaired IL-1β production and caspase-1 activation following infection by these pathogens (16-20). Given the ability of *P. aeruginosa* to reside and replicate within host cell, we examined whether AIM2 inflammasome was activated by *P. aeruginosa*. No impairment of IL-1β production and caspase-1 activation was found in AIM2-deficient macrophages. Similarly, the level of IL-1β secretion in the lungs and BALF of AIM2-deficient mice was not significantly impaired *in vivo* as well. Thus, *P. aeruginosa* possess unique property to induce IL-1β production and caspase-1 activation which is independent of AIM2.

The synthesis of pro-IL-1β is regulated by transcription factor NF-κB through the TLRs signaling pathway (37, 38, 41). Once the TLRs are activated by *P. aeruginosa*, a MyD88-dependent downstream signaling is triggered, leading to activation of NF-κB. The liberated NF-κB then moves into nucleus and activates NF-κB–mediated proinflammatory gene expression (37). No difference of NF-κB activation was found between AIM2-deficient and wild-type mice following *P. aeruginosa*, suggesting that TLR-NF-κB pathway was not affected by AIM2 deficiency. Previous studies have shown that inflammasome deficiency displays enhanced bacterial burden and attenuated neutrophil infiltration in airways in response to respiratory pathogen infection (10, 11). However, our results show that the bacterial clearance and neutrophil infiltration in the airways were not significantly impaired in AIM2-deficienct mice compared to wild-type mice. Accordingly, our findings from both *in vitro* and *in vivo* studies suggest that AIM2

inflammasome is functionally redundant in the inflammasome-mediated caspase-1 activation and IL-1β production during *P. aeruginosa* infection.

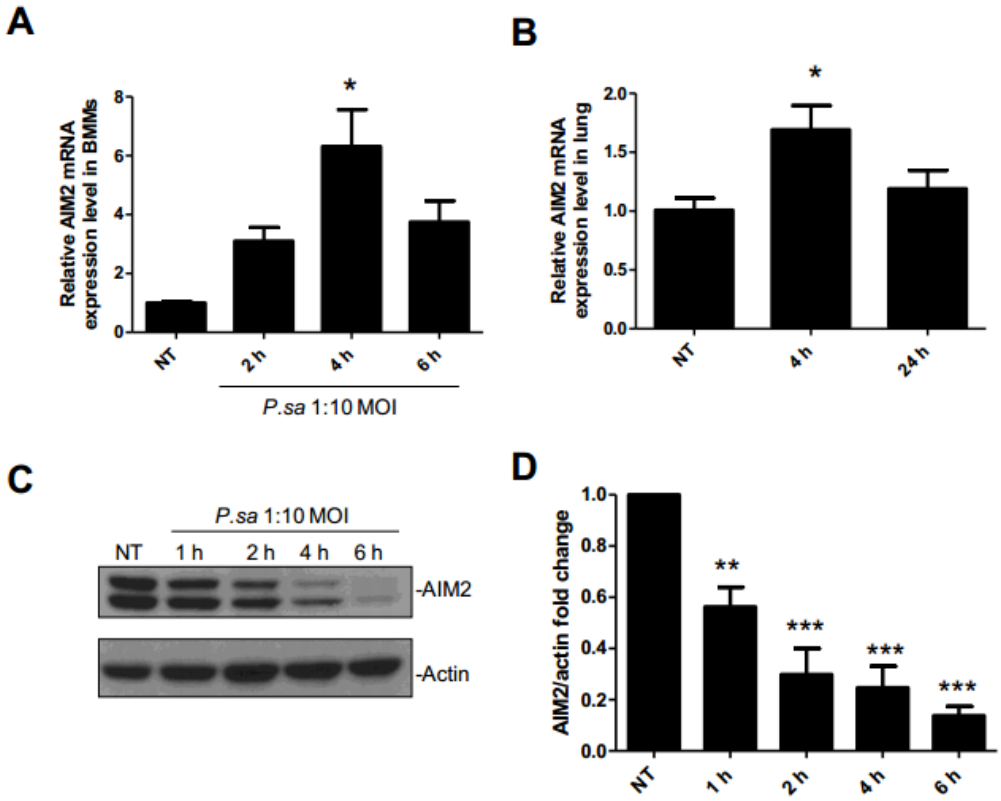

Figure 2.1: AIM2 mRNA expression is induced, but the AIM2 protein is reduced following *P. aeruginosa* infection. (A) Bone marrow-derived macrophages (BMMs) were infected with *P. aeruginosa* strain 8821 (*P.sa*) at a MOI of 1:10 for 2, 4, 6 h, or left untreated (NT). Total RNA isolated from these cells was reverse transcribed to cDNA and subjected to real-time quantitative PCR for AIM2. The AIM2 gene expression was normalized to endogenous control HPRT (n = 3 ± SEM, *p<0.05). (B) Wild-type (+/+) mice were infected intranasally with 1×10^7 (4 h) or 1×10^9 (24 h) CFU/mouse *P. aeruginosa* strain 8821 or equivalent volume of saline (NT). AIM2 gene expression in lung tissues was analyzed using real-time quantitative PCR and normalized to HPRT (n=3 ± SEM. *p<0.05). (C) Wild type (+/+) BMMs were infected with *P. aeruginosa* strain 8821 at a MOI of 1:10 for 1, 2, 4, 6 h, or left untreated (NT). Cell lysates were analyzed by Western blotting for AIM2 as well as actin loading control. (D) Scanning densitometry was performed for detecting AIM2 expression and data are expressed as fold change normalized to actin (n = 4 ± SEM, **p<0.01, ***p<0.0001).

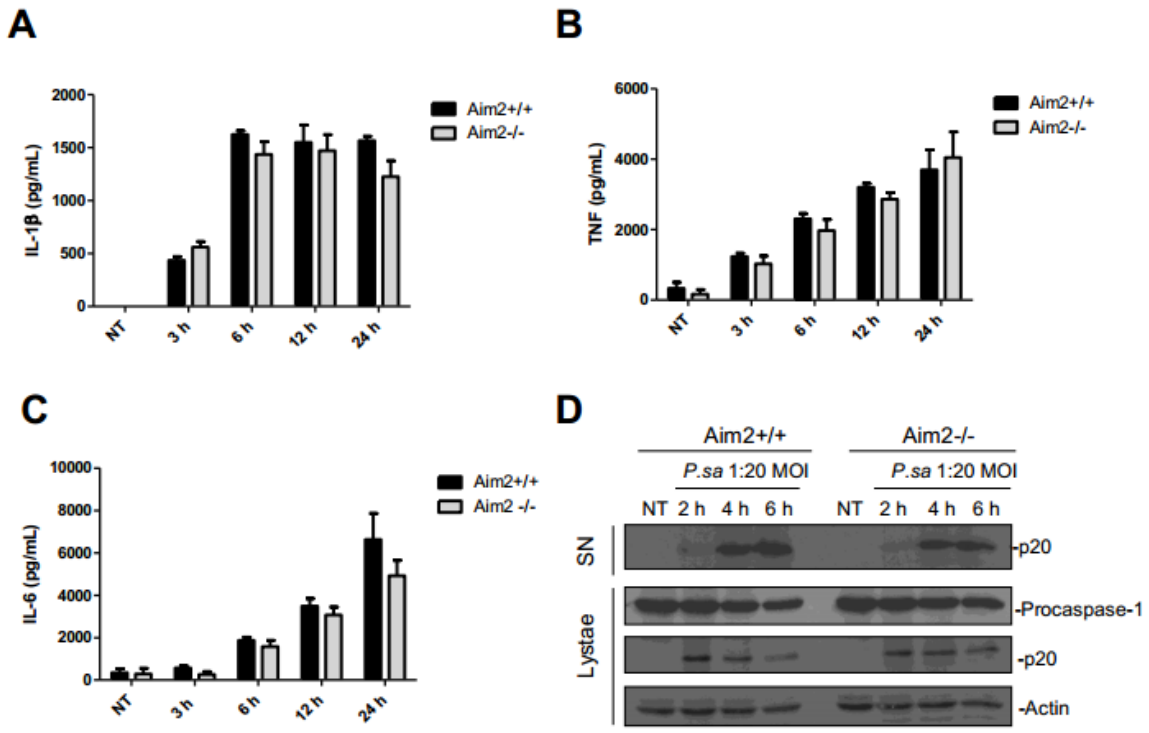

Figure 2.2: AIM2 deficiency does not significantly impair cytokine production or caspase-1 activation following *P. aeruginosa* infection *in vitro*. Wild type (+/+) and Aim2-deficient (-/-) mouse bone marrow-derived macrophages (BMMs) were infected with *P. aeruginosa* strain 8821 at a MOI of 1:10 for 3, 6, 12 or 24 h , or left untreated (NT). Cell-free supernatants were collected and assessed for IL-1β (A), TNF (B) and IL-6 (C) production by ELISA (n=3 independent experiments). (D) Lysates and cell supernatants were collected from Wild type (+/+) and Aim2-deficient (-/-) BMMs after treatment with *P. aeruginosa* strain 8821 (*P.sa*) at a MOI of 1:20 for 2, 4 or 6 h, or left untreated (NT). Procaspase-1, p20 and actin loading control in the cell lyastes and secreted p20 in the cell-free supernatants (SN) were detected by Western blotting. (one representative of 2 independent experiments).

