REGULATION OF INFLAMMATION DURING *PSEUDOMONAS AERUGINOSA*
LUNG INFECTION

by

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that is a common cause of nosocomial infections in immunocompromised individuals. The healthy individuals effectively clear *P. aeruginosa* infections through tightly controlled inflammatory responses. Dysregulated inflammation increases host susceptibility to *P. aeruginosa* infections and causes severe lung damage. The molecular mechanisms governing the inflammatory responses to *P. aeruginosa* infections remain incompletely defined. Herein, I identified a novel role of regulator of calcineurin-1 (RCAN1) in *P. aeruginosa* lipopolysaccharide (LPS)-activated TLR4 signaling, and a detrimental role of early growth response 1 (Egr-1) in host defense against *P. aeruginosa* lung infection. RCAN1, a small evolutionarily conserved protein that inhibits calcineurin phosphatase activity, functions as a negative regulator of inflammation. I found that RCAN1 downregulates myeloid differentiation primary response 88 (MyD88)-NF-κB pathway through inhibition of IκBα phosphorylation, and promotes activation of TIR-domain-containing adapter-inducing interferon-β (TRIF)-interferon-stimulated response element (ISRE) pathway through regulation of IRF7 activation and expression. Egr-1 is a zinc-finger transcription factor that controls inflammatory responses. I demonstrated that Egr-1 promotes inflammation associated with increased mortality, and negatively regulates nitric oxide production for bacterial clearance during *P. aeruginosa* lung infection. Together, these findings improve our understanding of regulatory mechanisms involved in host defense against *P. aeruginosa* infections in innate immunity, and suggest that RCAN1 and Egr-1 could be potential therapeutic targets to enhance bacterial clearance or reduce the risk of systemic inflammation.

List of Abbreviations Used

AHL	N-acyl homoserine lactone
ASK1	apoptosis signal-regulating kinase 1
ATF2	activating transcription factor 2
BIR	baculoviral inhibition of apoptosis repeat
BALF	bronchoalveolar lavage fluid
BMDC	bone marrow-derived dendritic cell
BMM	bone marrow-derived macrophage
BMK1	mitogen-activated protein kinase 1
CARD	caspase activation and recruitment domain
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony-forming unit
C4-HSL	N-butanoyl-L-homoserine lactone
CLR	C-type lectin receptor
CIITA	MHC-II transactivator
dsRNA	double-stranded RNA
DAMP	damage-associated molecular pattern
DMEM	Dulbecco's Modified Eagle Medium
DSCR1	critical region gene 1
DYRK1A	dual specificity tyrosine phosphorylation-regulated kinase 1A
Egr-1	early growth response 1
EGF	epidermal growth factor

EGFR	epidermal growth factor receptor
EMSA	electrophoretic mobility shift assay
EPS	extracellular polymeric substance
ERK	extracellular signal-regulated kinase
GADD153	growth arrest and DNA damage 153
GM-CSF	macrophage colony-stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
ISRE	interferon-stimulated response element
IKK	IκB kinase
LRRK2	leucine-rich repeat kinase 2
KC	keratinocyte chemoattractant
LBP	LPS binding protein
LB	Luria-Bertani
IFN	interferon
LIX	lipopolysaccharide-inducible CXC chemokine
LPS	lipopolysaccharide
IPAF	interleukin-converting enzyme protease-activating factor
IP-10	IFN-γ inducible protein-10
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory transcription factor
ISRE	IFN-stimulated response element

JNK	c-Jun N-terminal kinase
MyD88	myeloid differentiation primary response 88
MAL	MyD88-adaptor-like
MAPK	mitogen-activated protein kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MCIP1	myocyte-enriched calcineurin-interacting protein 1
MCP-1	monocyte chemoattractant protein 1
M-CSF	macrophage colony-stimulating factor
MD-2	myeloid differentiation protein-2
MEF2	myocyte enhancer factor 2
MLK	mixed-lineage kinase 2
MIP-1	macrophage inflammatory protein-1
MIP-2	macrophage inflammatory protein-2
MOI	multiplicity of infection
MPO	myeloperoxidase
MSK	mitogen- and stress-activated protein kinase
mTOR	mammalian target of rapamycin
NAB1	NGFI-A binding protein
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular trap
NFAT	nuclear factor of activated T cells
NLR	nucleotide binding and oligomerization domain-like receptors

NOS	reactive nitrogen species
TAK1	TGF-β-activated kinase 1
TAB	TAK1-binding protein
TAO	thousand-and-one amino acid
TBK1	TANK-binding kinase 1
TIRAP	TIR-associated protein
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethyl-benzidine dihydrochloride
TNF-α	tumor necrosis factor-alpha
Tollip	Toll interacting protein
T3SS	type III secretion system
Tpl2	tumor progression locus 2
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRAM	TRIF-related adaptor molecule
OprF	outer membrane protein F
OD	optical density
P. aeruginosa	*Pseudomonas aeruginosa*
PAMP	pathogen-associated molecular pattern
RANTES	regulated upon activation, normal T cell expressed and secreted
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
RIP1	receptor-interacting protein 1

PLC	phospholipase C
PKA	protein kinase A
PKC	protein kinase C
PRR	pattern recognition receptor
PYD	pyrin domain
RLR	retinoic acid-inducible gene-I-like receptor
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal RNA
3O-C12-HSL	N-(3-oxododecanoyl)-L-homoserine lactone
QS	quorum sensing
RCAN1	regulator of calcineurin 1
RIPA	radioimmunoprecipitation assay
ROS	reactive oxygen species
RT-qPCR	real time quantitative-PCR
SEM	standard error of the mean
STAT	signal transducer and activator of transcription
SH2	src homology 2
ssRNA	single-stranded RNA
TRAF6	tumor necrosis factor receptor associated factor 6
UBC-13	ubiquitin conjugating enzyme-13
UEV-1A	ubiquitin conjugating enzyme E2 variant-1

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Chapter One – Introduction

1.1 Overview

Pseudomonas aeruginosa is a Gram-negative bacterium that causes a wide range of life-threatening acute and chronic infections (Sadikot et al., 2005). *Pseudomonas aeruginosa* rarely causes infections in healthy individuals, but is an important opportunistic pathogen in immunocompromised individuals, leading to significant mortality and morbidity (Moradali et al., 2017). There is no vaccine currently available for this bacterial pathogen (Priebe and Goldberg, 2014). Eradication of *P. aeruginosa* has become a great challenge due to the ability of this bacterium to resist many of the currently available antibiotics (Lister et al., 2009). The infections caused by multidrug-resistant *P. aeruginosa* strains have emerged as a public health threat worldwide (Bassetti et al., 2018).

The innate immune system is the first line of defense against invading microorganisms (Albiger et al., 2007). Recognition of *P. aeruginosa* by innate immunity is mediated by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (Lavoie et al., 2011). PRRs are widely expressed by immune cells such as macrophages and dendritic cells, or non-immune cells such as epithelial cells and fibroblasts. Moreover, the PRR-mediated recognition of the pathogen-associated molecular patterns (PAMPs) on microorganisms initiates multiple signal transduction pathways, activating inflammatory responses that mediate host defense (Suresh and Mosser, 2013). Efficient clearance of pulmonary *P. aeruginosa* infections requires inflammatory cytokines and chemokines that direct immune cell recruitment to the site of infection (Lavoie et al., 2011). The levels of inflammatory cytokines and chemokines must be tightly regulated. Defective production of inflammatory mediators

results in persistent bacterial infections (Smith et al., 2009). By contrast, excessive production of inflammatory mediators is harmful to the host, which leads to severe tissue damage and systemic inflammation (Chen et al., 2018; Jaffer et al., 2010). Many positive and negative regulators of inflammation have been identified during *P. aeruginosa* infection in the past decades (Carrigan et al., 2010; Junkins et al., 2013a; Power et al., 2004; Yue et al., 2016). However, the complicated molecular mechanisms involved in regulation of inflammation during *P. aeruginosa* infection are not fully understood.

Regulator of calcineurin-1 (RCAN1) and early growth response 1 (Egr-1) have been identified as inflammatory regulators in the lung (Junkins et al., 2013a; Reynolds et al., 2006). In this work, I examined the role of RCAN1 and Egr-1 *in vitro* using macrophages, dendritic cells or neutrophils, and *in vivo* using a mouse model of acute bacterial pneumonia in response to *P. aeruginosa* infection or *P. aeruginosa* lipopolysaccharide (LPS) stimulation.

1.2 *Pseudomonas aeruginosa*

1.2.1 Pathogenesis of *P. aeruginosa* infection

Pseudomonas aeruginosa is an environmental ubiquitous bacterium that is harmless to healthy individuals but causes severe acute infections in immunocompromised patients and chronic infections in cystic fibrosis (CF) patients (Sadikot et al., 2005). It is one of the leading causes of nosocomial infections among patients with human immunodeficiency virus (HIV) infection, burn wounds, CF, cancer and organ transplants (Bodey et al., 1983).

1.2.1.1 Impact of *P. aeruginosa* infections in human diseases

1.2.1.1.1 Cystic fibrosis

CF is a genetic disorder that is caused by mutations in both copies of the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR), leading to formation of a thick, dehydrated and sticky mucus layer on the airway surfaces of CF patients, which hinders mucociliary clearance, reduces bacterial internalization by lung epithelial cells, and inhibits the functions of antimicrobial peptides (Davies, 2002). Thus, the thick mucus layer provides a favorable habitat for bacterial colonization and propagation. *Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacian* and *P. aeruginosa* are the most common bacterial pathogens in CF airways (Coutinho et al., 2008). Among these pulmonary bacteria, *P. aeruginosa* is the predominant bacterial pathogen causing chronic lung infection in CF patients (Bhagirath et al., 2016; Lipuma, 2010). The chronic infections caused by *P. aeruginosa* in the respiratory tract of CF patients leads to a decline in pulmonary function and ultimate mortality (Ciofu et al., 2013).

1.2.1.1.2 Immunocompromised host

An immunocompromised host is a patient who is not able to generate proper immune response because of impaired or weakened innate or adaptive immunity (Dropulic and Lederman, 2016). The innate immunodeficiency includes loss of physical barriers, defects in phagocytes and complement deficiency, whereas the adaptive immunodeficiency refers to defects in B and T lymphocytes (McCusker and Warrington, 2011). Immunocompromised patients are more susceptible to microbial infections, and the infections are usually acute and systemic, associated with organ failure and high mortality (George et al., 2014). For instance, *P. aeruginosa* caused wound infection in 14.5% of burn

patients (Coetzee et al., 2013). Bacteremia frequently occurred in *P. aeruginosa*-infected immunocompromised patients (Migiyama et al., 2016). A US burn center reported that the morality of the burn patients with *P. aeruginosa* bacteremia was 77% (McManus et al., 1985).

1.2.1.2 *P. aeruginosa* initial attachment to host

The pathogenesis of *P. aeruginosa* infection is multifactorial and complex. At initial attachment stage, *P. aeruginosa* utilizes two major adhesins, pili and flagella, to adhere lung epithelial cells through binding to the cell surface receptors (Bucior et al., 2012). The receptors for *P. aeruginosa* flagella and pili have been recognized as asialoglycolipids containing a disaccharide moiety, GalNAcβ1-4Gal, at terminal for binding (Thomas and Brooks, 2004). Among these asialoglycolipid receptors, asialoGM1 expression was found to be increased on the surface of CF respiratory epithelial cells (Saiman and Prince, 1993), and inhibition of asialoGM1 reduced *P. aeruginosa* adherence (Davies et al., 1999). Additionally, *P. aeruginosa* flagella and pili were shown to contribute to motility and biofilm formation (Conrad et al., 2011; O'Toole and Kolter, 1998).

1.2.1.3 *P. aeruginosa* virulence factors

Once initial foothold is established, *P. aeruginosa* employs several strategies to evade the host immune defense, including production of virulence factors, biofilm formation and phenotypic changes (Davies, 2002). *P. aeruginosa* virulence factors can be divided into two categories: cell surface and secreted molecules (Moradali et al., 2017). The cell surface virulence factors include flagella, pili, lipopolysaccharide (LPS), outer membrane protein F (OprF) and lectins (Sadikot et al., 2005). As mentioned above, *P. aeruginosa* flagella and pili are critical for initial stage of colonization (Bucior et al., 2012).

LPS, an important surface virulence factor of *P. aeruginosa*, consists of a lipid A, a core oligosaccharide, and a variable O-antigen polysaccharide, which activates host inflammatory response and induces cell death (Pier, 2007). *Pseudomonas aeruginosa* LPS was shown to play a protective role against serum-mediated lysis (Rocchetta et al., 1999). In addition, LPS has emerged as a phagocytic target. Pier et al. reported that lung epithelial cells used CFTR as a receptor for internalization of *P. aeruginosa* though binding to the core oligosaccharide of LPS (Pier et al., 1997). CF mice with ΔF508 mutation in CFTR displayed increased bacterial burden in lung compared to non-CF mice (Pier, 2000). The *P. aeruginosa* outer membrane protein OprF was found to bind to interferon (IFN)-γ, which resulted in production of virulence factors lectin LecA and pyocyanin (Wu et al., 2005). Lectins are bacterial outer membrane proteins that recognize host glycoconjugates and allow bacteria to adhere to the host tissues (Nilsson, 2003). *Pseudomonas aeruginosa* produces lectins LecA and LecB, which facilitated bacterial adhesion to lung epithelial cells and increased alveolar barrier permeability (Chemani et al., 2009). Moreover, *P. aeruginosa* lecB was identified to bind to exopolysaccharide Psl and contribute to biofilm structure (Passos da Silva et al., 2019)

The secreted virulence factors of *P. aeruginosa* include pyocyanin, pyoverdine, elastases, proteases, phospholipases, rhamnolipids, exotoxin A, effectors secreted by the type III secretion system (T3SS) and quorum sensing (QS) molecules, which can promote bacterial invasion and/or inhibit host immune response (Gellatly and Hancock, 2013). Pyocyanin, a blue redox-active secondary metabolite produced by *P. aeruginosa*, was found to damage host cell by inducing oxidative stress, disrupting host catalase and interfering with mitochondrial electron transport (Lau et al., 2004). Moreover, pyocyanin

has been found to induce neutrophil apoptosis and suppress acute inflammatory response *in vivo* (Allen et al., 2005). Pyoverdine is a siderophore of *P. aeruginosa* that acquires ferric iron from host cells (Kang et al., 2018). It was shown to upregulate production of pyoverdine itself and other virulence factors, such as exotoxin A and endoprotease (Lamont et al., 2002), and contribute to biofilm formation (Banin et al., 2005). *Pseudomonas aeruginosa* produces two elastases, LasA and LasB, which were critical for bacterial invasion by disrupting tight junctions between epithelial cells and immune evasion by degrading the components of the immune system, including immunoglobulin A and G, surfactant proteins, cytokines and complement protein C3 (Bastaert et al., 2018; Heck et al., 1990; Holder and Wheeler, 1984; Mariencheck et al., 2003; Nomura et al., 2014; Saint-Criq et al., 2018). Alkaline protease inhibited phagocytosis and killing of *P. aeruginosa* by neutrophils through cleavage of complement protein C2, and it prevented immune recognition by degrading exogenous flagellin (Casilag et al., 2016; Laarman et al., 2012). The *P. aeruginosa* phospholipase PLC has cytolytic activity, which disrupted host cell membrane (Titball, 1993). Rhamnolipid is a glycolipid biosurfactant that promoted infiltration of *P. aeruginosa* by disrupting the tight junctions between epithelial cells (Zulianello et al., 2006), and it also played a role in maintenance of biofilm architecture (Davey et al., 2003). Exotoxin A (ExoA) is a highly toxic virulence factor of *P. aeruginosa* that was secreted into the extracellular environment and inhibited host protein synthesis by catalyzing ADP-ribosylation of elongation factor 2 (Michalska and Wolf, 2015). T3SS is a major determinant of virulence that has a needle structure to directly inject cytotoxic effector proteins into the cytoplasm of host cells (Coburn et al., 2007). Four T3SS effectors, ExoU, ExoS, ExoT, and ExoY, have been identified in *P. aeruginosa* (Hauser, 2009).

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ExoU is a potent A2 phospholipase that causes a rapid and robust host cell lysis by cleaving the membrane phospholipids (Sato and Frank, 2004). ExoS and ExoT are bi-functional effector proteins that possess GTPase activating protein activity on N-terminal domain and an ADP-ribosyltransferase activity on C-terminal domain (Barbieri and Sun, 2004), and they have been found to induce apoptosis, block reactive oxygen species (ROS) production and inhibit phagocytic activity of neutrophils (Cowell et al., 2000; Rangel et al., 2014; Sun et al., 2012; Vareechon et al., 2017). ExoY, an nucleotidyl cyclase, increases intracellular accumulation of cyclic nucleotides, and it has been found to disrupt endothelial cell barrier integrity and vascular repair following lung injury by inducing microtubule breakdown (Morrow et al., 2017; Stevens et al., 2014). *Pseudomonas aeruginosa* secretes a number of QS molecules, including two N-acyl homoserine lactone (AHL) signal molecules, N-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL), and a *Pseudomonas* quinolone signal (PQS) molecule, 2-heptyl-3-hydroxy-4-quinolone (Willcox et al., 2008). The 3O-C12-HSL, C4-HSL and PQS bind to and activate their cognate transcription factors LasR, RhlR and PqsR respectively, inducing biofilm formation and expression of various virulence factors including elastases, proteases, pyocyanin, lectins, rhamnolipids and toxins (Lee and Zhang, 2015; Maura and Rahme, 2017; Rutherford and Bassler, 2012; Yang et al., 2009a). Furthermore, the PQS production was found to be negatively regulated by Rhl system and positively regulated by Las system, while the Rhl signal molecule C4-HSL was upregulated by both Las and PQS systems (Moradali et al., 2017; Wade et al., 2005).

1.2.1.4 *P. aeruginosa* biofilm formation and its impact on host defense

A biofilm is an aggregate of microorganisms that adhere to each other on a living or non-living surface, and are embedded within a self-produced matrix of extracellular polymeric substances (EPSs), including exopolysaccharides, proteins, metabolites and extracellular DNA (Das et al., 2013; Donlan, 2002). The development of bacterial biofilm comprises four stages: irreversible adherence of planktonic cells to a surface, formation of microcolonies in EPS matrix, maturation of biofilm structure, and dispersal of cells from the biofilm to other surfaces (Rasamiravaka et al., 2015). The regulation of *P. aeruginosa* biofilm mensuration mainly depends on QS systems and an intracellular second messenger c-di-GMP (Harmsen et al., 2010).

Bacterial biofilm plays a critical role in persistent infections (Chen and Wen, 2011). *Pseudomonas aeruginosa* biofilm prevents antimicrobial penetration, protects bacteria from host immune detection and induces persister cell formation (Mulcahy et al., 2014). Biofilm matrix not only decreases antibiotics penetration but also directly sequesters antibiotics (Hall and Mah, 2017). A study by Billings et al. demonstrated that Psl, a polysaccharide in *P. aeruginosa* biofilm matrix, physically sequestered polymyxin B and tobramycin through electrostatic interactions (Billings et al., 2013). Biofilm has been shown to protect bacteria from host immune response by reducing penetration and causing lysis of immune cells (Alhede et al., 2014). The *P. aeruginosa* rhamnolipid has been characterized to function as a biofilm shield inducing lysis of polymorphonuclear leukocytes (Van Gennip et al., 2009). Persister cells comprise about 1% of biofilm cells, and are slow-growing, metabolically inactive and highly tolerant to antibiotics (Lewis, 2010; Wood et al., 2013). The majority of *P. aeruginosa* cells can be killed by antibiotics;

however, persisters are able to remain viable and repopulate biofilms due to the existence of a dormant state that shuts down the synthesis of the antibiotic targets (Lewis, 2010; Van den Bergh et al., 2017).

Pseudomonas aeruginosa undergoes numerous physiological and phenotypic changes during biofilm formation (Drenkard, 2003). For example, in CF chronic infection, *P. aeruginosa* strains convert to a mucoid phenotype that displays upregulated alginate production driven by the CF microenvironment, allowing for formation of biofilm colonies (Pritt et al., 2007). As mentioned earlier, the *P. aeruginosa* flagellum is critical for initial attachment to lung epithelial cells (Bucior et al., 2012). However, after surface attachment, *P. aeruginosa* significantly downregulates flagellum expression and may also permanently lose the flagellum due to genetic mutations, which reduces activation of the host immune response, thereby allowing *P. aeruginosa* to evade immune detection and phagocytosis (Jyot et al., 2007). LPS elicits a robust inflammatory response through binding to TLR4 (Park and Lee, 2013). The mucoid *P. aeruginosa* strains have been identified to display modified lipid A or lose O-antigen expression, which prevents antibiotic targeting and contributes to immune evasion (Ernst et al., 2007; Maldonado et al., 2016).

1.2.1.5 Mechanisms of antibiotic resistance in *P. aeruginosa*

Treatment of *P. aeruginosa* infections has become a great challenge due to the ability of this bacterium to resist many of the currently available antibiotics (Lister et al., 2009). Generally, the major mechanisms of *P. aeruginosa* used to counter antibiotic attack can be classified into intrinsic, acquired and adaptive resistance. The intrinsic resistance of *P. aeruginosa* includes low outer membrane permeability, expression of efflux pumps that expel antibiotics out of the cell and the production of antibiotic inactivating enzymes. The

acquired resistance of *P. aeruginosa* can be achieved by either horizontal transfer of resistance genes or mutational changes (Breidenstein et al., 2011). The adaptive resistance of *P. aeruginosa* involves formation of biofilm in the lungs of infected patients where the biofilm serves as a diffusion barrier to limit antibiotic access to the bacterial cells (Drenkard, 2003). In addition, multidrug-tolerant persister cells in the biofilm are able to survive antibiotic attack; these cells are responsible for prolonged and recurrent infections in CF patients (Mulcahy et al., 2010).

1.3 Recognition of *P. aeruginosa* by pattern recognition receptors

The innate immune cells utilize PRRs to recognize the conserved molecular patterns on microorganisms, leading to initiation of inflammatory response through activation of multiple cellular signaling pathways (Akira et al., 2006). PRRs can be present either on the cell surface or within intracellular compartments. They can be classified into four categories: TLRs, nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I-like receptors (RLRs) and C-type lectin receptors (CLRs) (Takeuchi and Akira, 2010). Among these receptors, TLRs and NLRs have been identified to play a critical role in initiation of inflammatory response during *P. aeruginosa* infection (Alhazmi, 2018; McIsaac et al., 2012)

1.3.1 Toll-like receptors

TLRs are evolutionarily conserved glycoprotein receptors that localize to cell surface or intracellular compartments, such as endoplasmic reticulum, endosome and lysosome. They comprise of an extracellular domain with leucine-rich repeats for ligand binding, a transmembrane domain and an intracellular Toll/IL-1 receptor (TIR) domain for initiating signalling (Jin and Lee, 2008). TLRs are expressed widely on a variety of immune

cells, including macrophages, dendritic cells, mast cells, B cells and T cells, and non-immune cells such as epithelial cells and fibroblasts (Akira et al., 2006). A total of 13 TLR family members has been identified in mammals. The cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, which primarily recognize microbial membrane components such as LPS, lipids, lipoproteins and flagellin, whereas the intracellular TLRs, TLR3, TLR7, TLR8, TLR9 and TLR13, recognize the nucleic acids of bacteria and viruses (Kawasaki and Kawai, 2014).

1.3.1.1 TLR ligands

TLR2 forms a heterodimer with TLR1 or TLR6, and recognizes a board range of microbial cell wall components including peptidoglycan, lipoprotein and lipoteichoic acid (Oliveira-Nascimento et al., 2012). TLR3 recognizes double-stranded RNA (dsRNA), an intermediate that is generated during viral infections (Zhang et al., 2013). TLR4 binds to LPS, an outer membrane component of Gram-negative bacteria (Chow et al., 1999). TLR5 is activated by flagellin, a structure protein of bacterial flagella (Feuillet et al., 2006). TLR7 recognizes guanosine- and uridine-rich single-stranded RNA (ssRNA) from virus (Zhang et al., 2016). Similarly, TLR8 also binds to ssRNA, and it is preferentially activated by adenosine- and uridine-rich ssRNA (Forsbach et al., 2008). TLR9 recognizes the unmethylated CpG-DNA motifs in bacterial and viral DNA (Cornelie et al., 2004). TLR10 is a pseudogene in mouse whereas human TLR10 has been identified to cooperate with TLR2 to bind to bacterial lipoproteins. However, this binding did not initiate TLR signaling transduction, suggesting that TLR10 plays an anti-inflammatory role in host defence against bacterial infection (Jiang et al., 2016; Oosting et al., 2014). In contrast, Lee et al. found that TLR10 contributed to cytokine production in response to influenza A virus

infection (Lee et al., 2014). TLR11 interacts with the flagellin FliC of *Salmonella typhimurium* and *Escherichia coli*, and the profilin of *Toxoplasma gondii* (Mathur et al., 2012; Yarovinsky et al., 2005). TLR12 functions either as a homodimer or a heterodimer with TLR11 to recognize *Toxoplasma gondii* profilin (Raetz et al., 2013). TLR13 is the receptor for bacterial 23S ribosomal RNA (rRNA) (Li and Chen, 2012).

1.3.1.2 TLR signaling

Activation of TLRs triggers two distinct signaling pathways, MyD88-dependent and TRIF-dependent pathways (Kawasaki and Kawai, 2014). Upon ligand binding, the intracellular TIR domains of TLRs recruit adaptor molecules, MyD88, TIR-associated protein (TIRAP)/MyD88-adaptor-like (MAL), TRIF or TRIF-related adaptor molecule (TRAM) (Brown et al., 2011). All TLRs except TLR3 utilizes MyD88. TLR2, TLR4 and TLR9 require an additional adaptor TIRAP for signal transduction (Kawasaki and Kawai, 2014). MyD88 interacts with interleukin-1 receptor-associated kinase (IRAK)-4 via death domain, and IRAK-4 then activates IRAK-1, which undergoes auto-phosphorylation at several sites and disassociates from MyD88. IRAK4 associates with a E3 ubiquitin ligase, tumor necrosis factor receptor associated factor 6 (TRAF6), along with ubiquitin conjugating enzyme-13 (UBC-13) and ubiquitin conjugating enzyme E2 variant-1 (UEV-1A), and triggers K63-linked polyubiquitination of TRAF6 (Jain et al., 2014). The ubiquitinated TRAF6 activates a complex containing TGF-β-activated kinase 1 (TAK1), TAB1 (TAK1-binding protein 1), TAB2, and TAB3. The activated TAK1 disassociates from the complex and triggers activation of NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways (Wang et al., 2001). In NF-κB pathway, TAK1 interacts with IκB kinase (IKK) complex, comprising of IKK-α, IKK-β and IKK-γ (NEMO), and

phosphorylates IKK-α and IKK-β subunits (Israel, 2010). Activation of IKK complex leads to phosphorylation of IκBα, a NF-κB inhibitory protein. The phosphorylated IκBα rapidly undergoes ubiquitination and degradation, allowing NF-κB translocate into the nucleus and mediate inflammatory gene expression (Oeckinghaus and Ghosh, 2009). TAK1 is a member of the MAP kinase kinase kinase (MAPKKK) family, and is able to activate the MAPKs, extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK), which lead to activation of transcription factor activator protein 1 (AP-1) (Ajibade et al., 2013).

TLR3 and TLR4 activate TRIF-dependent pathway. TLR4 requires an additional adapter protein, TRIF related adapter molecule (TRAM) to recruit TRIF (Yamamoto et al., 2003). TRIF associates with TRAF6 and TRAF3. TRAF6 recruits kinase receptor-interacting protein 1 (RIP1), which activates TAK1 complex (Walsh et al., 2015), leading to activation of NF-κB and MAPK pathways (Walsh et al., 2015), whereas TRAF3 recruits the IKK-related kinases TANK-binding kinase 1 (TBK1) and IKKε, resulting in phosphorylation of interferon regulatory transcription factor (IRF) 3 and IRF7 (Pham and Tenoever, 2010). The activated IRF3 and IRF7 translocate into nucleus and mediate gene expression of type I interferons, IFN-α and IFN-β (Honda et al., 2006).