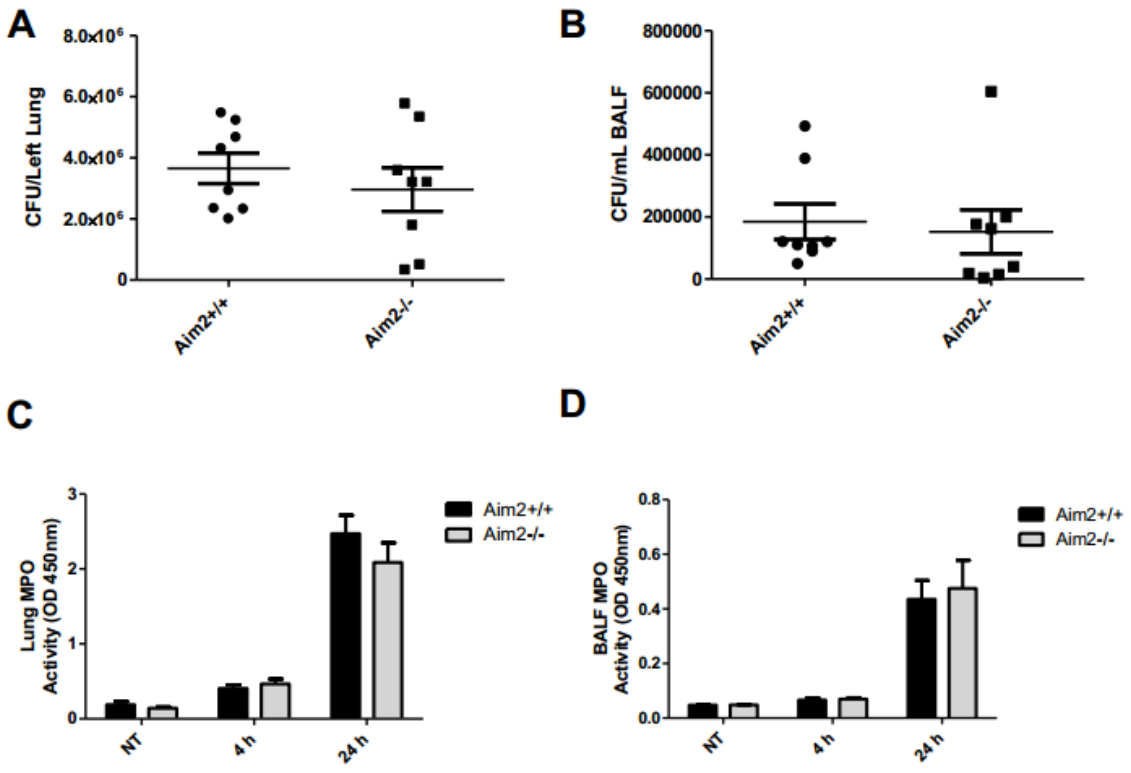

Figure 2.3: AIM2 deficiency has no effect on bacterial clearance and neutrophil infiltration in lung following *P. aeruginosa* infection. Wild type (+/+) and Aim2-deficient (-/-) mice were intranasally infected with of *P. aeruginosa* strain 8821 (1×10^7 CFU/mouse for 4 h or 1×10^9 CFU/mouse for 24 h) or treated with saline as a control (NT). Bacterial burden was examined by colony counting for the mice infected with *P. aeruginosa* for 24 h (lung tissues: A and BALF: B). MPO activity was measured in the lung (C) and BALF (D) lysates (n = 8-11 independent experiments).

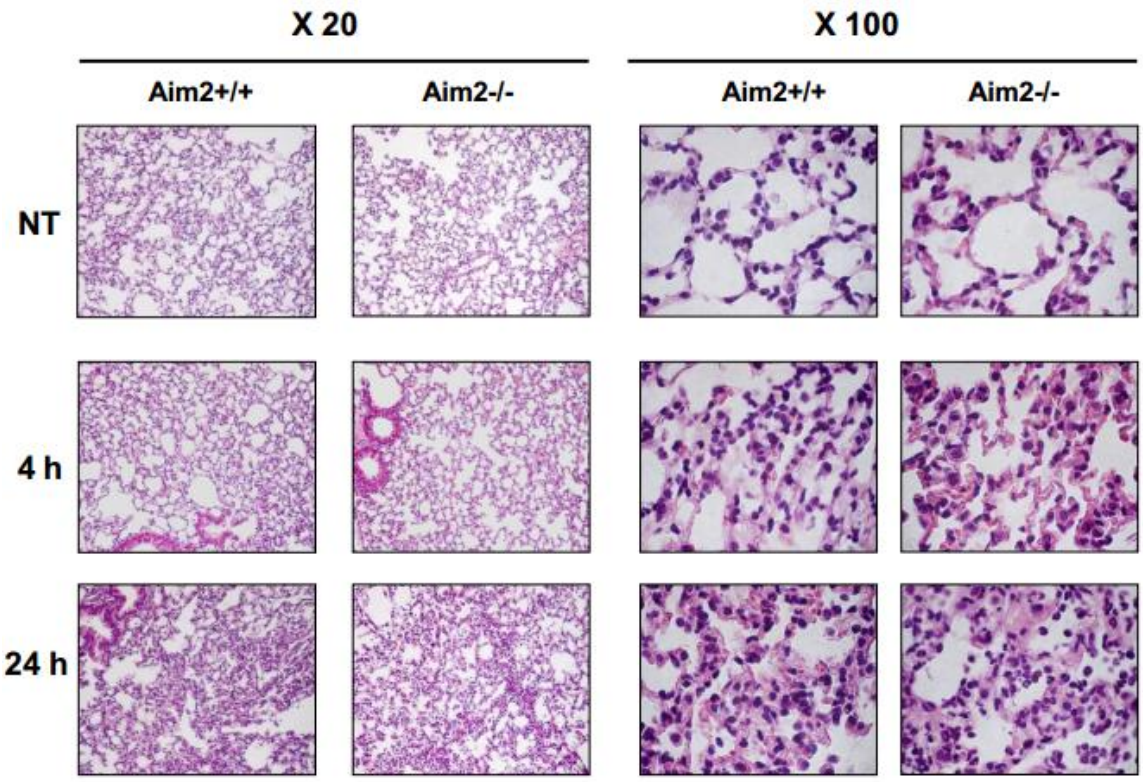

Figure 2.4: AIM2 deficiency has little effect on inflammatory cell infiltration into the airways following *P. aeruginosa* lung infection. Wild type (+/+) and Aim2-decificent (Aim2-/-) mice were intranasally infected with *P. aeruginosa* strain 8821 (1×10^7 CFU/mouse for 4 h or 1×10^9 CFU/mouse for 24 h) or treated with saline as a control (NT). The upper lobe of the left lung was collected for H&E staining (original magnification X 20 or X 100). Pictures are representative of 6 mice from each group.

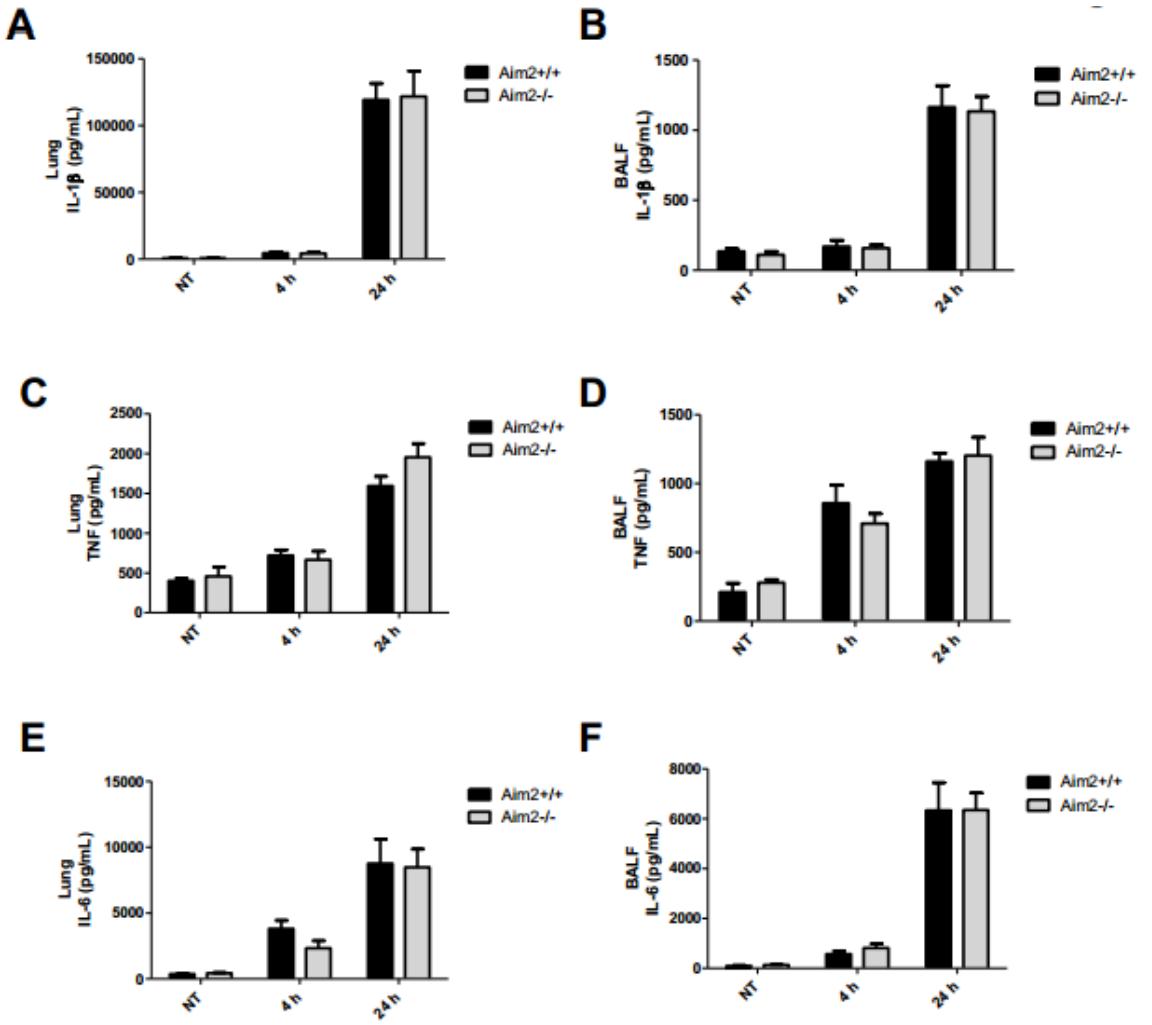

Figure 2.5: AIM2 deficiency does not significantly impair cytokine production following *P. aeruginosa* lung infection *in vivo*. Wild type (+/+) and AIM2-deficient (-/-) mice were infected intranasally with 1 X 10^7 (4 h) or 1 X 10^9 (24 h) CFU/mouse *P. aeruginosa* strain 8821 or equivalent volume of saline (NT). After 4 or 24 h, lung tissues and BALF were collected for the determination of IL-1β (A, B), TNF (C, D) and IL-6 (E, F) by ELISA (n = 8-11 independent experiments).

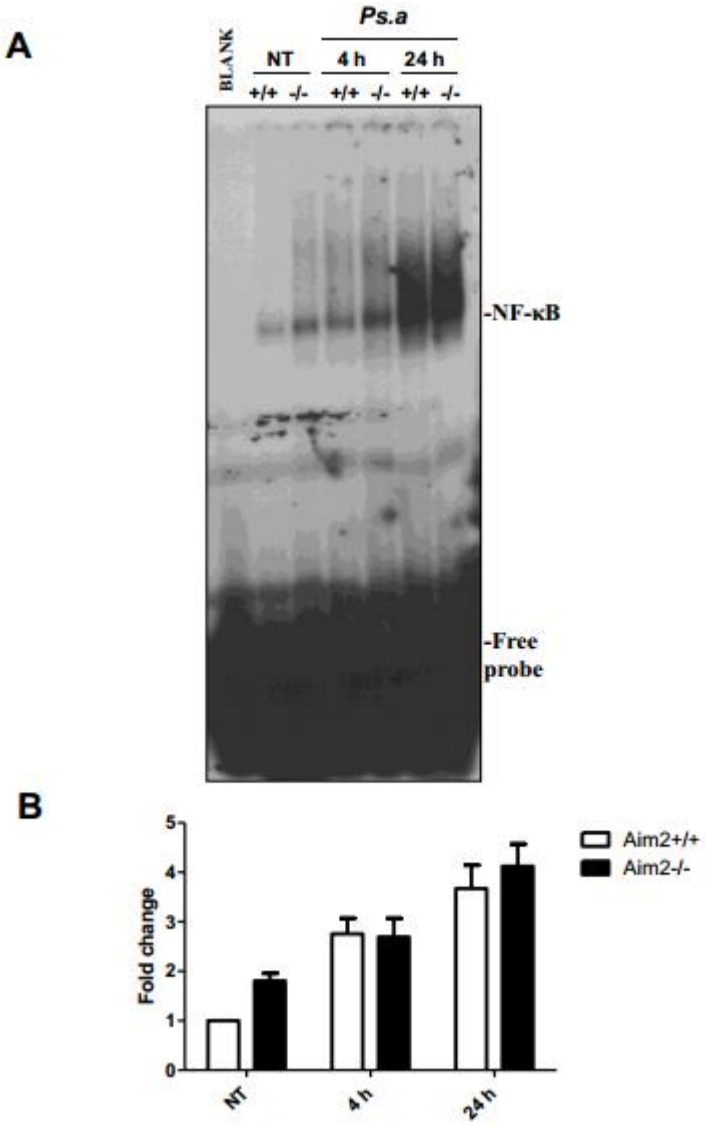

Figure 2.6: Aim2-deficiency does not significantly affect NF-κB activation *in vivo* following *P. aeruginosa* infection. Wild type (+/+) and Aim2-deficient (-/-) mice were infected intranasally with 1 X 10^7 (4 h) or 1 X 10^9 (24 h) CFU/mouse *P. aeruginosa* strain 8821 or equivalent volume of saline (NT). Nuclear proteins were extracted from long tissues and subjected to EMSA by incubation with ^{32}P-labeled NF-κB DNA probes (A). Scan densitometry was performed for analysis (B), and data are expressed as fold change versus wild-type untreated lung (n = 6 independent experiments).

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CHAPTER 3: REGULATOR OF CALCINEURIN 1 NEGATIVELY REGULATES TLR-MYD88-DEPENDENT SIGNALING

3.1 Abstract

Toll-like receptors (TLRs) are expressed in a wide range of cells. TLRs recognize the conserved molecular patterns in microorganisms. Activation of TLRs triggers myeloid differentiation factor 88 (MyD88)-dependent and/or -independent pathways. However, the molecular mechanisms involved in regulation of TLR signaling pathways are still under investigation. In this study, we identified regulator of calcineurin 1 (RCAN1) as a novel negative regulator of TLR-MyD88-dependent signaling in a mouse model of acute pneumonia using *P. aeruginosa* LPS. RCAN1-deficient mice display prolonged NF-κB activity associated with enhanced proinflammatory cytokine production and neutrophil recruitment upon *P. aeruginosa* LPS treatment in lung. The MyD88-independent pathway is less affected by RCAN1. Moreover, we found that the phosphorylation of IκBα is enhanced in RCAN1-deficient bone-marrow derived dendritic cells, which leads to upregulated NF-κB activity. Together, our findings reveal that RCAN1 play a negative role in regulation of TLR-MyD88-dependent signaling.

3.2 Introduction

Toll-like receptors (TLRs) are a family of pattern-recognition receptors (PRRs) that play an essential role in activation of innate immunity by binding to the pathogen-associated molecular patterns (PAMPs) (1). They are located on cell membranes or

intracellular vesicle membranes, and a total of 12 functional TLRs has been identified in animals (2). Activation of TLRs by specific ligands triggers two distinct downstream signaling pathways, myeloid differentiation primary-response protein 88 (MyD88) pathway and Toll/IL-1R domain-containing adaptor protein inducing interferon-β (TRIF) pathway that induce expression of various genes (1, 3). The MyD88-dependent signaling pathway ultimately leads to activation of NF-κB through phosphorylation and degradation of the inhibitory protein IκB. This phosphorylation results in the dissociation of IκB from NF-κB. The liberated NF-κB then translocates into the nucleus and activates expression of NF-κB–dependent proinflammatory genes, including IL-β, IL-6, TNF-α (4, 5). Moreover, the MyD88-dependent signaling pathway has been found to be essential for the host defense against the infections of microbial pathogens, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (6, 7). The TRIF-dependent signaling pathway activates interferon regulatory factors (IRF)-3 and IRF-7, which regulate the gene expression of IFN-α and -β (4). This MyD88-independent signaling pathway also plays a role in host defense against microbial infections by mediation of proinflammatory cytokine production and neutrophil recruitment (8-10).

TLR signalling must be tightly regulated. Unrestrained production of proinflammatory mediators may be harmful to the host, leading to development of autoimmune diseases (11). A novel negative regulator of proinflammatory responses, regulator of calcineurin-1 (RCAN1), was recently identified (12-14). The human *RCAN1* gene, previously known as *DSCR1* (Down syndrome critical region 1) (15), *MCIP1* (myocyte-enriched calcineurin interacting protein 1) (16), *Adapt78* (17) and *calcipressin*

(18), is located on chromosome 21 within the Down syndrome critical region. It is highly expressed in various tissues, including the brain, heart, muscle, liver, kidney, lung and testis (15, 19, 20). The *RCAN1* gene consists of seven exons, and the exon 1 to 4 can be alternatively spliced into 4 different transcripts (isoforms *RCAN* 1.1 to *RCAN* 1.4). RCAN 1.1 and RCAN 1.4 are two predominant isoforms that play a regulatory role in inflammatory responses (21).

Nuclear factor of activated T cells (NFAT) pathway has been implicated in production of a variety of proinflammatory cytokines, including IL-2, IL-3, IL-4, GM-CSF and TNF-α (22-26). RCAN1 is able to suppress the NFAT-mediated gene expression through inhibition of calcineurin (18). Calcineurin is a heterodimeric protein, consisting of catalytic subunit calcineurin A and a regulatory subunit calcineurin B for calcium binding (27). RCAN1 interacts with calcineurin A and inhibits the calcineurin-dependent phosphatase activity, preventing NFAT activation (18).

RCAN1 is also able to negatively regulate the NF-κB signalling pathway through enhancing the stability of IκB, which would remain bound to the NF-κB (14). A previous study in our lab showed that RCAN1 down-regulates the inflammatory responses during *P. aeruginosa* acute lung infection. RCAN1 deficiency elevates the NF-κB activation and production of a variety of proinflammatory cytokines (13). However, the molecular mechanisms involved in the regulation of *P. aeruginosa*-induced TLR signaling pathway by RCAN1 have not yet been elucidated.