1.3.1.3 Recognition of *P. aeruginosa* by TLRs

TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9 has been identified to recognize *P. aeruginosa* (Fig 1.1) (McIsaac et al., 2012). *Pseudomonas aeruginosa* infection was reported to induce upregulation of TLR2 expression (Power et al., 2004; Shin et al., 2013). However, TLR2-deficient mice had partially reduced cytokine production but no difference in neutrophil recruitment and bacterial clearance compared to wild-type mice in a mouse

model of *P. aeruginosa* pneumonia (Skerrett et al., 2007). It suggests that TLR2 is redundant in the host defense against to *P. aeruginosa* lung infection. TLR4 requires accessory proteins LPS binding protein (LBP) and CD14 to recognize LPS (Park and Lee, 2013). LPS first binds to LBP in serum, and is then transferred to CD14, which presents LPS to a TLR4-myeloid differentiation protein-2 (MD-2) complex (Maeshima and Fernandez, 2013; Wright et al., 1990). TLR4 has been shown to play an critical role in host defense against *P. aeruginosa* infections (Faure et al., 2004; Huang et al., 2006; Morris et al., 2009; Zhang et al., 2005b). Huang et al. demonstrated that TLR4-deficient mice were susceptible to *P. aeruginosa* corneal infection, displaying increased bacterial burden, and impaired iNOS and β-Defensin-2 production (Huang et al., 2006). Faure et al. showed that TLR4 deficiency increased lung epithelial injury, impaired cytokine production and reduced bacterial clearance, accompanying with increased level of bacteremia and mortality, in a mouse model of *P. aeruginosa* pneumonia (Faure et al., 2004). *Pseudomonas aeruginosa* flagellin, the ligand of TLR5, has been found to be sufficient to activate TLR-dependent inflammatory responses in alveolar macrophages and airway epithelial cells (Raoust et al., 2009). Moreover, the flagellin-deficient *P. aeruginosa* strains were not able to activate NF-κB in human airway epithelial cells (Zhang et al., 2005b). The flagellin-TLR5-MyD88-dependent signaling was reported to be responsible for early clearance of *P. aeruginosa* in lung (Anas et al., 2016), and this TLR5-mediated bacterial clearance was efficient at low-inoculum of *P. aeruginosa* (Morris et al., 2009). In contrast to TLR2, TLR4 and TLR5, TLR9 play a detrimental role in host defense against *P. aeruginosa* infections (Benmohamed et al., 2014). Benmohamed et al. demonstrated that TLR9-deficient mice had increased survival rate, associated with enhanced bacterial

clearance and elevated proinflammatory cytokine production, during *P. aeruginosa* lung infection (Benmohamed et al., 2014). Another study by Huang et al. silenced TLR9 in a mouse model of *P. aeruginosa* keratitis, and showed that TLR9 deficiency reduced the level of inflammation and caused less perforated corneas in infected mice (Huang et al., 2005).

1.3.2 NOD-like receptors

NLRs are intracellular sensors that initiate innate immune response by recognizing the PAMPs from microorganisms and the damage-associated molecular patterns (DAMPs) from host (Kim et al., 2016). There are 22 NLRs identified in human, and the NLR family proteins consists of a C-terminal leucine-rich repeats for ligand sensing, a central domain that facilitates self-oligomerization, and a N-terminal effector domain for protein-protein interaction (Saxena and Yeretssian, 2014). The NLRs can be classified into four subfamilies based on their N-terminal structure: the acidic transactivation domain (NLRA), the baculoviral inhibition of apoptosis repeat (BIR) domain (NLRB), the caspase activation and recruitment domain (CARD/NLCR), and the pyrin domain (PYD/NLRP). The NLRA subfamily includes only one member, MHC-II transactivator (CIITA). The NLRB subfamily also contains only one member, NAIP. The NLRC subfamily includes NOD1 (NLRC1), NOD2 (NLRC2), NLRC3, NLRC4, NLRC5 and NLRX1. The NLRP subfamily has 14 members, NLRP1-14 (Kim et al., 2016).

1.3.2.1 NLR functions

CIITA functions as a transactivator for MHC II gene expression (Choi et al., 2011). NOD1 and NOD2 are cytosolic sensors for bacterial peptidoglycan. NOD1 recognizes γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), a peptidoglycan component found in

most Gram-negative bacteria (Chamaillard et al., 2003). NOD2 recognizes muramyl dipeptide (MDP) found in the peptidoglycan of both Gram-positive and Gram-negative bacteria (Girardin et al., 2003). Upon ligand binding, NOD1 and NOD2 recruit RIP2 through CARD-CARD interactions, leading to activation of NF-κB and MAPK signaling pathways (Moreira and Zamboni, 2012). The other NLR members, NLRP1, NLRP2, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4, and NAIP, assemble into a multiprotein complex termed inflammasome, which is primarily expressed in myeloid cells, and recruits the inactive caspase-1 precursor and cleaves it into active form, in response to various PAMPs and DAMPs, including microbial components, ATPs, uric acid, alum, asbestos, silica, UV radiation and skin irritants (Fig 1.2) (Kim et al., 2016). Activation of caspase-1 results in maturation of two proinflammatory cytokines IL-1β and IL-18, which are produced as inactive pro-forms through NF-κB pathway (Fig 1.2) (Sahoo et al., 2011). Furthermore, inflammasome also induces a programmed cell death termed pyroptosis (Aachoui et al., 2013). Pyroptosis is caspase-1-dependent. The activated caspase-1 rapidly induces pore formation on plasma membrane, which disrupts cellular osmotic homeostasis, causing water influx, cell swelling and osmotic lysis ultimately (Bergsbaken et al., 2009). Pyroptosis is critical for eliminating the niches for intracellular bacterial replication and releasing inflammatory cellular contents to extracellular environment to induce inflammation (Jorgensen and Miao, 2015).

1.3.3.2 *P. aeruginosa* recognition by NLRs

Although *P. aeruginosa* is an extracellular bacterium, it has been found to survive and multiply inside epithelial cells and macrophages by producing a virulence factor MgtC (Belon et al., 2015; Bernut et al., 2015; Fleiszig et al., 1995). NOD1, NLRC4 and NLRP3

has been identified to play a role in recognition of the intracellular *P. aeruginosa* (Alhazmi, 2018). NOD1 is activated by *P. aeruginosa* peptidoglycan, and it induces NF-κB activation and mediates intracellular bacterial killing (Travassos et al., 2005). Furthermore, Irving et al. used *P. aeruginosa* outer membrane vesicles to deliver peptidoglycan into epithelial cells, and demonstrated that NOD1 interacted with peptidoglycan in early endosomes and promoted RIP2-dependent autophagy activity (Irving et al., 2014). Autophagy is an evolutionarily conserved process that degrades and recycles cellular organelles and long-lived proteins in eukaryotic cells (Yin et al., 2016). Autophagy has been identified to play an essential role in clearance of *P. aeruginosa* in macrophages and mast cells (Junkins et al., 2013b; Yuan et al., 2012). NLRC4, also known as interleukin-converting enzyme protease-activating factor (IPAF), was shown to recognize *P. aeruginosa* flagellin and activate caspase-1 in a T3SS-dependent manner (Miao et al., 2008). Sutterwala et al. reported that *P. aeruginosa*-induced caspase-1 activation was NLRC4-dependent but flagellin-independent, suggesting that the other *P. aeruginosa* molecular components may activate NLCR4 (Sutterwala et al., 2007). Furthermore, Miao et al. indicated that PscI, a T3SS rod protein of *P. aeruginosa*, was able to activate caspase-1 through NLRC4 (Miao et al., 2010). The mechanisms were further explored by Grandjean et al., showing that NLRC4 recognized the *P. aeruginosa* PscI and a T3SS needle protein PscF in combination with NAIP (Grandjean et al., 2017). NLRP3 is a well-characterized NLR family member that is capable of recognizing a broad range of stimuli, including both microbial and non-microbial ligands (Swanson et al., 2019). NLRP3 uses a N-terminal PYD domain to interact with the PYD domain of an adapter protein apoptosis associate speck-like containing a CARD domain (ASC), which recruits pro-caspase-1 via CARD-CARD

interactions (Abderrazak et al., 2015). NLRP3 has been found to be activated by *P. aeruginosa* (Deng et al., 2016; Rimessi et al., 2015). Rimessi et al. found that *P. aeruginosa*-dependent mitochondrial perturbation induced NLRP3 activation by accumulation of mitochondrial Ca^{2+}, promoting inflammatory response in CF airway epithelial cells (Rimessi et al., 2015). However, a study by Deng et al. reported that NLRP3 played a deleterious role in response to *P. aeruginosa* infection, which suppressed the macrophage-mediated intracellular killing of *P. aeruginosa* by enhancing autophagy activity. This finding is contradictory to the previous studies, which showed that autophagy enhanced bacterial killing (Junkins et al., 2013b; Yuan et al., 2012).

1.4 Airway epithelial cells in *P. aeruginosa* recognition and clearance

Airway epithelial cells are the first line of defense against invading pathogens by acting as a physical barrier that prevents penetration of pathogen into body, and sentinels that initiates inflammatory response, mediating recruitment and activation of immune cells to the infection sites (Eisele and Anderson, 2011). The airway epithelial cells can be divided into three types, ciliated cells, secretory cells and basal cells (Crystal et al., 2008). These cells are connected by junctional complexes consisting of tight junctions, adherens junctions, desmosomes and gap junctions, which form a barrier that separates host internal milieu from the external environment (Tsuji et al., 1969). The ciliated cells account for over 50% of epithelial cells in the airway, and have approximately 200 to 300 cilia on luminal surface of each cell (Tilley et al., 2015). The major function of ciliated cells is to sweep away the mucus-trapped foreign particles from the lung to the pharynx. This process is termed mucociliary clearance (Ganesan et al., 2013). Epithelial cells also function as a biochemical barrier by producing antimicrobial peptides that directly kill the invading

microorganisms (Nochi and Kiyono, 2006). The epithelial antimicrobial peptides include β-defensins, cathelicidin LL-37, calprotectin, secretory leukocyte protease inhibitor, adrenomedullin and neutrophil gelatinase-associated lipocalin; these molecules not only have antimicrobial activity but also enhance wound healing and regulate immune response by either promoting or inhibiting proinflammatory cytokine production, recruiting phagocytes and modulating adaptive immune functions (Bals and Hiemstra, 2004; McCormick and Weinberg, 2010).

Airway epithelial cells express most TLR family members, including TLR1-10, and the NLR family members NOD1 and NOD2 (Gomez and Prince, 2008). Upon microbial infection, airway epithelial cells are able to generate appropriate signaling to induce production of chemokines and cytokines that recruit and activate phagocytic cells to the infected airways (Eisele and Anderson, 2011). TLR5, the receptor of flagellin, has been shown to play a predominant role in recognition of *P. aeruginosa* and activation of NF-κB in human airway epithelial cells (Zhang et al., 2005b). As mentioned earlier, the mucoid *P. aeruginosa* strains lack flagellin. The CFTR of airway epithelial cells were reported to take the responsibility for recognition and clearance of mucoid *P. aeruginosa* by binding to LPS and initiating phagocytosis (Campodonico et al., 2008; Schroeder et al., 2002). Furthermore, the MyD88-dependent IL-1R signaling in airway epithelial cells was found to be critical for controlling *P. aeruginosa* lung infection through mediating production of cytokine IL-6, and chemokine macrophage inflammatory protein-1β (MIP-1β/CCL4), keratinocyte chemoattractant (KC/CXCL1), macrophage inflammatory protein-2 (MIP-2/CXCL2), which are important for neutrophil recruitment (Mijares et al., 2011).

1.5 Innate immune cells in *P. aeruginosa* recognition and clearance

Although epithelial cells greatly contribute to recognition and clearance of *P. aeruginosa* in early stage of infection, immune system requires professional immune cells such as macrophages, dendritic cells, mast cells and neutrophils to effectively combat this bacterium.

1.5.1 Alveolar macrophages

Alveolar macrophages are lung-resident sentinel cells found in pulmonary alveolus, and are one of the first responders to invading pathogens (Byrne et al., 2015). Alveolar macrophages are primary phagocytes that internalize and kill bacterial pathogens in uninflamed lower airways. Importantly, they also play a role in immunomodulation by producing proinflammatory cytokines and chemokines upon stimulation (Arango Duque and Descoteaux, 2014). *Pseudomonas aeruginosa* LPS and flagellin were found to activate the TLR4 and TLR5 of alveolar macrophages, and induce production of cytokines, tumor necrosis factor-alpha (TNF-α) and IL-6, and chemokine KC (Raoust et al., 2009). Depletion of alveolar macrophages from *P. aeruginosa*-infected mice reduced MIP-2 and KC production, leading to delayed neutrophil recruitment and decreased bacterial clearance (Kooguchi et al., 1998). In addition, the *P. aeruginosa*-induced autophagy in alveolar macrophages enhanced bacterial clearance *in vitro* (Yuan et al., 2012).

1.5.2 Dendritic cells

Dendritic cells are professional antigen presenting cells, bridging innate and adaptive immunity (Paul, 2011). Similar to alveolar macrophages, the lung-resident dendritic cells are located in the respiratory mucosa and alveoli (Kopf et al., 2015), and they uptake and process inhaled foreign antigens to activate naïve T cells (Condon et al.,

2011). Dendritic cells also play an important role in maintaining lung homeostasis and orchestrating host immune responses (Qian and Cao, 2018). *Pseudomonas aeruginosa* infection was reported to increase the number of dendritic cells in lung, and induce expression of co-stimulatory molecules CD80 and CD86 on the dendritic cell surface (Damlund et al., 2016). A study by Worgall et al. showed that the mice immunized with *P. aeruginosa*-pulsed dendritic cells had proliferative response of CD4^{+} T-cell and decreased mortality following a lethal intrapulmonary challenge with *P. aeruginosa* (Worgall et al., 2001). Pène et al. examined the role of dendritic cells during *P. aeruginosa* lung infection in a mouse model of post-sepsis. In this study, the mice with cecal ligation puncture-induced sepsis had functional defects in dendritic cells, and they displayed severe lung damage, disrupted IL-12/IL-10 balance and significant mortality followed by a secondary pulmonary infection with *P. aeruginosa*. Furthermore, administration of normal dendritic cells improved survival and restored cytokine balance (Pene et al., 2008).

1.5.3 Mast cells

Mast cells are found in mucosa and connective tissues throughout the body. Mast cell progenitors are released from bone marrow into bloodstream, and migrate to connective tissue, where they complete differentiation and become resident cells (Hallgren and Gurish, 2011). Mast cell granules contain various chemical and biological mediators, including histamine, heparin, cytokines, proteoglycans and neutral proteases. Upon stimulation, mast cells release preformed granule contents and newly synthesized mediators to extracellular environment, which is important for regulating vascular homeostasis, inflammation, angiogenesis and cell proliferation (Krystel-Whittemore et al., 2015). Mast cells are abundant in respiratory tracts, and act as sentinels of innate immunity

due to the presence of all types of PRRs, including TLRs, NLRs, CLRs and RLRs (Agier et al., 2018; Campillo-Navarro et al., 2014).

Mast cells have been identified to be involved in host response to *P. aeruginosa* infection (Junkins et al., 2014; Le et al., 2012). Mast cells generated different biological effects in response to *P. aeruginosa* LPS and whole-cell *P. aeruginosa*. Le et al. showed that mast cells were the pivotal sources of inflammatory cytokines, TNF-α, IL-1β, and IL-6, in response to *P. aeruginosa* LPS pulmonary stimulation in rats, and the LPS-activated mast cells reduced the gene expression of tight junction proteins claudin-1 and occludin in lung tissues, suggesting that mast cells may enhance epithelial permeability (Le et al., 2012). In contrast, Junkins et al. found that mast cell-deficient mice displayed increased airway epithelial permeability following *P. aeruginosa* lung infection. They further used a transwell co-culture model to identify that mast cell did not affect tight junction integrity but secreted an unknown factor to decrease epithelial cell apoptosis and TNF production (Junkins et al., 2014). Additionally, mast cells contributed to *P. aeruginosa* clearance by producing neutrophil chemoattractants, KC, MIP-2 and lipopolysaccharide-inducible CXC chemokine (LIX/CXCL5), promoting neutrophil transendothelial migration, and directly internalizing *P. aeruginosa* (De Filippo et al., 2013; Lin et al., 2002; Lukacs et al., 1998).

1.5.4 Neutrophils

Neutrophils, also known as polymorphonuclear leukocytes, are most abundant white blood cells in human circulation. They are important innate effector cells for controlling bacterial and fungal infections (Kruger et al., 2015). Patients with neutropenia, a disorder with low number of neutrophils, are highly susceptible to microbial infections (Barg et al., 2015). Neutrophils develop in bone marrow from haematopoietic stem cells,

and the mature neutrophils enter blood circulation. The circulating neutrophils are recruited to the site of infection by chemoattractants secreted by epithelial cells or tissue-resident leucocytes (Kim and Luster, 2015). Three major antimicrobial functions of neutrophils have been characterized: phagocytosis, degranulation of antimicrobial compounds, and release of neutrophil extracellular traps (NETs) to capture microbes extracellularly (Rosales, 2018). In addition, the neutrophil-produced serine proteases, ROS and reactive nitrogen species (NOS) are critical for bacterial killing (Teng et al., 2017). Neutrophils are short-lived with a half-life of 6 to 8 h (Summers et al., 2010). After clearance of infection, neutrophils spontaneously undergo apoptosis, and are cleared by resident macrophages and dendritic cells through phagocytosis (Greenlee-Wacker, 2016).

Pulmonary infection with *P. aeruginosa* was found to induce massive recruitment of neutrophils to the airways (Craig et al., 2009). *Pseudomonas aeruginosa* ExoU enhanced neutrophil transepithelial migration by inducing production of neutrophil-derived leukotriene B4 (Pazos et al., 2017). Neutrophils are critical for protecting host from *P. aeruginosa* lung infection. Neutrophil elastase is a serine protease stored in primary granule, and it has been found to mediate *P. aeruginosa* killing by degrading the outer membrane protein OprF (Hirche et al., 2008). Koh et al. showed that the mice with neutrophil depletion were very susceptible to *P. aeruginosa* infection even with low-dose inoculation, and reconstitution of neutrophil by granulocyte colony-stimulating factor (G-CSF) greatly increased survival rate (Koh et al., 2009). *Pseudomonas aeruginosa* also employs self-protective mechanisms to fight against neutrophil attack. As mentioned early, *P. aeruginosa* pyocyanin induced neutrophil apoptosis and suppressed acute inflammatory response *in vivo* (Allen et al., 2005). Moreover, *P. aeruginosa* ExoS has been found to

inhibit ROS production in human neutrophils by blocking nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity (Vareechon et al., 2017).

1.6 Cell signaling pathways in regulation of *P. aeruginosa*-induced inflammation

A tightly controlled inflammation is critical for host to effectively clear bacterial infection and maintain tissue homeostasis (Liu and Cao, 2016). Innate immune system employs multiple signaling pathways including NF-κB, TRIF-IRF3/7, NFAT/Calcineurin, MAPK and JAK/STAT pathways to regulate *P. aeruginosa*-induced inflammatory responses.

1.6.1 NF-κB pathway

NF-κB is a family of transcription factors that is expressed in almost all cell types and plays a pivotal role in regulation of diverse cellular processes, including inflammation, immune response, cell differentiation, proliferation, and survival (Oeckinghaus and Ghosh, 2009). NF-κB is comprised of five family members p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB and c-Rel, which are sequestered in cytosol by IκBs in the resting state (Christian et al., 2016). NF-κB is activated by a variety of stimuli, including environmental stress, and the ligands of cytokine receptors, TLRs, NLRs, TNF receptor superfamily, T-cell receptor and B-cell receptor (Liu et al., 2017). Upon stimulation, the NF-κB family proteins translocate to nucleus and bind to the κB enhancer of target genes as heterodimers or homodimers, and the p50/RelA and p50/c-Rel dimers are predominant NF-κB complexes in regulation of inflammatory response (Oeckinghaus and Ghosh, 2009). *Pseudomonas aeruginosa* lung infection strongly activated NF-κB, and the *P. aeruginosa*-induced NF-κB played an essential role in production of proinflammatory cytokines and

chemokines, neutrophil recruitment and bacterial clearance (Chen et al., 2008a; Mijares et al., 2011; Power et al., 2004).

1.6.2 TRIF-IRF3/7 pathway

The family of IRFs are transcription factors consisting of 9 members in mammals, and each IRF has a N-terminal DNA-binding domain with a helix-turn-helix structure recognizing a DNA consensus sequence known as IFN-stimulated response element (ISRE) and a C-terminal IRF association domain for interacting with other family members or other transcriptional factors (Yanai et al., 2012). IRF family proteins are originally identified as transcriptional factors of the Type I IFNs for antiviral defense, but the subsequent studies have revealed that IRFs are critical for modulating innate and adaptive immunity through regulating the development, survival and function of immune cells (Ozato et al., 2007; Taniguchi et al., 2001). IRF3 and IRF7 share a similar structure, and they are activated by TBK1 and IKKε in TRIF-dependent pathway, which contributes to host immune response to *P. aeruginosa* lung infection (Power et al., 2007). Once IRF3 and IRF7 are activated, they form either a homodimer or a heterodimer, and bind to ISRE DNA sequences (Taniguchi et al., 2001). IRF3 and IRF7 have been shown to promote neutrophil recruitment in *P. aeruginosa*-infected mice by inducing the production of specific chemokines (Carrigan et al., 2010; Yue et al., 2016). Carrigan et al. identified that the *P. aeruginosa*-induced production of chemokines RANTES (regulated upon activation, normal T cell expressed and secreted/CCL5) and IP-10 (IFN-γ inducible protein-10/CXCL10) was significantly reduced in IRF3-deficient mice, leading to impaired neutrophil recruitment and bacterial clearance (Carrigan et al., 2010). Furthermore, another study by

Yue et al. showed that IRF7 also contributed to RANTES and IP-10 production in response to *P. aeruginosa* lung infection (Yue et al., 2016).

1.6.3 NFAT/Calcineurin pathway

The nuclear factor of activated T cells (NFAT) family of transcription factors play an important role in T-cell development and function, which includes calcineurin-dependent (NFATc1, NFATc2, NFATc3, and NFATc4) and -independent (NFAT5) family members (Hogan et al., 2003). The NFAT family proteins shares a N-terminal NFAT-homology region containing regulatory motifs, a core REL homology region containing a transactivation domain and DNA-binding motifs, and a C-terminal domain (Macian, 2005). In canonical NFAT activation pathway, an increase of intracellular levels of calcium obtained from endoplasmic reticulum or the extracellular environment leads to activation of the calmodulin-dependent serine/threonine phosphatase calcineurin. The activated calcineurin dephosphorylates the serine residues in the regulatory domain of NFAT, leading to exposure of a nuclear localization signal in NFAT for nuclear translocation (Fig.1.3) (Vaeth and Feske, 2018). Once in the nucleus, NFAT mediates gene expression of immunomodulatory cytokines, important for immune cell activation and homeostasis (Fric et al., 2012). In the context of *P. aeruginosa* infection, we have identified that *P. aeruginosa* infection rapidly and transiently induced activation of NFATc1 and NFATc3 (Pang et al., 2017). Calcineurin consists of a catalytic A (CnA) and regulatory B (CnB) subunit. The CnA subunit has three isoforms: CnAα, CnAβ and CnAγ. CnAα and CnAβ are ubiquitously expressed while CnAγ is found primarily in the brain and testes (Sakuma and Yamaguchi, 2010). CnAβ deficiency impaired activation of NFAT activation, and production of proinflammatory cytokines IL-1β, TNF and IL-6 and chemokine MIP-2 upon

P. aeruginosa lung infection. We also found that deficiency of calcineurin-NFAT pathway diminished NF-κB activation, and inhibition of NFAT reduced NF-κB DNA binding activity, suggesting NFAT and NF-κB cooperatively regulate inflammatory gene expression (Pang et al., 2017).

1.6.4 MAPK pathways

MAPKs are a family of serine/threonine kinases involved in cell proliferation, differentiation, motility and survival in response to a variety of extracellular stimuli such as cytokines, mitogens, growth factors and environmental stresses (Morrison, 2012). MAPK pathway contains a three-tiered kinase cascade in which a MAPKKK (also known as MAP3K) phosphorylates MAP kinase kinase (MAPKK or MAP2K) which in turn activates the MAPK (Kim and Choi, 2010). ERK1/2, JNK and p38 are well-characterized MAPK family proteins in mammals. ERK1/2 is primarily activated through Ras-Raf-MEK1/2 cascade in response to growth factors and mitogens. The activated ERK1/2 translocates to nucleus where they activate multiple transcription factors such as Elk-1, Ets1, Sap-1a, c-Myc, c-Jun and signal transducer and activator of transcription 3 (STAT3), which are important for cell growth and differentiation (Mebratu and Tesfaigzi, 2009). JNK family members include JNK1, JNK2, and JNK3, and they are activated by cytokines, growth factors, environmental stresses such as heat stress, oxidative stress and DNA damage (Johnson and Nakamura, 2007). The stresses induce recruitment of Rho family GTPases, Rac, Rho, Cdc42, which activates MAPKKKs, including MEKK1, MEKK4, mixed-lineage kinase 2 (MLK2), MLK3, apoptosis signal-regulating kinase 1 (ASK1), TAK1, and tumor progression locus 2 (Tpl2). These MAPKKKs are responsible for phosphorylation of the JNK MAPKKs, MKK4 and MKK7 (Morrison, 2012; Zeke et al.,

2016). JNK activates transcription factors such as c-Jun, activating transcription factor 2 (ATF2), Elk-1, p53, Sap-1a, STAT3, NFAT4 and NFATc1, important for cell proliferation, differentiation, apoptosis and inflammation (Zhang and Liu, 2002). Similarly, p38 is also activated by cytokines, growth factors and environmental stresses, and involved in cell proliferation, differentiation and inflammation. However, the MAPKKs for p38 are MKK3 and MKK6, which are activated by MEKK1, MEKK2, MEKK3, MLK2, MLK3, ASK1, Tpl2, TAK1, thousand-and-one amino acid 1 (TAO1) and TAO2. The nuclear substrates for p38 include ATFs, myocyte enhancer factor 2 (MEF2), Elk-1, Ets1, growth arrest and DNA damage 153 (GADD153), p53, mitogen- and stress-activated protein kinase 1 (MSK1) and MSK2 (Zarubin and Han, 2005).

Pseudomonas aeruginosa infection has been found to activate ERK1/2, JNK and p38 through TLR-TAK1 or epidermal growth factor receptor (EGFR) signaling (Azghani et al., 2014; Farias and Rousseau, 2015; Lagoumintzis et al., 2008; Xu et al., 2013). These MAPKs were shown to be involved in host response to *P. aeruginosa* infection. ERK1/2 has been found to mediate production of TNF, IL-8 and IL-33, and promote bacterial internalization in response to *P. aeruginosa* infection (Chai et al., 2014; Evans et al., 2002; Farias and Rousseau, 2015; Lagoumintzis et al., 2008; Ratner et al., 2001). The *P. aeruginosa*-activated JNK regulated production of IL-6 and IL-8, and induced apoptosis (Jia et al., 2003; Zhang et al., 2005a). Activation of p38 by *P. aeruginosa* was TLR5 signaling-dependent, and was essential for neutrophil recruitment and production of TNF, IL-6, cyclooxygenase-2 and IL-8 (Lagoumintzis et al., 2008; Zhang et al., 2005a; Zhang et al., 2007).

1.6.5 JAK/STAT pathway

The Janus kinase (JAK)/STAT pathway mediates a variety of cellular processes including proliferation, differentiation, migration, apoptosis, and cell survival in response to cytokines and growth factors (Harrison, 2012). Cytokine receptors are the most common cell surface receptors for activating this pathway. JAKs are tyrosine kinases that bind to the cytoplasmic domains of cytokine receptors. In mammals, the JAK family consists of four members: JAK1, JAK2, JAK3 and Tyk2 (Yamaoka et al., 2004). Upon ligand binding, the receptors undergo multimerization and induce JAK phosphorylation. The activated JAKs subsequently recruit and phosphorylate the downstream transcription factors STATs. The phosphorylated STATs dimerize through interaction with their conserved Src homology 2 (SH2) domain, and translocate to nucleus. Once in the nucleus, the dimerized STATs bind to the promoter sequences of target genes and regulate the gene transcription (Rawlings et al., 2004). The JAK/STAT pathway has been found to crosstalk with MAPK and phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathways (Harrison, 2012). Several studies reported that JAK2 was critical for ERK activation through mediating Ras activation (Abe and Berk, 1999; Winston and Hunter, 1995). Moreover, ERK was found to phosphorylates STAT3 at serine 727, leading to activation of STAT3 (Ceresa et al., 1997; Chung et al., 1997; Gough et al., 2013). The PI3K/AKT/mTOR pathway is important for cell growth, proliferation, metabolism and survival, and is activated by extracellular stimuli such as insulin, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (Yu and Cui, 2016). JAK1 and JAK2 have been found to phosphorylate the insulin receptor substrate -1 and -2, and the p85 regulatory subunit of PI3K (Gual et al., 1998; Yamauchi et al., 1998). Moreover, Abell et

al. demonstrated that STAT3 regulated expression of the PI3K regulatory subunits, p55α and p50α (Abell and Watson, 2005).