To examine the regulatory role of RCAN1 in TLR signaling, we use a lung infection model of acute pneumonia that employs *P. aeruginosa* lipopolysaccharide (LPS), which triggers potent inflammatory responses through activation of TLR4 (22). We found that RCAN1 deficiency leads to enhanced neutrophil recruitment in lung and MyD88 pathway-mediated proinflammatory cytokine production both *in vitro* and *in vivo* following *P. aeruginosa* LPS stimulation. The increased production of proinflammatory cytokines was associated with enhanced IκBα phosphorylation and a prolonged NF-κB activation in RCAN1-deficient mice. These findings suggest that RCAN1 is a negative regulator of TLR-MyD88-dependent signalling.

3.3 Methods and Materials

Animals

The RCAN1 gene was targeted for deletion by standard homologous recombination in embryonic stem cells (Sv129 strain), followed by generation of chimeric mice, which were subsequently bred to pass the targeted allele into the germline in the C57BL/6 genetic background, as described elsewhere (28). These mice were originally provided by Dr. Jeffery Molkentin (Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, OH). C57/BL6 mice were purchased from Charles River Laboratories and bred in the same facility as RCAN1 deficient mice were used as wild-type controls. All animal protocols were approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with guidelines of the Canadian Council on Animal Care.

Antibodies and reagents

Abs to total IKKα/β, total p38 MAPK, actin, and all HRP-linked secondary Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Abs to phospho-IκB, total IκB, phospho-IKKα/β, phospho-ERK, total ERK, phospho-JNK, total JNK, phospho-p38 were purchased from Cell Signalling Technology (Danvers, MA). *P. aeruginosa* LPS was purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and activation

Bone marrow-derived dendritic cells (BMDCs) were used for *in vitro* study. Bone marrow cells were flushed from C57BL/6 or RCAN1$^{-/-}$ mice femurs and tibias and cultured in complete medium (RPMI 1640 containing 10% FBS, 1% Penicillin/Strep and 5% X63-conditioned supernatant). Media were changed every 2-3 days by placing half initially. Cells were cultured for 7 days until they are mature, and then treated with *P. aeruginosa* LPS for various time points. After incubation, cell-free supernatants were collected for measuring cytokines by ELISA. Cell pellets were lysed for Western blotting.

Cytokine production

The concentrations of IL-1β, IL-6, TNF, MIP2, RANTES and IP-10 in the lungs, bronchoalveolar lavage fluid (BALF) and cell-free supernatants were determined by ELISA as described previously using Ab pairs from R&D System (Minneapolis, MN), and developed with an amplification system (Invitrogen) (29).

Western blotting

Cells were lysed in RIPA buffer supplemented with a cocktail of protease and phosphatase inhibitors. Cells lysates (30 μg) were subjected to electrophoresis in 12% SDS-polyacrylamide gels. Gels were transferred to polyvinylidene difluoride (PVDF) membrane, blocked with 5% non-fat milk powder, blotted with primary and secondary antibodies, and detected by an ECL detection system (Western Lightning Plus-ECL; PerkinElmer).

Luciferase assay

Luciferase assay was performed as previously described (30). Briefly, 4 X 10^6 BMDCs were cotransfected with 3 μg of pNF-κB-Luc (Agilent Technologies) and 1 μg of pRL-TK (Promega) using mouse dendritic cell nucleofector kit (Amaxa) and the Amaxa Nucleofector Device. After electroporation, BMDCs were plated in culture medium and allowed to recover for 24 h. Subsequently, the cells were stimulated with 2 μg/ml *P. aeruginosa* LPS for 8 h. Firefly and Renilla activities were quantified using a dual-luciferase reporter assay system (Promega).

Lung stimulation with *P. aeruginosa* LPS, collection of lung and BALF

Mice were intranasally stimulated with *P. aeruginosa* LPS at 5 μg/g of body weight for 4 h or 24 h. After intranasal administration for 4 h or 24 h, BALF was obtained by lavaging the lung with 3 X 1 ml of PBS containing soybean trypsin inhibitor (100 μg/ml). The lung tissues were obtained for detection of cytokines and myeloperoxidase (MPO).

Lung tissues were homogenized at maximum speed for 10s in 50 mM HEPES buffer (4 μl/mg lung) containing soybean trypsin inhibitor (100 μg/ml). For cytokines and MPO

assays, the homogenate was centrifuged at 4^{o}C for 30 min at 18000 × g. The supernatants were stored at -80^{o}C for later cytokine analysis. The pellets were resuspended and homogenized in 0.5% cetyltrimethylammonium chloride (CTAC) (4 µl/mg lung) and centrifuged again at 18000 × g for 30 min. The clear extracts were collected for MPO assay.

BALF was centrifuged at 480 × g for 5 min at 4^{o}C for detection of cytokines and MPO activity. The supernatants were used for cytokine analysis. The cell pellets were resuspended in 1 ml of NH_4Cl buffer to lyse erythrocytes and centrifuged. The supernatants were discarded. The pellets were resuspended in 0.5% CTAC (250 µl/mouse) and centrifuged again. The clear extracts were collected for MPO assay.

MPO assay

The MPO assay was used to determine the infiltration of neutrophils into the lungs of the mice as described previously (31). Briefly, samples in duplicate (75 µl) were mixed with equal volumes of the substrate (3,3′,5,5′-tetramethyl-benzidine dihydrochloride, 3 mM; Resorcinol, 120 µM; and H_2O_2, 2.2 mM) for 2 minutes. The reaction was stopped by adding 150 µl of 2 M H_2SO_4. The optical density was measured at 450 nm.

EMSA analysis

Nuclear protein extracts were obtained using a nuclear extract kit (Active Motif, Carlsbad, CA), according to the manufacturer's protocol. EMSA performed as previously described (32). Briefly, 10 µg of extracted nuclear protein was added into 10 µl of binding buffer (Promega E358A) with 1 µg of poly(deoxyinosinic-deoxycytidylic acid)

(GE healthcare) and incubated for 15 min at room temperature. The labeled double-stranded oligonucleotide was added into each reaction mixture, which was incubated for 30 min at room temperature and separated by electrophoresis on a 6% polyacrylamide gel in 0.5 X Tris–boric acid–EDTA buffer. Gels were vacuum dried and subjected to autoradiography. The following synthesized double-stranded oligonucleotides were used: NF-κB binding consensus sequence, 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (Promega, Madison, WI) and ISRE binding consensus sequence, 5′-TTTCAGTTCTCCCTTTCAGTTTTC-3′.

Statistics

Data are presented as the mean ± SEM of the indicated number of experiments. Statistical significance was determined by assessing means with ANOVA and the Tukey-Kramer multiple comparison tests, or by using an unpaired *t* test. Differences were considered significant at $p < 0.05$.

3.4 Results

RCAN1-deficent mice display enhanced neutrophil infiltration *in vivo* following *P. aeruginosa* LPS stimulation. TLR signaling is essential for recruitment of neutrophil to the infection site following *P. aeruginosa* infection (7, 9). To determine whether RCAN1 has an effect on TLR-mediated neutrophil recruitment, RCAN1-deficient and wild-type mice were infected intranasally with *P. aeruginosa* LPS at 5 μg/g body weight for 4 h or 24 h. Lung tissues and BALF were collected for detection of neutrophil infiltration by MPO assay. The MPO activity of RCAN1-deficient mice was greatly enhanced in lung at

4 h and 24 h (Fig. 3.1A). However, no significant differences of MPO activity were observed in the BALF at both time point (Fig. 3.1B). These results suggest that RCAN1-decifiency leads to increase of neutrophil recruitment into the lungs.

RCAN1-deficent mice have increased proinflammatory cytokine production in response to *P. aeruginosa* LPS stimulation. Activation of TLR4 by LPS triggers two distinct signaling pathways, MyD88- and TRIF-dependent pathways (3). The MyD88-dependent signaling pathway activates NF-κB, which mediates the production of various proinflammatory cytokines and chemokines (4, 5). The TRIF-dependent pathway is able to activate IRF-3 and IRF-7. IRF-3 has been found to regulate gene expression of RANTES (CCL5) and IP-10 (CXCL10) in addition to IFN-β (33, 34). To examine whether RCAN1 affects the TLR-mediated proinflammatory cytokine production, lung and BALF supernatants were collected for determination of IL-1β, IL-6, TNF, and MIP2, that are mediated through MyD88-dependent pathway, as well as RANTES (Fig. 3.2I and 3.2J) and IP-10 (Fig. 3.2K and 3.2L) that are regulated through TRIF-dependent pathway. We observed that the levels of IL-1β (Fig. 3.2A), IL-6 (Fig. 3.2C), TNF (Fig. 3.2E) and MIP2 (Fig. 3.2G) were significantly increased at 4 h or 24 h in the lungs of RCAN1-deficient mice compared with that in wild-type mice. Similar result was also found in BALF except no significant difference of IL-1β was observed, which is due to little amount of IL-1β produced in the BALF of both RCAN1-deficient and wild-type mice (Fig. 3.2B, D, F, H). In contrast, the production of RANTES and IP-10 in lungs and BALF were not significantly enhanced in RCAN1-deficient mice compared with that in wild-type mice except the RANTES production in BALF at 24 h (Fig. 3.2I-L). These data

suggest that RCAN1 plays a negative regulatory role in the proinflammatory cytokine production mediated through TLR-MyD88 pathway, but has minor effect on TRIF pathway.