STAT protein family has seven members, of which STAT1, STAT2, STAT3, STAT4 and STAT6 are involved in modulation of inflammatory responses (Pfitzner et al., 2004). STATs can be activated by both pro- and anti-inflammatory cytokines, and each individual STAT protein has a specific function. STAT1 is activated by type I (IFN-α and -β), type II (IFN-γ), and type III (IFN-λ) IFNs, whereas STAT2 is only activated by type I and type III IFNs (Kaplan, 2013; Zhou et al., 2007). STAT3 can be activated by both pro-inflammatory cytokines such as IL-6, and anti-inflammatory cytokines such as IL-10 (Braun et al., 2013). STAT4 mediates IL-12 signaling, which is important for Th1 differentiation and IFN-γ production (Lund et al., 2004). STAT6 regulates Th2 differentiation in response to IL-4 or IL-13 stimulation (Bao and Reinhardt, 2015).

STAT1, STAT3 and STAT4 have been found to contribute to host defense against *P. aeruginosa* infection (Ding et al., 2017; Parker et al., 2012; Saint-Criq et al., 2018). The CF epithelial cells with CFTR mutations displayed impaired type I IFN-STAT1 signaling, which caused diminished dendritic cell activation (Parker et al., 2012). *Pseudomonas aeruginosa* elastase LasB was shown to degrade CFTR and IL-6, leading to inhibition of IL-6-STAT3 signaling-mediated epithelial cell repair (Saint-Criq et al., 2018). Furthermore, Ding et al. identified that SOCS3-surpressed STAT3 signaling in lung CD4+ T cells resulted in impaired Th17 response and neutrophil recruitment in response to *P. aeruginosa* infection (Ding et al., 2017). O'Sullivan et al. indicated that STAT4 deficiency impaired production of the proinflammatory cytokines IL-1β, TNF and the chemokine MIP-2, but it did not affect bacterial clearance and neutrophil recruitment following *P. aeruginosa* lung

infection. The contribution of STAT4 to cytokine production was independent of IL-12 signaling (O'Sullivan et al., 2008).

1.7 Cytokine and chemokine production induced by *P. aeruginosa*

Upon *P. aeruginosa* infection, immune cells produce a variety of proinflammatory cytokines including IL-1β, IL-6, IL-12, IL-17, IL-18 and TNF, and chemokines such as MIP-2, RANTES, IP-10, KC and LIX to promote inflammation (Carrigan et al., 2010; Hazlett et al., 2002; Huang et al., 2002; Junkins et al., 2013a; Power et al., 2004; Xu et al., 2014). IL-1β, IL-6 and TNF are primary proinflammtory cytokines, which produce fever, enhance inflammation, cause tissue destruction, initiate acute phase responses and induce sepsis (Chaudhry et al., 2013; Dinarello, 2000; Morrow et al., 2017). Production of IL-1β, IL-6 and TNF was strongly activated by *P. aeruginosa* infection, and these cytokines were critical for *P. aeruginosa* clearance (Al Moussawi and Kazmierczak, 2014; Cole et al., 2003; Yu et al., 2000). IL-12 and IL-18 were shown to contribute to host defence against *P. aeruginosa* infection by inducing IFN-γ production (Hazlett et al., 2002; Huang et al., 2002). IFN-γ is considered as a proinflammatory cytokine for its ability to augment TNF activity and induce nitric oxide production (Dinarello, 2000). IL-17 is primarily produced by Th17 cells, and it induces production of other cytokines and chemokines such as IL-6, GM-CSF, MIP-2, KC, LIX, MCP-1, promoting recruitment of neutrophils and macrophages (Jin and Dong, 2013). IL-17 has been found to play a protective role in host defense against both acute and chronic pulmonary *P. aeruginosa* infection in mice (Bayes et al., 2016; Liu et al., 2011). Chemokines are a family of chemoattractant cytokines that direct migration of leucocyte from the circulation into the infected tissues and activate leucocytes by binding to their cognate chemokine receptors, which triggers activation of

multiple signaling pathways, including JAK/STAT, MAPK, PI3K/AKT and phospholipase C (PLC)/protein kinase C (PKC) pathways (Griffith et al., 2014; Ono et al., 2003). The chemokines MIP-2, RANTES, IP-10, KC and LIX have been identified to be essential for neutrophil recruitment and bacterial clearance in response to *P. aeruginosa* infection (Bryant-Hudson and Carr, 2012; Carrigan et al., 2010; Tsai et al., 2000b).

1.8 Regulator of calcineurin 1 in inflammation

Regulator of calcineurin 1 (RCAN1), also known as down syndrome critical region gene 1 (DSCR1), myocyte-enriched calcineurin-interacting protein 1 (MCIP1), Adapt78 or calcipressin-1, is a highly conserved protein that suppresses the NFAT pathway by inhibition of calcineurin phosphatase activity (Fuentes et al., 2000). The *RCAN1* gene is located on chromosome 21 in the Down syndrome critical region, and is highly expressed in various tissues including brain, heart, muscle, liver, kidney, lung and testis (Fuentes et al., 1997; Fuentes et al., 1995; Rothermel et al., 2000). It has seven exons which can be alternatively spliced to render 4 different transcript isoforms (*RCAN1-1*, *RCAN1-2, RCAN1-3* and *RCAN1-4*) (Fuentes et al., 1997). RCAN1 is overexpressed in Down syndrome patients and causes a variety of immunodeficiency disorders (Fuentes et al., 2000; Ram and Chinen, 2011). Importantly, the Down syndrome patients have high risk of respiratory tract infections (Guffroy et al., 2019; Selikowitz, 1992).

In addition to regulation of NFAT activity, RCAN1 is also able to inhibit NF-κB activation by affecting IκBα phosphorylation through calcineurin-dependent (Alzuherri and Chang, 2003; Frantz et al., 1994; Steffan et al., 1995; Wu et al., 2013) or -independent mechanisms (Kim et al., 2006; Liu et al., 2015), which are poorly characterized. Furthermore, RCAN1 has been found to play a negative regulatory role in inflammation

during *P. aeruginosa* lung infection, which suppresses proinflammatory cytokine production and NF-κB activation (Junkins et al., 2013a). However, the molecular mechanisms involved in RCAN1-regulated TLR signaling during *P. aeruginosa* infection remain undefined.

1.9 Early growth response 1 in inflammation

Early growth response 1 (Egr-1) is a zinc-finger transcription factor that is rapidly and transiently induced by a broad range of extracellular stimuli, and binds to the GC-rich consensus sequences in the promotor of target genes, important for cell growth, migration, differentiation and apoptosis (Dinkel et al., 1998; Min et al., 2008; Pignatelli et al., 2003; Thiel and Cibelli, 2002). Egr-1 protein consists of an activation domain on the N-terminus, a highly conserved DNA-binding domain composed of three zinc finger motifs, and a repression domain containing binding sites for the transcriptional co-repressor proteins NGFI-A binding protein 1 (NAB1) and NAB2 (Thiel and Cibelli, 2002). The expression of Egr-1 was previously reported to be mediated mainly through MEK-ERK1/2-Elk-1 pathway upon stimulation (de Klerk et al., 2017; Guha et al., 2001; Rossler and Thiel, 2009; Shin et al., 2006). The functional activation of Egr-1 is poorly characterized, which may be modulated by kinase phosphorylation or triggered through dissociation from co-repressor NAB proteins. A study by Rössler et al. indicated that the newly synthesized Egr-1 protein was biologically active in resveratrol-treated HEK293 cells, which may be due to the lack of intrinsic inhibition by NAB1 and NAB2 (Rossler et al., 2015). In contrast, the mechanisms involved in inhibition of Egr-1 transcriptional activity is well-characterized, which is mediated by NAB1 and NAB2, binding to the Egr-1 inhibitory domain (Qu et al., 1998; Russo et al., 1995). In addition, Egr-1 has been found to

cooperatively regulate gene expression with other transcription factors such as NF-κB, NFAT and AP-1 through physical interactions (Chapman and Perkins, 2000; Decker et al., 2003; Ma et al., 2009; Nakashima et al., 2003). However, the mechanisms and binding sites of the interactions are not well-defined.

Egr-1 has been found to promote inflammation by upregulating production of inflammatory mediators, including IL-1, IL-6, TNFα, IL-8, monocyte chemoattractant protein 1 (MCP-1), MIP-1 MIP-2, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in response to various stimuli (Ho et al., 2016; Prince et al., 2007; Reynolds et al., 2006; Saben et al., 2013; Schmidt et al., 2008). However, several studies reported that the contribution of Egr-1 to inflammation was deleterious to host, which caused excessive production of the inflammatory mediators, leading to tissue damage, fibrosis and septic shock (Ho et al., 2016; Prince et al., 2007; Reynolds et al., 2006). *Pseudomonas aeruginosa* has been found to induce Egr-1 expression in human pharyngeal epithelial cells and macrophages (de Klerk et al., 2017; Wu et al., 2018b). However, the role of Egr-1 in host inflammatory response to *P. aeruginosa* lung infection is not elucidated yet.