RCAN1-deficient dendritic cells display enhanced production of proinflammatory cytokines following treatment with *P. aeruginosa* LPS. Dendritic cells (DCs) are resident immune cells in the airways important for host defense against pathogen infection and linking the host innate and adaptive immunity (35). To examine the effect of RCAN1 on TLR-mediated proinflammatory cytokine production *in vitro*, the BMDCs from RCAN1-deficient and wild-type mice were treated with 1 μg/ml *P. aeruginosa* LPS for 3 h and 6 h. Cell free supernatants were collected for detection of IL-1β, IL-6, TNF, MIP2, RANTES and IP-10 by ELISA. *P. aeruginosa* LPS-induced IL-1β is not detected in both RCAN1-deficient and wild-type BMDCs, whereas the other cytokines and chemokines started to increase at 3 h following stimulation. The levels of IL-6 (Fig. 3.3B), TNF (Fig. 3.3C), MIP2 (Fig. 3.3D), RANTES (Fig. 3.3E) and IP-10 (Fig. 3.3F) were significantly enhanced in the supernatants of RCAN1-deficient BMDCs compared with wild type BMDCs at 3 h or 6 h. This result suggests that RCAN1 negatively regulates the proinflammatory cytokine production mediated through both MyD88 and TRIF pathways *in vitro*.

RCAN1 deficiency leads to increase of *P. aeruginosa* LPS-induced IκBα phosphorylation *in vitro*. To determine the regulatory mechanisms of RCAN1 on TLR signalling *in vitro*, we further set out to characterize the phosphorylation levels of IκB kinase (IKK) and IκBα. The IKK is in the downstream of MyD88-dependent pathway.

Activation of IKK leads to phosphorylation and degradation of the IκB, which results in the dissociation of IκB from NF-κB. The liberated NF-κB then translocates into the nucleus and activates NF-κB–dependent proinflammatory gene expression (3). BMDCs from wild-type and RCAN1-deficient mice were stimulated with 1 μg/ml *P. aeruginosa* LPS for various time points and subjected to Western blot analysis. The *P. aeruginosa* LPS-induced IKKα/β phosphorylation was increased and reached its highest level around 30 min (Fig. 3.4A). However, no significant differences of IKKα/β phosphorylation levels were found between RCAN1-deficient and wild-type BMDCs (Fig. 3.4B). IκBα is a downstream protein of IKK. The IκBα phosphorylation levels in RCAN1-deficient BMDCs were significantly enhanced at 20 min and 30 min compared with that in wild-type BMDCs (Fig. 3.4A and 3.4C). These findings suggest that RCAN1 can decrease the phosphorylation of IκBα.

TLR signalling pathway is also able to activate mitogen activated protein kinases (MAPK) family, including p38, ERK, and JNK, through activation of TNF receptor-associated factor 6 (TRAF6) (3). To determine whether RCAN1 plays a role on MAPK pathways, the cell lysates were subjected to Western blotting for phospho-ERK1/2 phospho-JNK, and phospho-p38, as well as their total protein levels. Results show that phosphorylation of the three MAPK proteins were induced by *P. aeruginosa* LPS but no significant differences were observed between wild-type and RCAN1-deficient BMDCs (Fig. 3.4D), suggesting that RCAN1 does not affect the TLR-mediated MAPK pathways.

Enhanced proinflammatory cytokine production in RCAN1-deficient mice is associated with prolonged NF-κB activation following *P. aeruginosa* LPS lung

infection. The TLR-mediated MyD88 and TRIF pathways activate transcriptional factor NF-κB and IRF3 respectively (4). IRF3 induces transcription through binding to the interferon-stimulated response element (ISRE) within the promoters (36). We next assessed the *P. aeruginosa* LPS-induced NF-κB and ISRE activity in the lungs of RCAN1-deficient and wild-type mice by EMSA. The activity of NF-κB was greatly increased at 4 h in the lungs of both RCAN1-deficient and wild-type mice. Interestingly, the NF-κB activity in the lungs of wild type mice was decreased and almost returned to the base line level at late 24 h time point, whereas the moderate decrease of NF-κB activity was observed in the lungs of RCAN1-deficient mice (Fig. 3.5A and Fig. 3.5C). The NF-κB activity was further confirmed in BMDCs upon *P. aeruginosa* LPS treatment by luciferase assay. BMDCs from wild-type and RCAN1-deficient mice were transfected with an NF-κB luciferase reporter plasmid and an internal control plasmid. Cells were then treated with 2 μg/ml *P. aeruginosa* LPS for 8 h. The activity of NF-κB was significantly enhanced in RCAN1-deficient BMDCs compared with wild type BMDCs (Fig 3.5E). The ISRE activity in lungs of RCAN1-deficient and wild-type mice was greatly induced by *P. aeruginosa* LPS at both 4 h and 24 h (Fig. 3.5B). However, no statistically significant difference was found between RCAN1-deficient and wild-type mice (Fig. 3.5D).

3.5 Discussion

Innate immune system employs a variety of PRRs to sense the conserved molecular pattern on pathogen, initiating inflammatory responses (37). TLRs are highly conserved PRRs that possess an extracellular domain for ligand binding, a transmembrane domain

and an intracellular domain for signalling (38). After binding to specific ligands, the intracellular domain of TLRs recruits adaptor molecules, MyD88, MyD88-adaptor-like (MAL), TRIF and TRIF-related adaptor molecule (TRAM), which triggers two distinct signaling pathways, MyD88 and TRIF pathway (39). Both signaling pathways are essential for host defense against microbial infection (6-10). TLR signaling must be tightly controlled to avoid inappropriate activation or suppression, which can be fatal to the host (11). The molecular mechanisms involved in the regulation of TLR signalling are still under investigation. We previously demonstrated that a novel protein RCAN1 overexpressed in Down Syndrome (DS) patients functions as a negative regulator of inflammation during *P. aeruginosa* lung infection (13), which could explain the increased severity and persistent respiratory tract infections in DS individuals (40). In this study, we directly examined the regulatory role of RCAN1 on TLR4 signaling using *P. aeruginosa* LPS stimulation both *in vivo* and *in vitro*. Our data suggest that RCAN1 negatively regulates the TLR-MyD88 signaling pathway.

Neutrophils play an essential role in bacterial clearance from the lung. Recruitment of neutrophils to infection site is mediated by chemokines produced through TLR signaling pathway (41, 42). We found that *P. aeruginosa* LPS induced-neutrophil recruitment in the lung is significantly enhanced in RCAN1-decificant mice, which is associated with increased production of cytokines and chemokines, including IL-1β, IL-6, TNF and MIP2. The chemokine MIP2 directly attracts neutrophils to the infection site, and the cytokines, IL-1β and TNF, have found to be able to indirectly recruit neutrophils (43, 44). The production of RANTES and IP-10 in the lungs had no statistically

significant difference between RCAN1-deficient and wild-type mice. Although the RANTES and IP-10 production was found to be enhanced in RCAN1-deficient BMDCs, the overall amount of RANTES and IP-10 produced in the lungs have no difference between the two type of cells due to involvement of various immunes cells sensing *P. aeruginosa* LPS. These findings suggest that RCAN1 negatively regulates the TLR-MyD88-dependent pathway mediated cytokine and chemokine production, leading to the increase of neutrophil recruitment in airways.

IL-1β is a key proinflammatory cytokine synthesized as an inactive pro form upon TLR pathway activation, and requires cleavage to become active by a cytoplasmic protease called caspase-1 (45). We found that IL-1β is produced in the lungs in response to *P. aeruginosa* LPS stimulation. LPS can extracellularly activate TLR4 on cell membrane but how it activates caspase-1 in cytosol remains unclear. One possible explanation is that the LPS-induced inflammatory responses cause host lung tissue damage, which releases cellular contents including caspase-1 and pro-IL-1β into extracellular environment. Caspase-1 is then activated by LPS and cleaves pro-IL-1β into mature form. Caspase-1 has been previously shown to be released into extracellular environment, and the activity of caspase-1 can be detected *in vitro* (46-48).

We further investigated the molecular mechanisms involved in the regulation of MyD88 signaling pathways by RCAN1. The phosphorylation level of IκBα was enhanced in RCAN1-deficient BMDCs, whereas the phosphorylation level of IKKα/β was not affected. This finding is consistent with a previous study which shows RCAN1 increases the stability of IκBα, leading to down-regulated NF-κB activation (14). The regulation of

IκB by RCAN1 can be achieved through calcineurin-independent mechanisms as well. Calcineurin has been shown to increase the activity of NF-κB through inhibition of IκB function (49). Thus, the negative regulatory effect of RCAN1 on calcineurin decreases the NF-κB activation. Our data suggest that RCAN1 has a negative effect on IκBα phosphorylation but not on its upstream signaling molecule IKK.

After disassociation from IκB, NF-κB migrates into nucleus and mediates proinflammatory gene expression (1). We found that a prolonged NF-κB activity at late 24 h in the lungs of RCAN1-deficient mice in response to *P. aeruginosa* LPS stimulation, suggesting that the NF-κB activity is persisted without regulation by RCAN1. This further confirmed *in vitro* using luciferase assay. The NF-κB activity is significantly enhanced in RCAN1-deficient BMDCs at 8 h.