1.10 Objectives and significance

The objectives of this study are to examine the regulatory role of RCAN1 in *P. aeruginosa* LPS-activated TLR4 signaling and the role of Egr-1 in innate immune response to *P. aeruginosa* lung infection. Characterization of the role and impact of the two inflammation regulators in host defense against *P. aeruginosa* infection could contribute to the potential therapeutic inventions that effectively eradicate *P. aeruginosa* by boosting inflammatory responses or prevent tissue damage and septic shock by reducing inflammatory level.
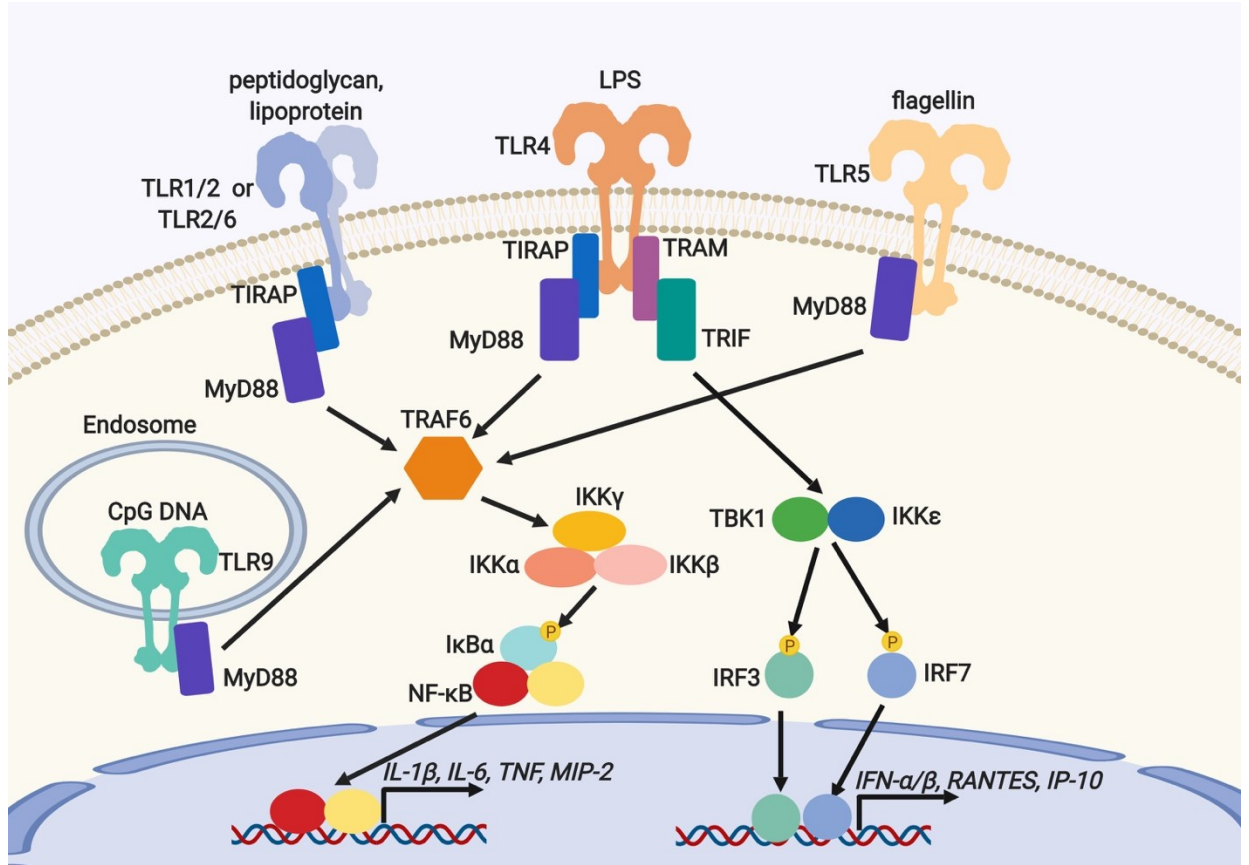
Fig 1.1. *P. aeruginosa* **activates TLR signaling pathways.** TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9 recognize *P. aeruginosa*. Upon ligand binding, TLR2, TLR5 and TLR9 activate MyD88-dependent pathways, whereas TLR4 activates both MyD88- and TRIF-dependent pathways. The MyD88-dependent pathway leads to phosphorylation and degradation of IκBα, allowing NF-κB translocate into the nucleus and mediate gene expression of cytokines and chemokines, including IL-1β, IL-6, TNF and MIP-2. The TRIF-dependent pathway induces phosphorylation and activation of IRF3 and IRF7. The activated IRF3 and IRF7 drive transcription of IFN-α and IFN-β, and the chemokines RANTES and IP-10.
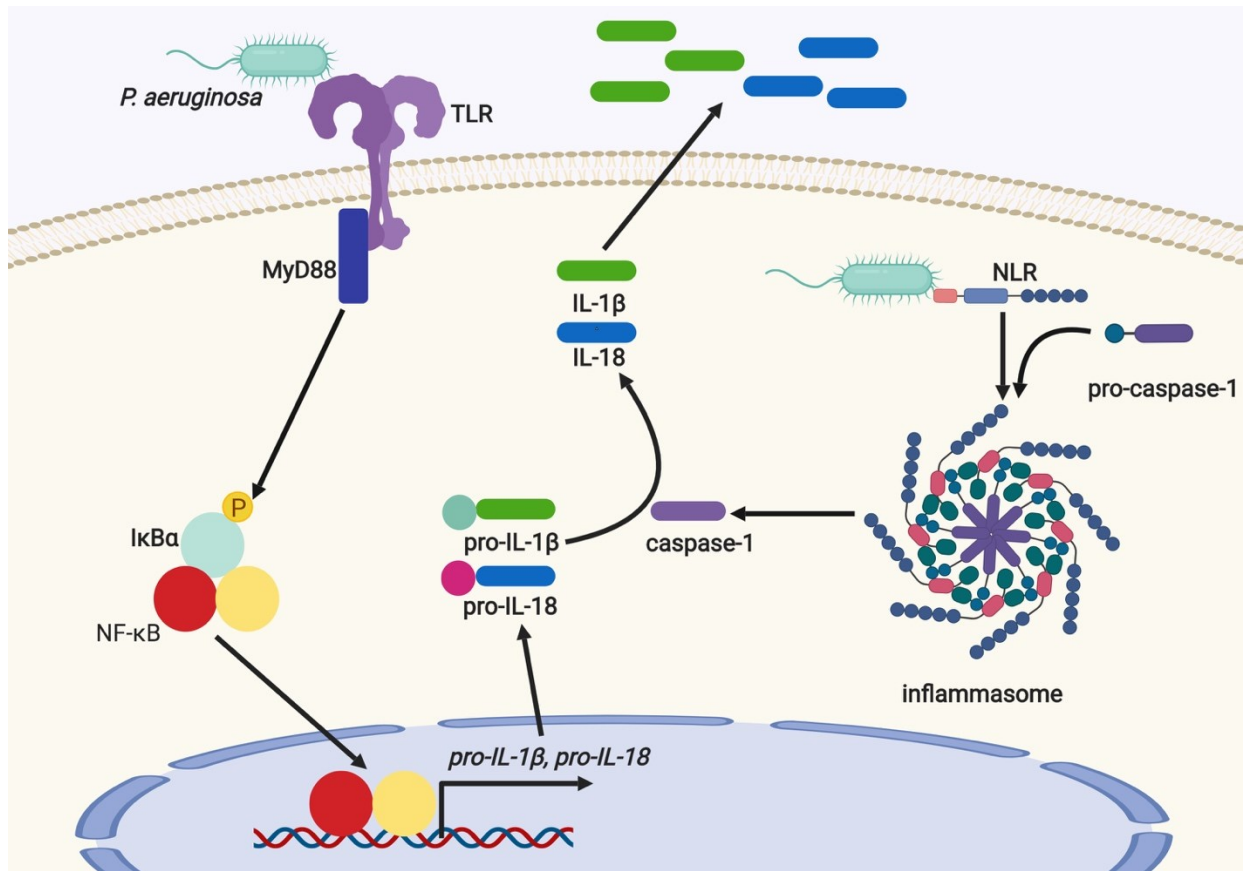
Fig 1.2. *P. aeruginosa* activates inflammasome pathway. The NLR family members such as NLRP3 and NLRC4 are activated by intracellular *P. aeruginosa*, and assemble into the multimeric complexes inflammasomes, which recruit and cleave the inactive caspase-1 precursor into active form. The active caspase-1 cleaves the preforms of IL-1β and IL-18 produced through NF-κB pathway into their mature forms.
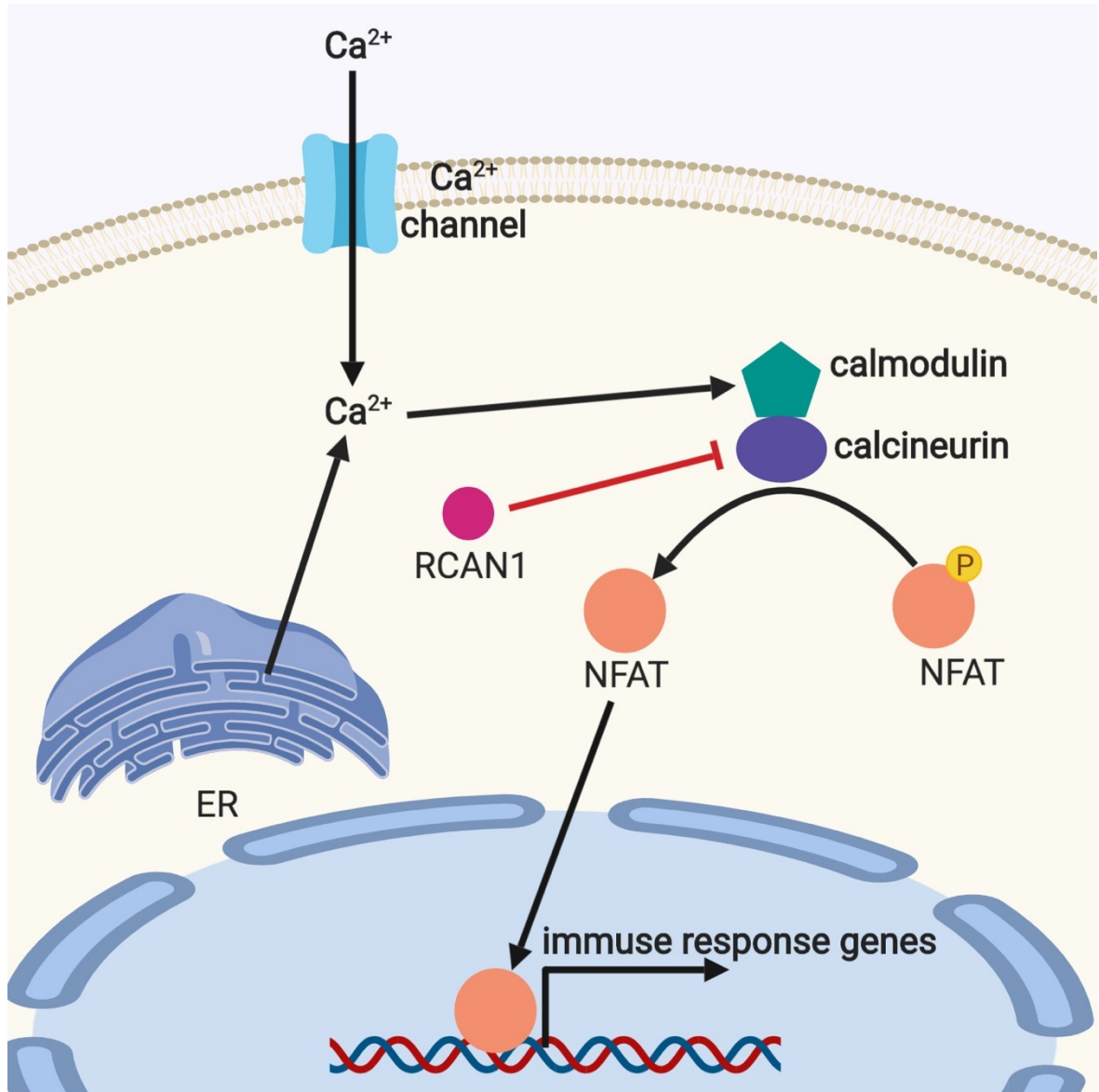
Fig 1.3. The NFAT/calcineurin pathway. An increase of intracellular levels of calcium obtained from endoplasmic reticulum or the extracellular environment leads to conformational change and activation of the calmodulin-dependent phosphatase calcineurin. The activated calcineurin dephosphorylates the NFAT, leading to NFAT nuclear translocation and target gene expression. RCAN1 suppresses NFAT pathway through inhibition of calcineurin activity.

Chapter Two – Materials and Methods

2.1 Animals

RCAN1-deficient mice with a genetic background of C57BL/6 were generated as described previously with a deletion of exons 5 and 6 leading to deficiency of *Rcan1* products (Rcan1-1 and Rcan1-4) (Sanna et al., 2006), and were provided by J. Molkentin (Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, OH). C57BL/6 mice were purchased from Charles River Laboratories and were used as wild-type controls. Heterozygous Egr-1 mice (+/-) with a genetic background of C57BL/6 were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). Heterozygous breeders were used to establish separate wild-type (+/+) and Egr-1-deficient (-/-) breeding colonies, which were maintained in the same specific pathogen-free facility. 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 (protocol number: 18-002).

2.2 Antibodies

Antibodies for phospho-IκBα (2859), total IκBα (9242), phospho-IKKα/β (2697), total IKKα (2682), total IKKβ (2684), phospho-ERK (9101), total ERK (9102), phospho-p38 (9211), total p38 (8690), phospho-JNK (9251), total JNK (9252), Egr-1 (4153), and iNOS (13120), were purchased from Cell Signaling. Antibody for IRF7 (ab109255) was purchased from Abcam. Antibodies for NF-κB p65 (sc-109), IRF3 (sc-9082, sc-9082X), IRF7 (sc-9083, sc-9083X), actin (sc-1616) and all secondary antibodies were purchased from Santa Cruz Biotechnology. Antibody for PerCP-Cy™5.5 anti-mouse Ly6G (560602)

was purchased from BD Biosciences. Antibody for APC anti-mouse F4/80 (17-4801-82) was purchased from eBioscience. Antibodies for Brilliant Violet 650™ anti-mouse CD45 (103151) and FITC anti-mouse CD64 (139316) were purchased from BioLegend.

2.3 Bacterial preparation

Pseudomonas aeruginosa strain 8821, a mucoid strain isolated from a cystic fibrosis patient, was a gift from Ananda M. Chakrabarty, University of Illinois, Chicago, IL. *Pseudomonas aeruginosa* was cultured as described previously (Power et al., 2007). Briefly, suspension cultures were grown in Luria-Bertani (LB) broth overnight at 37 °C with shaking at 225 rpm in a shaking incubator (New Brunswick Scientific Innova 4080) to reach the early stationary phase (an optical density (OD) between 2.5 and 3.0 at 600 nm). Bacteria were washed in phosphate-buffered saline (PBS) (PH 7.4) and resuspended in saline (0.9 % NaCl) for *in vivo* experiments or PBS for *in vitro* assays.

2.4 Lung infection with *P. aeruginosa* or stimulation with LPS and collection of lung and bronchoalveolar lavage fluid (BALF)

P. aeruginosa LPS (cat#L8643) was purchased from Sigma-Aldrich. Mice were anesthetized with 50 μl ketamine/xylazine combination (ketamine: 60 mg/ml, xylazine: 12 mg/ml) via intraperitoneal injection, and intranasally infected with 1×10^9 colony-forming unit (CFU) *P. aeruginosa* resuspended in 20 μl saline or administered with 1 μg *P. aeruginosa* LPS per gram of body weight dissolved in 20 ul saline for 4 h or 24 h. After stimulation, mice were euthanized with 150 μl ketamine/xylazine combination through intraperitoneal injection. To remove the intrapulmonary blood from lung tissues, 10 ml PBS was infused to right atrium using a butterfly needle, and the lungs turned from pink to white. BALF was obtained by lavage of lungs with 3×1 ml PBS containing soybean

trypsin inhibitor (100 µg/ml) (Sigma-Aldrich, cat#T6522). The postcaval lobe of lung was saved for histology, and the rest of lung tissues was homogenized in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (4 µl per mg of lung) containing soybean trypsin inhibitor (100 µg/ml). For counting bacterial CFUs, 10 µl of lung homogenates were serially-diluted, plated onto an LB agar dish, and incubated for 24 h at 37 °C. The remaining lung homogenates were centrifuged at 4 °C for 20 min at 18,000 X g. The supernatants were stored at −80 °C for later cytokine analysis and nitric oxide assay. The pellets were resuspended and homogenized in 0.5% cetyltrimethylammonium chloride (4 µl per mg of lung) and centrifuged at 4 °C for 10 min at 18000 X g. Cleared supernatants were used for nitric oxide assay and myeloperoxidase (MPO) assay.

BALF (10 µl) was serially-diluted, plated onto an LB agar dish and incubated for 24 h for CFU counting. The remaining BALF was centrifuged at 480 X g for 5 minutes at 4°C. The supernatants were used for cytokine and nitric oxide analysis. The pellets were resuspended in 1 ml NH$_4$Cl (0.15 M) and centrifuged at 480 X g for 5 min to lyse red blood cells. The supernatants were discarded, and the pellets were resuspended in 0.5% cetyltrimethylammonium chloride (250 µl/mouse) and then centrifuged at 480 X g for 5 min. The cleared supernatants were used for nitric oxide assay and MPO assay.

2.5 Animal survival and disease scores

To determine the animal survival and disease progression, 10 wild-type and 10 Egr-1-deficient mice were infected intranasally with 1×10^9 CFU *P. aeruginosa* per mouse. Disease scores were recorded for 10 days, according to the following scoring system based on physical appearance, posture, activity/behavior, appetite, hydration, body weight and body temperature (ventral surface temperature). The scoring system was summarized in

Table 2.1. Total possible score was 21. Animals were euthanized when the score reached 15 or higher.

2.6 Macrophage culture, dendritic cell culture and *P. aeruginosa* infection

For macrophage culture, bone marrow cells were flushed from femurs and tibias of wild-type (+/+), Egr-1-deficient (-/-) and RCAN1-deficient (-/-) mice. Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) media (Life Technologies, cat#11995) supplemented with 10% fetal bovine serum (Life Technologies, cat#12484), 100 U/ml penicillin/streptomycin (Life Technologies, cat#15140) and 30% L929 supernatant containing macrophage colony-stimulating factor (M-CSF). For dendritic cell culture, bone marrow cells were cultured in RPMI (Roswell Park Memorial Institute)-1640 media (Life Technologies, cat#11875) supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin and 5% X-63 supernatant containing GM-CSF. Media were changed every 2 - 3 days by replacing half of the initial volume. After 7 days, cells were treated with *P. aeruginosa* 8821 at a multiplicity of infection (MOI) of 10, 200 ng/ml *P. aeruginosa* LPS or mock-control. At different time-points post-treatment, cell-free supernatants were collected for measuring cytokine and chemokine production; whereas cell pellets were processed for measuring levels of RNAs or proteins by real time quantitative-PCR (RT-qPCR) or immunoblotting, respectively. A portion of cell pellets was reserved for transcription factor activation analysis by electrophoretic mobility shift assay (EMSA).

2.7 BALF alveolar macrophage collection and LPS stimulation

Alveolar macrophage collection from BALF was described previously (Cheung et al., 2000). Briefly, BALF was obtained by lavage of lungs with 1 ml of PBS for 3 times.

BALF cells were spun down and resuspended in 2 ml DMEM media containing 10% FBS and 100 U/ml penicillin/streptomycin. Cells were incubated at 37 °C for 1 h in 6-well plates, which allowed alveolar macrophages to adhere to the plate; poorly attached and unattached cells were removed by washing with PBS. The purity of alveolar macrophage preparations was examined using a Diff-Quik stain set (Siemens Healthcare Diagnostics, DE, cat#B4132-1A). Subsequently, wild-type and RCAN1-deficient alveolar macrophages were stimulated with 200 ng/ml *P. aeruginosa* LPS for 6 h or left untreated, and supernatants were subjected to enzyme-linked immunosorbent assay (ELISA) for determining cytokine and chemokine production.

2.8 Cytokine production analysis

Concentrations of IL-1β, IL-6, TNF, MIP-2, RANTES, IP-10, KC and LIX in lungs, BALF and culture supernatants were determined by ELISA as described previously using antibody pairs from R&D Systems (Minneapolis, MN) (Lin et al., 2002). IFN-β levels were measured using VeriKine-HS Mouse IFN-β ELISA Kit (PBL Assay Science, Piscataway, NJ, cat#42410) according to the manufacturer's instructions. Briefly, 96-well ELISA microplates (Thermo Fisher Scientific, Cat#44-2404-21) were coated with capture antibody dissolved in bicarbonate buffer (0.1 M $NaHCO_3$, 0.5 M NaCl) and incubated at 4 °C overnight. Next day, the plates were washed with 0.01% Tween-20 in PBS, and blocked with 2% BSA in PBS for 1 h at room temperature. After blocking, the plates were washed as described above, and 50 μl of standards or samples were added into each well and incubated at room temperature for 2 h. The plates were then washed, and 50 μl detection antibody dissolved in 0.2% BSA and 0.05% Tween-20 in PBS were added to each well and incubated at room temperature for 1 h. After incubation, the plates were washed and 50 μl

streptavidin-HRP (R&D Systems, cat#DY998) diluted in 0.2% BSA and 0.05% Tween-20 in PBS (1:200) were added to each well and incubated at room temperature for 30 min. Subsequently, plates were washed, and 50 ul of 3,3',5,5'-tetramethyl-benzidine dihydrochloride (TMB) ELISA substrate solution (eBioscience, cat#00-4201-56) were added to each well and incubated at room temperature for 10-20 min. Reaction was stopped by adding 50 ul of 0.3 M H_2SO_4. The absorbance was measured on a microplate reader (SpectraMax 190, Molecular Devices) at 450 nm.

2.9 Myeloperoxidase (MPO) assay

The MPO assay was used to determine the infiltration of neutrophils into the lungs of the mice as described previously (Schneider and Issekutz, 1996). Briefly, samples in duplicate (75 µl) were mixed with equal volumes of the substrate (TMB (Sigma-Aldrich cat#T8768), 3 mM; Resorcinol (Sigma-Aldrich, cat# 398047), 120 µM; and H_2O_2 (Fisher Scientific, cat#H325), 2.2 mM) for 2 min at room temperature. The reaction was stopped by adding 150 µl of 2 M H_2SO_4. The OD was measured on a microplate reader (SpectraMax 190, Molecular Devices) at 450 nm.

2.10 Flow cytometry analysis

Cells extracted from mouse lung tissues and BALF were resuspended in 0.15 M NH_4Cl buffer to lyse erythrocytes, and stained with Fixable Viability Stain (FVS) 510 (BD Biosciences) at 1:1000 dilution in PBS at 4 °C for 20 min. Cells were washed and resuspended in PBS with 1% BSA, and incubated with Fc block (BD Biosciences, cat#553141) for 15 min at 4 °C. After blocking, the cells were stained with the antibodies for Ly6G, CD45, F4/80 and CD64 at 1:200 dilution for 30 min at 4 °C. The cells were then fixed with 1% paraformaldehyde for 20 min at 4 °C and resuspended in PBS with 1% BSA.

Antibody-stained cells were acquired on CytoFLEX flow cytometer (Beckman Coulter), and the data were analyzed using FCS Express 6 Flow Cytometry Software (De Novo Software). The gating strategies for neutrophils and macrophages were described in a previous study (Misharin et al., 2013). Neutrophils were characterized as $CD45^+$, $F4/80^-$ and $Ly6G^+$. Macrophages were characterized as $CD45^+$, $F4/80^+$, $CD64^+$ and $Ly6G^-$.

2.11 Measurement of nitric oxide production

Neutrophils were isolated from mouse bone marrow using a Mouse Neutrophil Enrichment Kit (STEMCELL Technologies Inc., cat#19762). Neutrophils and macrophages were seeded in 12-well plates and infected with *P. aeruginosa* at a MOI of 10 for 6 h or left untreated. After infection, the plates were centrifuged at 480 X g for 5 min, and the cell pellets were lysed in 0.1% Triton X-100 (Sigma-Aldrich cat#x100). Cell-free supernatants and cell lysates were collected for measuring the extracellular and intracellular nitric oxide levels, respectively, using a Griess Reagent Kit (Thermo Fisher Scientific, cat#7921). Briefly, N-(1-naphthyl) ethylenediamine was mixed with equal volume of sulfanilic acid to form Griess Reagent. A total of 20 μl Griess Reagent were mixed with 150 μl cell supernatants or cell lysates and 130 μl of deionized H_2O in a 96-well plate. The mixture was incubated at room temperature for 30 min. The OD was measured on a microplate reader (SpectraMax 190, Molecular Devices) at 548 nm.

2.12 Western blotting

Bone marrow-derived macrophages (BMMs) were lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, cat# R0278) supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, cat#87786), and centrifuged at 10000 X g for 10 min. The protein concentration of the

cleared lysates was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, cat#23225). A total of 30 μg protein for each sample was electrophoresed in 10% SDS polyacrylamide gels. Gels were transferred to polyvinylidene difluoride membrane (Bio-Rad, cat# 1620177), blocked with 5% non-fat milk in TBST (Tris-buffered saline, 0.05% Tween-20), probed with specific primary and corresponding secondary antibodies, and detected by an ECL-detection system (Western Lightning Plus-ECL; PerkinElmer, cat #NEL103E001EA) on BioMax film (Kodak). Blots were scanned using a scanner (Canon CanoScan LiDE120) and quantified using ImageJ software.

2.13 Real-time quantitative PCR

The *P. aeruginosa*-infected or *P. aeruginosa* LPS-stimulated BMMs or lung tissues were processed using Trizol (Invitrogen, cat#15596018), and RNA was purified using RNeasy kit (Qiagen, cat#74104) and RNase-free DNase set (Qiagen, cat#79254). The total RNA (1000 ng) was reverse transcribed into cDNA using RNA to cDNA EcoDry™ Premix (Takara Bio, cat#639549). Primers were designed by Primer-BLAST (National Center for Biotechnology Information, NCBI), and the primer sequences were summarized in Table 2.1. RT-qPCR assay were conducted in triplicate using SYBR Green method on a CFX Connect Real-Time PCR Detection System (Bio-Rad) according to manufacturer's instructions. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a housekeeping control mRNA. Data was analyzed using the relative standard curve method according to the manufacturer's protocol.

2.14 Immunoprecipitation

BMMs were infected with *P. aeruginosa* 8821 at a MOI of 10 for 1 h or left untreated. Cell lysates (500 μg) were prepared as described above in the 'Western Blotting'

section, and incubated with 20 μl protein A/G linked agarose (Santa Cruz Biotechnology, sc-2003) and 2 μl mouse or rabbit IgG (Santa Cruz Biotechnology, sc-2025 and sc-3888) for 30 min at 4 °C to exclude non-specific binding. The pre-cleared lysates were incubated with 1 μg of anti-Egr-1 or NF-κB p65 antibody overnight at 4 °C. Next day, the samples were incubated with 20 μl protein A/G agarose beads for 2 h at 4 °C. IgG was used as the negative control. Beads were washed with 4 X 500 ml RIPA buffer after incubation, and dissolved in 2 X SDS-PAGE loading buffer. The presence of Egr-1 or NF-κB p65 were detected by western blot using Egr-1 and NF-κB p65 antibodies.

2.15 Phagocytosis and intracellular killing assay

Phagocytosis and intracellular killing assays were described previously (Yang et al., 2014). Macrophages or neutrophils were infected with *P. aeruginosa* at a MOI of 10. For the phagocytosis assay, cells were collected to enumerate the internalized bacteria after 1 h of incubation. For intracellular killing assay, 100 μg/ml gentamycin was added to the cell culture medium after 1 h post-infection to eliminate the extracellular bacteria. Cells were allowed to incubate for another 2 h to evaluate the intracellular killing of bacteria. For both the phagocytosis and intracellular killing assays, cells were then washed with PBS, pelleted by centrifuging at 480 X g for 5 min, and lysed in 0.1% Triton X-100. Cell lysates were serially-diluted, plated on LB agar plates and incubated at 37 ºC for 24 h for CFU counting. Phagocytic activity was analyzed based on the CFU data obtained 1 h after infection. Intracellular killing efficiency was calculated as the number of CFU at 3 h post-infection subtracted from the number of CFU at 1 h post-infection (1 h CFU – 3 h CFU).

2.16 Nuclear extract preparation and electrophoresis mobility shift assay (EMSA)

EMSA was performed as previously described (Power et al., 2004). Briefly, nuclear protein extracts were prepared using a nuclear extract kit (Active Motif, Carlsbad, CA, cat# 40410), following the manufacturer's protocol. Probe labeling was accomplished by mixing 2 ul probe (1.75 pmol/ul) with 1 ul T4 kinase (10 U) (Life Technologies, cat# EK0031), 10 X T4 Kinase buffer (Promesa, cat#C1318) and 1 ul ^{32}P adenosine triphosphate (Perkin Elmer, Waltham, MA, cat#NEG002A250UC) in 37 °C water bach for 30 min. The reaction was stopped by adding 1 ul EDTA (0.5 M) to the mixture, and the volume was adjusted to 100 ul by adding 89 ul TE buffer (Invitrogen, cat# 12090015). The labeled oligonucleotides were purified by loading onto a Sephadex G-25M column (GE Healthcare, Pittsburgh, PA, cat#27-5325-01) and centrifuged at 2800 rpm for 2 min. The flow-through were collected, which contains the purified oligonucleotides. Nuclear protein (10 μg) was added to a 10 μl volume of gel shift binding buffer (Promega, cat#E358A) supplemented with 1 μg poly(dI:dC) (Sigma-Aldrich, cat#81349) for 15 min at room temperature. Labeled double-stranded oligonucleotide was added to each reaction mixture that was incubated at room temperature for 30 min 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 consensus sequence on the IL-6 promoter, 5'-AGTTGAGGGGACTTTCCCAGGC-3' (Promega, Madison, WI), and NFAT-binding consensus sequence on mouse IL-13 promoter, 5′-AAGGTGTTTCCCCAAGCCTTTCCC-3′ (Sigma-Aldrich). ISRE-binding consensus sequence on mouse IFN-β promoter, 5'-GAAAACTGAAAGGGAGAACTGAAA-3' (Zhao et al., 2011).

Supershift assay was performed as described previously (Yang et al., 2009b). Briefly, samples were prepared as described above and then incubated with 2 µg of the indicated antibody on ice for 45 minutes prior to incubation with ^{32}P-labeled double-stranded DNA probes. Samples were resolved and developed as described above. The antibodies for IRF3 (sc-9082X) and IRF7 (sc-9083X) from Santa Cruz Biotechnology were applied in supershift assay.

2.17 Measurement of IRF7 activation by ELISA

IRF7 activity in cell nuclear extracts was determined using transcription factor ELISA (TransAM IRF7 kit, Active Motif, Carlsbad, CA), according to the manufacturer's instruction. Briefly, nuclear extracts were added into a 96-well plate pre-coated with oligonucleotides containing the IRF7 consensus binding sites, followed by sequential incubations with IRF7 antibody and HRP-labeled secondary antibody. Results were read on a spectrophotometer at 450 nm.

2.18 Statistical analysis

Data are presented as means ± standard error of the mean (SEM) of the indicated number of experiments. Statistical significance between multiple treatments was determined by one-way analysis of variance and post-hoc Tukey's honest significant difference test. Alternatively, when two independent variables were analyzed, a two-way analysis of variance and a Bonferroni multiple-comparison test were used. Statistical analysis was performed using GraphPad Prism software version 5.04 (GraphPad Software Inc., La Jolla, CA).