In summary, our results suggest that RCAN1 plays a negative regulatory role on TLR-MyD88 dependent signaling pathway. Following *P. aeruginosa* LPS stimulation, RCAN1 deficiency enhances neutrophil recruitment, proinflammatory cytokine production and NF-κB activation. RCAN1 could be a potential therapeutic target for treatment of respiratory infections.

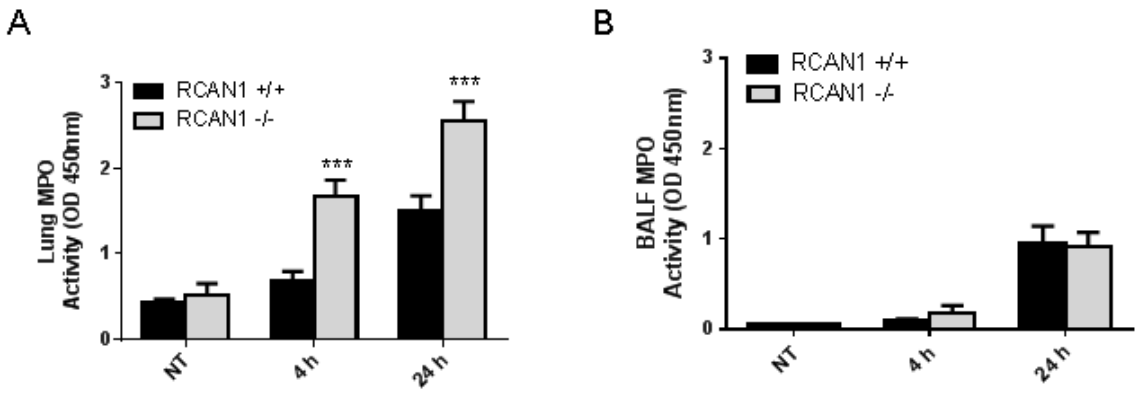
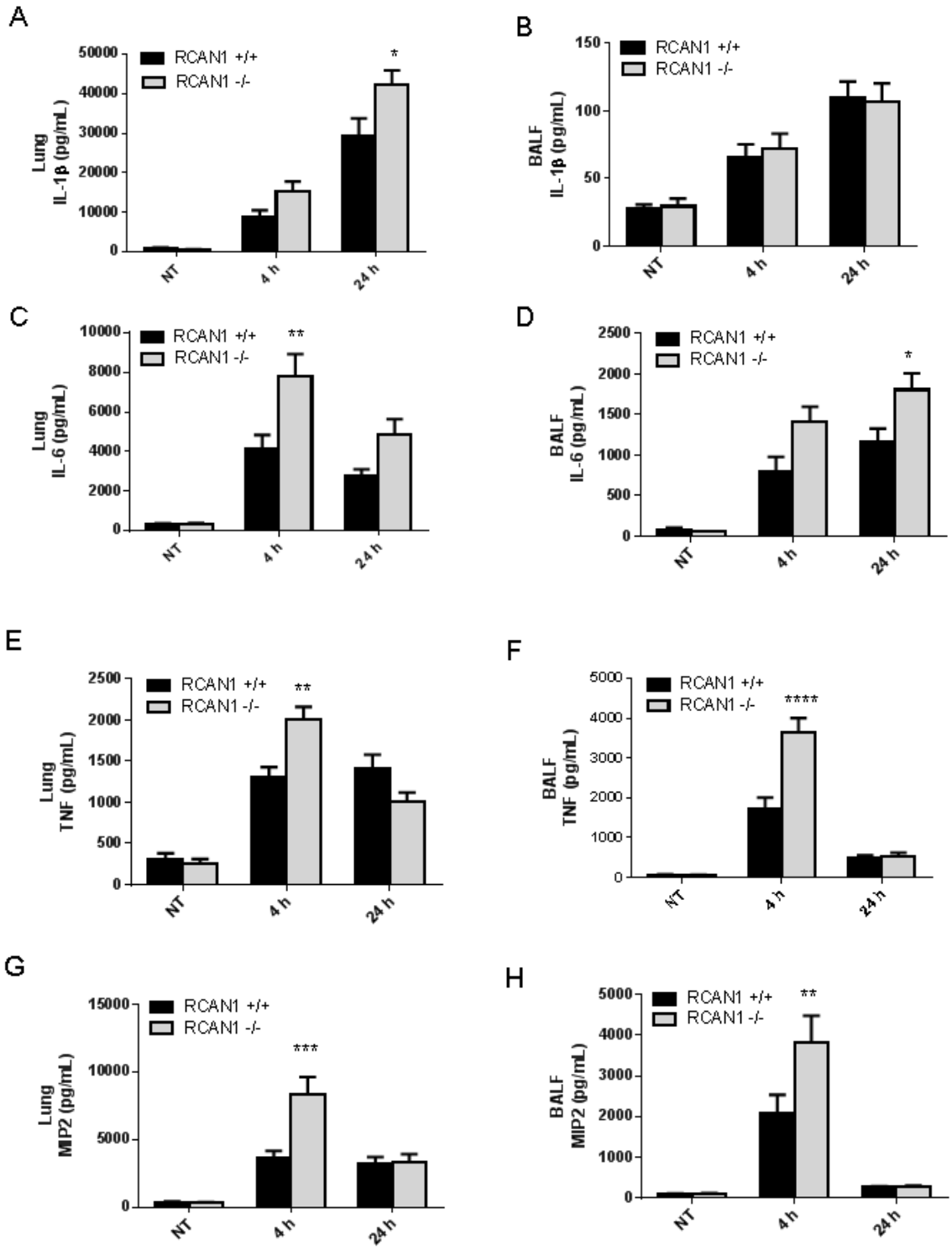


Figure 3.1: RCAN1-deficient mice display enhanced neutrophil infiltration in lung following *P. aeruginosa* LPS stimulation. Wild-type (+/+) and RCAN1-deficient (-/-) mice were infected intranasally with *P. aeruginosa* LPS at 5 µg/g of body weight, or an equivalent volume of saline as a control (NT) for 4 h or 24 h. Lungs and BALF were collected after 4 h or 24 h. MPO activity were measured in the Lung (A) and BALF (B) lysate (n=9 ± SEM, ***p<0.0001).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Lung
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RCAN1 -/-
BALF
IL-1β (pg/mL)
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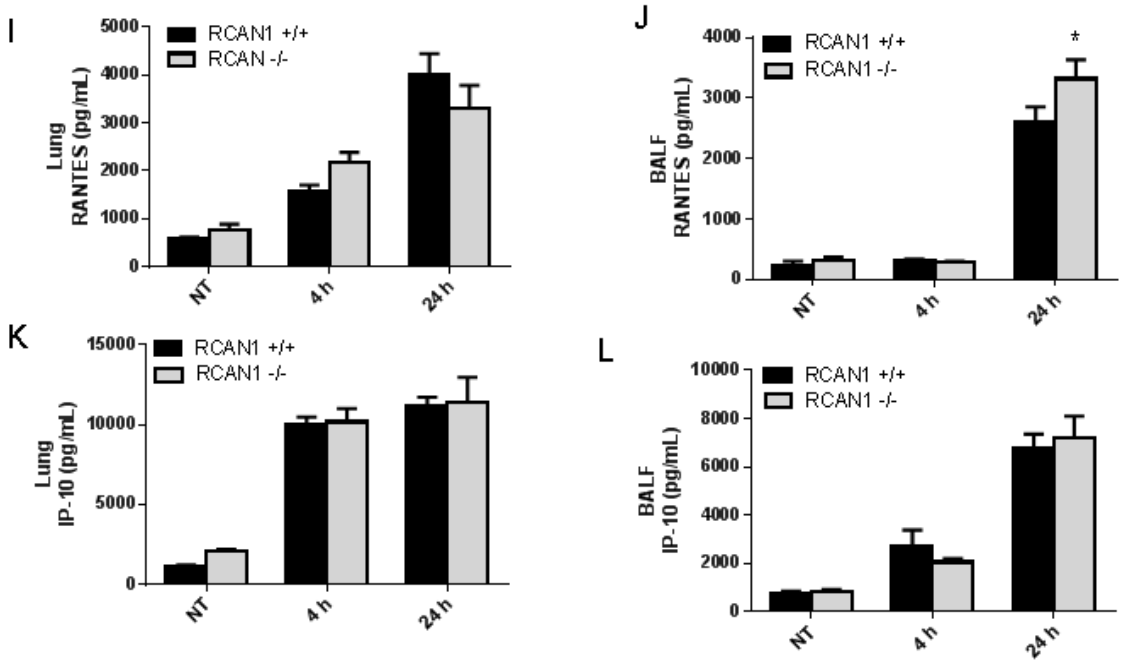

Figure 3.2: RCAN1-deficient mice display enhanced production of proinflammatory cytokines in the lung in response to *P. aeruginosa* LPS stimulation. Wild-type (+/+) and RCAN1-deficient (-/-) mice were infected intranasally with *P. aeruginosa* LPS at 5 µg/g of body weight, or an equivalent volume of saline as a control (NT) for 4 h or 24 h. After 4 h or 24 h, lung tissues and BALF were collected for the determination of IL-1β (A, B), IL-6 (C, D), TNF (E, F), MIP2 (G, H), RANTES (I, J) and IP-10 (K, L) production by ELISA . (n=9 ± SEM, *p<0.05, **p<0.001, ***p<0.0001, ****p<0.00001).

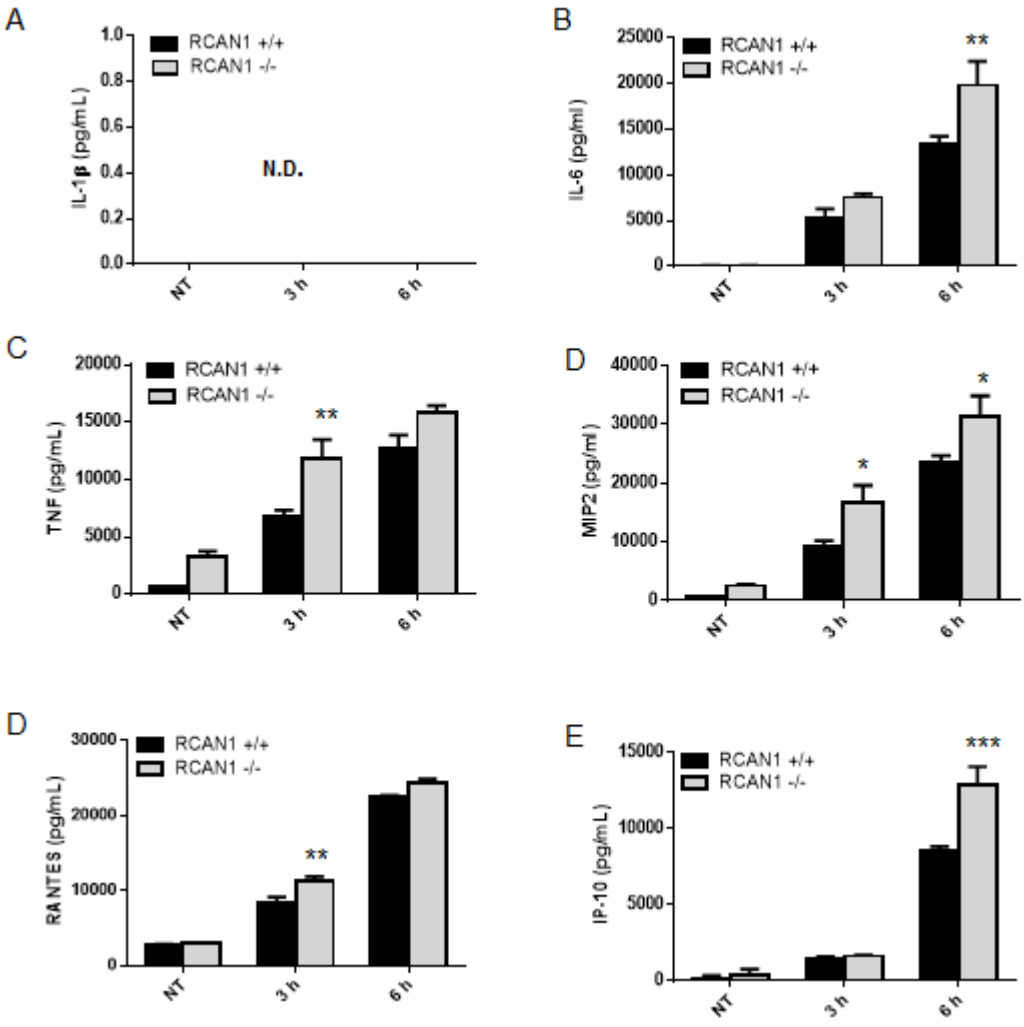

Figure 3.3: RCAN1-deficient dendritic cells display enhanced production of proinflammatory cytokines following *P. aeruginosa* LPS stimulation. Wild-type (+/+) and RCAN1-deficient (-/-) BMDCs were treated with 1 ug/ml *P. aeruginosa* LPS for 3 h, 6 h or left untreated (NT). Cell supernatants were collected for the determination of IL-6 (A) TNF (B), MIP2 (C), RANTES (D) and IP-10 (E) secretion by ELISA. (n=3 ± SEM, *p<0.05, **p<0.001, **p<0.0001).

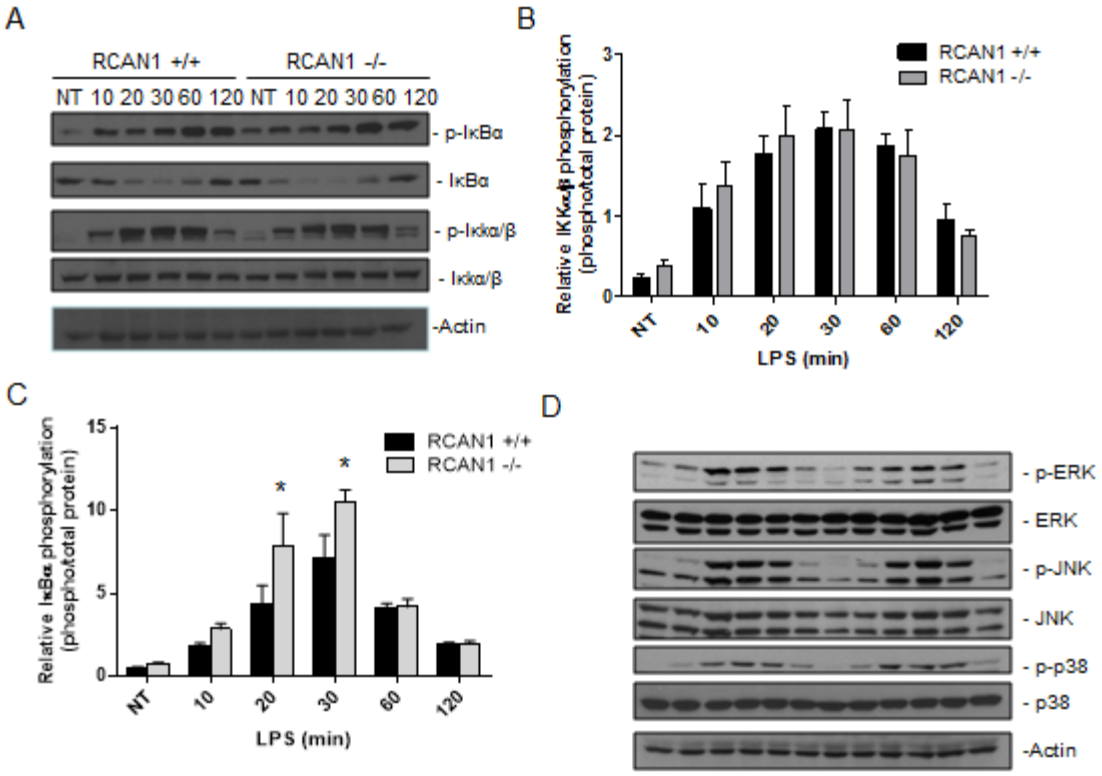

Figure 3.4: RCAN1 deficiency increases *P. aeruginosa* LPS-induced IκBα phosphorylation *in vitro*. Wild-type (+/+) and RCAN1-deficient (-/-) BMDCs were treated with 1 ug/ml *P. aeruginosa* LPS for indicated times or left untreated (NT). Cell lysates were subjected to Western blot for phospho- and total IKKα/β, IκBα, as well as actin as loading control. Blots are representative of three independent experiments. Densitometry analysis of phosphorylated IKK α/β (B) and IκBα (C) were normalized to their total protein respectively (n=3 ± SEM, *p<0.05). RCAN1 deficiency has no effect on MAPK signaling. Cell lysates were analyzed by Western blot for the indicated phosphorylated and total proteins. Data shown is a representative from three independent experiments (D).

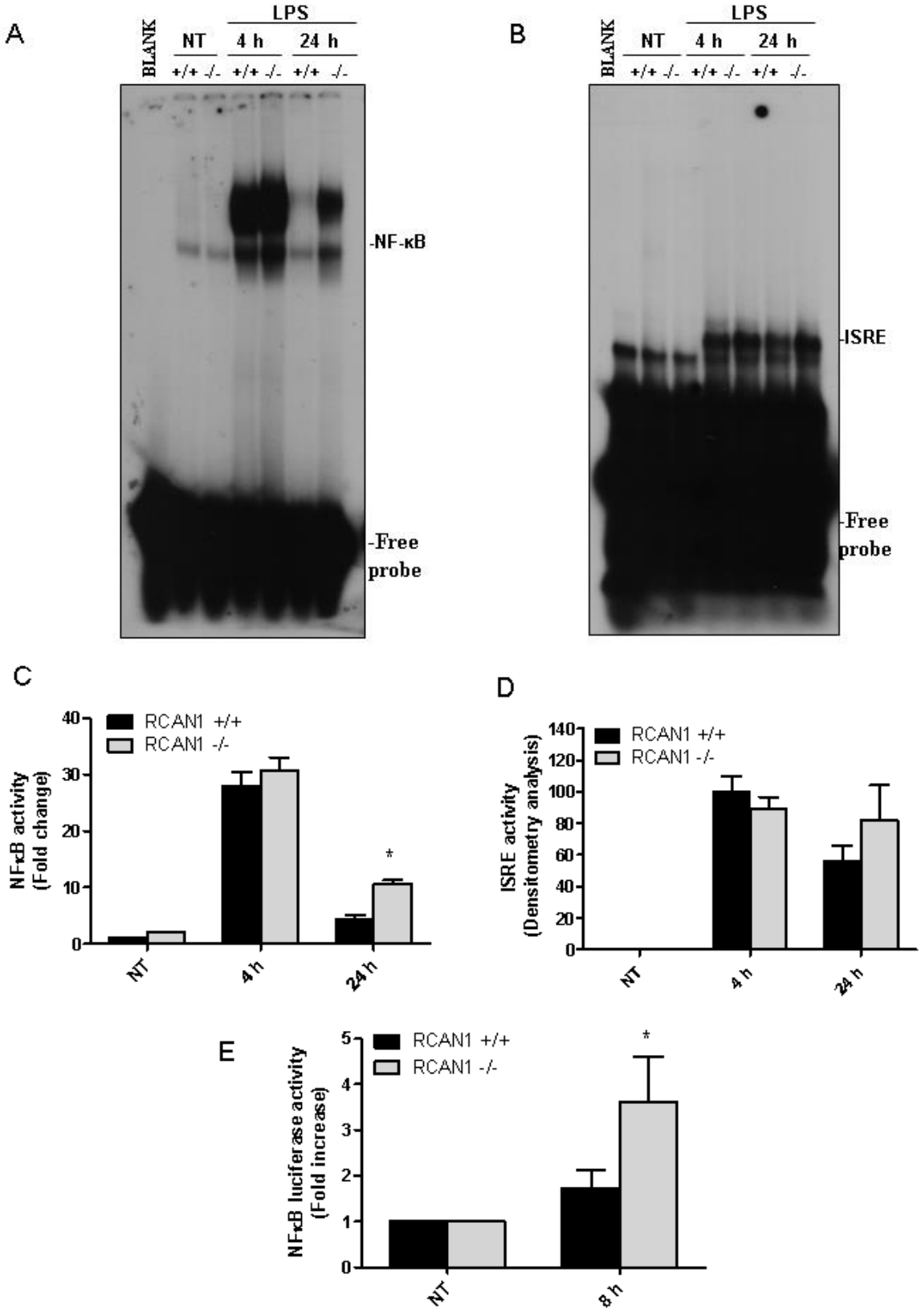
A
BLANK
NT
LPS
4 h
24 h
+/+ -/- +/+ -/- +/+ -/-
-NF-κB
-Free probe
B
BLANK
NT
LPS
4 h
24 h
+/+ -/- +/+ -/- +/+ -/-
-ISRE
-Free probe
C
NFκB activity (Fold change)
RCAN1 +/+
RCAN1 -/-
40
30
20
10
0
*
NT
4 h
24 h
D
ISRE activity (Densitometry analysis)
RCAN1 +/+
RCAN1 -/-
140
120
100
80
60
40
20
0
NT
4 h
24 h
E
NFκB luciferase activity (Fold increase)
RCAN1 +/+
RCAN1 -/-
5
4
3
2
1
0
*
NT
8 h

Figure 3.5: Effects of RCAN1-deficiency mice on NF-κB and ISRE activation following *P. aeruginosa* LPS stimulation. Wild-type (+/+) and RCAN1-deficient (-/-) mice were administered intranasally with *P. aeruginosa* LPS at 5 μg/g of body weight, or an equivalent volume of saline as a control (NT) for 4 h or 24 h. Nuclear proteins were extracted from long tissues and subjected to EMSA by incubation with ^{32}P-labeled NF-κB (A) or IRSE DNA probes (B). Data are representative of six individual experiments. Scan densitometry was performed for analysis of NF-κB (C) and ISRE (D) activation, and data are expressed as fold change versus wild-type untreated lung (n=6 ± SEM, *p<0.05). (E) BMDCs were cotransfected with pNF-κB–Luc and the control reporter plasmid pRL-TK. After transfection, cells were left untreated (NT) or stimulated with LPS (2 ug/ul) for 8 h. Firefly and Renilla activities were quantified sequentially using a dual luciferase reporter–assay system (n=5 ± SEM, *p<0.05).

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CHAPTER 4: DISCUSSION

4.1 Crosstalk between TLRs and NLRs

Recognition of microbial pathogens is essential for initiation of innate immune responses. Host immune system employs a variety of PRRs including TLRs, NLRs, RIG-like receptors (RLRs) and C-type lectin receptors (CLRs) to detect the conserved structures on pathogens, known as pathogen-associated molecular patterns (PAMPs) (1). Upon binding to the PAMPs, the PRRs trigger various downstream signaling pathways that are necessary for elimination of invading pathogens from host. The immune processes usually require activation of at least two different PRRs (2). The crosstalk between these PRRs confers a more efficient and integrated defense mechanism for host immunity. TLR and NLR signalling have found to act synergistically in both innate and adaptive immune system (3-8).

In host innate immunity, nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2, the members of NLR family, synergize with TLRs in production of various cytokines to enhance host defense against bacterial infection and activation of the antigen-presenting cell, such as DC (3, 4, 7). Another subfamily of NLR, NALPs, cooperate together with TLRs for production and maturation of IL-1β during microbial infection (9). In addition, the crosstalk between TLRs and NLRs has been identified in pathogenesis of type 2 diabetes (5). In host adaptive immunity, activation of NOD1 and NOD2 has found to be able to promote the TLR-induced B cell proliferation (8).

One of the well-documented examples is the cooperation between TLRs and NLRs for mediation of IL-1β production. IL-1β is a key proinflammatory cytokine that plays a critical role in mediation of innate and adaptive immune responses (10, 11). Production of IL-1β requires activation of both TLRs and NLRs. TLRs mainly recognize the PAMPs of extracellular microbial pathogens, and trigger the NF-κB-mediated gene expression of pro-form of IL-1β. NLRs are responsible for detecting the components of microorganisms released into host cytosol. Activation of NLRs leads to assemble into inflammasomes, which cleaves the pro-IL-1β synthesized via TLR signaling pathway into active form (9). Additionally, some studies revealed that the TLR-induced NF-κB up-regulates the gene expression of NLRs, and activation of TLR signaling enhances NLR responses (12, 13). Our lab is interested in studying the molecular mechanisms of host defense against *P. aeruginosa* infection. To date, TLR1, -2, -4, -5, -6 and -9 have been found to be involved in the recognition of *P. aeruginosa in vivo* and *in vitro* (14-17). NLRC4 is the most well characterized NLR that is activated by *P. aeruginosa* flagellin with a T3SS-dependent manner (18). Deficiency in either of the two pathways can cause failure of IL-1β production during *P. aeruginosa* infection (16, 18). Thus, the cooperation of TLRs and NLRs is essential for mediation of IL-1β production in the host defense against *P. aeruginosa* infection.

4.2 RCAN1 is involved in regulation of IL-1β production

RCAN1 is a well characterized inhibitor of NFAT signaling through interaction with calcineurin and suppressing its phosphatase activity (19). RCAN1 is also able to inhibit the NF-κB signaling through stabilization of IκB-NF-κB interactions, which can be

calcineurin-dependent and calcineurin-independent (20-23). Overexpression of RCAN1 attenuates NF-κB activity by increasing IκB stability in a calcineurin-independent manner (20). For the calcineurin-dependent mechanisms, calcineurin has found to be able to inactivate IκB and elevate NF-κB activation (21-23). It is possible that RCAN1 inhibits the calcineurin activity, leading to reduction of NF-κB activation. Additionally, the RCAN1 gene transcription is induced by NF-κB, and it is independent of calcineurin-NFAT signaling (24). This negative feedback loop NF-κB-RCAN1-IκB avoids excessive activation of TLR signaling in host immunity. We identified the negative regulatory role of RCAN1 in TLR-MyD88-dependent pathway using a lung infection model of acute pneumonia that employs *P. aeruginosa* LPS. The RCAN1-deficient mice display enhanced IL-1β production in lungs compared to wild-type mice following *P. aeruginosa* LPS treatment. We found that *P. aeruginosa* LPS induced-phosphorylation of IκBα is enhanced in RCAN1-deficient mice. These data suggest that RCAN1 may directly or indirectly affect the phosphorylation of IκBα. However, the specific mechanism remains to be explored. The molecular target of RCAN1 on TLR-MyD88 signalling pathway will be further investigated in our future studies.

4.3 A redundant role of AIM2 inflammasome in host defense against *P. aeruginosa* infection

In Chapter 2, we set out to examine the role of AIM2 inflammasome during *P. aeruginosa* infection. In order to minimize activation of NLRC4 inflammasome, a mucoid strain of *P. aeruginosa* 8821 which has reduced expression of flagellin was used both *in vitro* and *in vivo* experiments. We found that *P. aeruginosa* infection induces

AIM2 mRNA expression but degradation of AIM2 protein, suggesting a potential involvement of AIM2 in *P. aeruginosa* infection. The degradation of AIM2 was detected on Western blotting. Our study did not investigate the mechanisms involved in degradation of AIM2. Autophagy may down-regulate the level of AIM2 during *P. aeruginosa* infection. Recently, our laboratory discovered induction of autophagy activity by *P. aeruginosa*. One recent study found that the destruction of AIM2 inflammasome is mediated by increased autophagy activity (25, 26).

Surprisingly, no significant differences in cytokine production, neutrophil recruitment and bacterial clearance were found between wild-type and AIM2-deficient mice following *P. aeruginosa* infection. It suggests that AIM2 inflammasome plays a redundant role in host defense against *P. aeruginosa* infection. AIM2 inflammasome may have a minor contribution in IL-1β production. However, this study can be improved by examining the infection induced pyroptotic cell death by cytotoxic assay and AIM2 activation in cells by immunofluorescence.

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