Table 2.1 Disease scoring system

Characteristics	0	1	2	3
Physical appearance	Normal	Lack of grooming	Rough hair coat	Very rough hair coat
Posture	Normal	Sitting in hunched position	Hunched posture, head resting on floor	Lying prone on cage floor/unable to maintain upright posture
Activity/behavior	Normal	Somewhat reduced/minor changes in behavior	Above plus change in respiratory rate or effort	Moves only when stimulated
Appetite	Normal	Reduced appetite	Not eating since last check point	Not eating for last two check points
Hydration	Normal	Mildly dehydrated	Moderately dehydrated	Severely dehydrated
Body weight	< 5% change from preinfection weight	< 10% weight change	10% ~ 15% weight change	15% ~ 19% weight change
Body temperature (ventral surface temperature)	33 °C ~ 34 °C	28 °C ~ 32.5 °C	25 °C ~ 27.5 °C	< 24.5 °C

Table 2.2 Sequences of oligonucleotides

qPCR	Forward 5'-3'	Reverse 5'-3'
RCAN1-1	GTTCGTGGACGGCCTGTG	AAGGGGTTGCTGAAGTTTATCC
RCAN1-4	TGCTTGTGTGGCAAACGATG	AGGAACTCGGTCTTGTGCAG
Egr-1	CGCTTTTCTCGCTCGGATG	GCGGATGTGGGTGGTAAGGT
iNOS	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG
IL-1β	TGCCACCTTTTGACAGTGATGA	TGCCTGCCTGAAGCTCTTGT
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
TNF	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
MIP-2	CCACTCTCAAGGGCGGTCAA	GGTACGATCCAGGCTTCCCG
KC	TGCAGACCATGGCTGGGATT	AGCCTCGCGACCATTCTTGA
LIX	GCGGTTCCATCTCGCCATTC	TCCGTTGCGGCTATGACTGA
IFN-β	GCCTTTGCCATCCAAGAGATGC	ACACTGTCTGCTGGTGGAGTTC
RANTES	CCTGCTGCTTTGCCTACCTCTC	ACACACTTGGCGGTTCCTTCGA
IP-10	ATCATCCCTGCGAGCCTATCCT	GACCTTTTTTGGCTAAACGCTTTC
IRF3	CGGAAAGAAGTGTTGCGGTTAGC	CAGGCTGCTTTTGCCATTGGTG
IRF7	ACAGGGCGTTTTATCTTGCG	TCCAAGCTCCCGGCTAAG
HPRT	CACAGGACTAGAACACCTGC	GCTGGTGAAAAGGACCTCT

Chapter Three – Regulator of calcineurin 1 differentially regulates TLR-dependent MyD88 and TRIF signaling pathways

3.1 Introduction

TLRs are a family of transmembrane receptors that recognize diverse molecular patterns derived from microbes (Takeda and Akira, 2004). Upon binding of ligands, TLRs dimerize and recruit adapter proteins to their cytoplasmic Toll/IL-1 receptor domain to initiate downstream signaling (Kawasaki and Kawai, 2014). TLR4 binding to LPS activates two distinct signalling pathways; the MyD88 pathway and the TRIF pathway (Akira and Takeda, 2004). MyD88-dependent signal transduction activates NF-κB via phosphorylation and ubiquitin-mediated degradation of its inhibitory protein IκBα, which allows NF-κB nuclear translocation and transactivation of a multitude of proinflammatory cytokines and chemokines, including IL-6, TNF and MIP-2 (Blackwell and Christman, 1997; Kawasaki and Kawai, 2014). In the parallel TRIF-dependent pathway, LPS activates TBK1 and IKKε, leading to phosphorylation and activation of IRF3 and IRF7 (Honda and Taniguchi, 2006b; Solis et al., 2007). Activated IRF3 and IRF7 drive transcription of IFN-α and IFN-β, and the chemokines RANTES and IP-10 (Fitzgerald et al., 2003; Taniguchi et al., 2001). Both MyD88- and TRIF-dependent pathways have been found to contribute to host defense against the microbial infection (Naiki et al., 2005; Power et al., 2007; Ruiz et al., 2015; Takeuchi et al., 2000).

TLR signaling is tightly regulated. Unrestrained production of proinflammatory mediators through TLR signaling can disrupt the balance between pro- and anti-inflammatory responses and cause severe inflammatory and autoimmune diseases (Cario, 2010; Li et al., 2009). Many negative regulators of TLR signaling have been identified in

the past decade (Kondo et al., 2012). We previously identified RCAN1 as a central negative regulator of inflammation during *P. aeruginosa* infection *in vivo*; RCAN1-deficient mice displayed aberrant NF-κB activation and increased levels of inflammatory cytokines, which correlated with increased mortality (Junkins et al., 2013a). RCAN1 has not yet been linked to regulation of TLR signaling.

The *RCAN1* gene is located on chromosome 21 in the Down syndrome critical region, and is highly expressed in various tissues including brain, heart, muscle, liver, kidney, lung and testis (Fuentes et al., 1997; Fuentes et al., 1995; Rothermel et al., 2000). It has seven exons which can be alternatively spliced to render 4 different transcript isoforms (*RCAN1-1*, *RCAN1-2, RCAN1-3* and *RCAN1-4*) (Fuentes et al., 1997). RCAN1 was previously shown to inhibit calcineurin phosphatase activity by direct interaction with the catalytic subunit of calcineurin, leading to suppression of NFAT activation and signaling axis (Fuentes et al., 2000).

In this study, we used LPS from *P. aeruginosa*, a potent activator of TLR4, to examine the role of RCAN1 in both MyD88- and TRIF-dependent signaling *in vivo* and *in vitro*. We found that RCAN1 deficiency significantly enhanced MyD88-NF-κB-mediated cytokine production (IL-6, TNF and MIP-2) and NF-κB activity both *in vivo* and *in vitro*. Moreover, we found that RCAN1-deficient macrophages displayed increased IκBα phosphorylation and no significant change on IKKα/β phosphorylation compared to wild-type macrophages. By contrast, RCAN1 deficiency downregulated the ISRE-mediated cytokine production (IFN-β, RANTES, IP-10) and TRIF-IRF7-ISRE pathway activation *in vitro*. Interestingly, RCAN1 deficiency had limited effects on the TRIF-IRF-ISRE pathway *in vivo*. These findings suggest that RCAN1 is a negative regulator of the TLR-MyD88-

NF-κB signaling pathway through targeting IκBα, and to our knowledge, provide the first line of evidence that RCAN1 plays a role in mediating TLR-TRIF-IRF7-ISRE signaling pathway activation.

3.2 Results

3.2.1 RCAN1 deficiency upregulates MyD88-NF-κB-mediated cytokine and chemokine production but downregulates TRIF-IRF-ISRE-mediated cytokine and chemokine production in macrophages following *P. aeruginosa* LPS stimulation

Macrophages play an important role in host defense and mediation of inflammatory responses, and they express a variety of TLRs to detect invading microbial pathogens (Arango Duque and Descoteaux, 2014; Fujiwara and Kobayashi, 2005). Treatment of macrophages with *P. aeruginosa* LPS led to a significant induction of RCAN1-4 mRNA at 2 h. By contrast, RCAN1-1 mRNA expression was not induced by *P. aeruginosa* LPS. This finding suggests that the upregulated RCAN1-4 may be involved in regulation of TLR signaling (Fig 3.1). Deletion of exons 5 and 6 from mouse *RCAN1* gene leads to deficiency of all *RCAN1* products. To assess the effect of RCAN1 on the cytokine production regulated through the MyD88-NF-κB pathway, we stimulated wild-type and RCAN1-deficient BMMs with 200 ng/ml of *P. aeruginosa* LPS for 3 h, 6 h, 12 h and 24 h. Cell supernatants were collected to detect the production of cytokines and chemokines including IL-6, TNF and MIP-2, which are largely regulated through MyD88-NF-κB pathway during *P. aeruginosa* infection (Power et al., 2004). We found that the *P. aeruginosa* LPS-induced production of IL-6 (Fig 3.2A), TNF (Fig 3.2B) and MIP-2 (Fig 3.2C) was significantly enhanced in RCAN1-deficient BMMs compared to wild-type BMMs, suggesting that RCAN1 negatively regulates MyD88-NF-κB-mediated cytokine and chemokine

production. To confirm this finding, the *P. aeruginosa* LPS-induced mRNA levels of IL-6, TNF and MIP-2 in wild-type and RCAN1-deficient BMMs were examined by RT-qPCR. We discovered that RCAN1-deficient BMMs displayed elevated mRNA expression of IL-6 (Fig 3.3A), TNF (Fig 3.3B) and MIP-2 (Fig 3.3C) compared to wild-type BMMs in response to *P. aeruginosa* LPS stimulation.

IRF3 and IRF7 are transcription factors that translocate to the nucleus upon activation of the TRIF-dependent pathway, and stimulate transcription of IFN-β, RANTES and IP-10, as well as other cytokine genes (Honda and Taniguchi, 2006a). The supernatants from wild-type and RCAN1-deficient BMMs challenged with *P. aeruginosa* LPS were collected to measure the production of IFN-β, RANTES and IP-10. In contrast to IL-6, TNF and MIP-2, RCAN1-deficient BMMs displayed impaired production of IFN-β (Fig 3.2D), RANTES (Fig 3.2E) and IP-10 (Fig 3.2F), suggesting a positive role of RCAN1 in the regulation of these cytokines and chemokines *in vitro*. Furthermore, the levels of *P. aeruginosa* LPS-induced IFN-β (Fig 3.3D), RANTES (Fig 3.3E) and IP-10 (Fig 3.3F) mRNAs were significantly reduced in RCAN1-deficient BMMs compared to wild-type BMMs.

To examine how lung-resident macrophage response to *P. aeruginosa* LPS, the alveolar macrophages were collected from the BALF of wild-type and RCAN1-deficient mice and stimulated with 200 ng/ml of *P. aeruginosa* LPS for 6 h or left untreated. Cell supernatants were collected for determining the cytokine and chemokine production. We discovered that the cytokine production pattern of alveolar macrophages was similar to BMMs except IL-6 (Fig 3.4).

3.2.2 RCAN1 deficiency leads to increased *P. aeruginosa* LPS-induced IκBα phosphorylation *in vitro*

In canonical MyD88-NF-κB signal transduction, the tripartite IKK complex liberates the NF-κB transcription factor by phosphorylating IκBα, thereby stimulating IκBα poly-ubiquitination and degradation by the 26S proteasome; following IκBα degradation, NF-κB translocates to the nucleus where it transactivates genes that regulate immunity, inflammation and cell fate (Israel, 2010). To understand the molecular mechanisms of RCAN1 regulation of the MyD88-dependent pathway, we characterized the phosphorylation levels of IKK complex subunits IKKα and IKKβ, as well as IκBα, in wild-type and RCAN1-deficient BMMs following *P. aeruginosa* LPS challenge at various time points (3 h, 6 h, 12 h and 24 h) by western blotting (Fig 3.5A). The *P. aeruginosa* LPS-induced phosphorylation of IκBα was markedly enhanced in RCAN1-deficient BMMs compared to wild-type BMMs, whereas no significant differences of IKKα and β phosphorylation levels were observed between wild-type and RCAN1-deficient BMMs (Fig 3.5B, C and D), suggesting that IκBα is a potential target site of RCAN1 in *P. aeruginosa* LPS-induced MyD88 pathway.

3.2.3 RCAN1-deficient BMMs display enhanced NF-κB activity in response to *P. aeruginosa* LPS stimulation

The transcription factor NF-κB is a master regulator of inflammatory responses (Oeckinghaus and Ghosh, 2009). To determine whether RCAN1 deficiency has an impact on NF-κB activation *in vitro*, nuclear extracts from *P. aeruginosa* LPS-challenged or untreated wild-type and RCAN1-deficient BMMs were subjected to EMSA to analyze NF-κB activity. NF-κB activity was greatly enhanced in RCAN1-deficient BMMs compared

to wild-type BMMs (Fig 3.6). This finding corroborates our observation of increased IκBα phosphorylation in RCAN1-deficient BMMs, and suggests that RCAN1 negatively regulates the *P. aeruginosa* LPS-induced NF-κB activity *in vitro*.

3.2.4 RCAN1 deficiency impairs TRIF-IRF-ISRE pathway activation in macrophages in response to *P. aeruginosa* LPS stimulation

To further demonstrate the regulatory role of RCAN1 in TRIF-IRF-ISRE pathway, we analyzed the *P. aeruginosa* LPS-induced mRNA and protein expression of IRF3 and IRF7 in wild-type and RCAN1-deficient BMMs by RT-qPCR and Western blot respectively. There was no significant differences observed in the *P. aeruginosa* LPS-induced IRF3 mRNA levels between wild-type and RCAN1-deficient BMMs (Fig 3.7A). By contrast, RCAN1-deficient BMMs displayed diminished IRF7 mRNA levels compared to the wild-type BMMs following *P. aeruginosa* LPS challenge (Fig 3.7B). Similarly, the IRF3 protein was found to be constitutively expressed during LPS treatment, whereas the IRF7 protein levels were significantly elevated at 3 h and RCAN1-deficient BMMs displayed reduced IRF7 protein expression compared to wild-type BMMs (Fig 3.7C, D and E). These results suggest that RCAN1 facilitates IRF7 expression *in vitro*.

After nuclear translocation, IRF3 and IRF7 induce transcription through binding to ISRE sites in the promoters of target genes (Honda and Taniguchi, 2006b). Using EMSA, we tested the *P. aeruginosa* LPS-induced ISRE binding activity in wild-type and RCAN1-deficient BMMs. Wild-type and RCAN1-deficient BMMs were treated with 200 ng/ml *P. aeruginosa* LPS for 2, 4, 6 and 8 h or left untreated. ISRE binding activity was significantly reduced but not abolished in the RCAN1-deficient BMMs at 2 h, compared to wild-type BMMs (Fig 3.8A and C). Because both IRF3 and IRF7 can contribute to ISRE binding

activity, supershift assays for IRF3 and IRF7 were performed on nuclear extracts from 2 h LPS-stimulated wild-type BMMs, using IRF3 and IRF7 antibodies to determine whether the two proteins contribute to ISRE binding. The IRF3 and IRF7 antibodies employed in these supershift assays were previously validated by us and others (Eguchi et al., 2008; Yue et al., 2016). Both IRF3 and IRF7 antibodies blocked ISRE binding activity, and the IRF7 antibody resulted in a greater reduction of ISRE binding activity than the IRF3 antibody (Fig 3.8B and D). Moreover, the binding specificity of nuclear proteins to ISRE DNA sequence was verified through competitive binding by 50 X non-radioisotope labeled ISRE probes (Fig 3.8B). These findings suggest that RCAN1 contributes to ISRE binding activity and IRF7 is predominant in binding of ISRE in response to *P. aeruginosa* LPS stimulation. To further demonstrate RCAN1-regulated IRF7 activation, the nuclear extracts from non-treated (NT) and 2 h LPS-stimulated wild-type and RCAN1-deficient BMMs were examined by transcription factor ELISA for IRF7. Consistent with the EMSA results, reduced IRF7 activation at 2 h was observed in RCAN1-deficient BMMs compared with wild-type BMMs (Fig 3.8E).

3.2.5 RCAN1 differentially regulates the activation of mitogen-activated protein kinases

TLR signaling is able to activate mitogen-activated protein kinases (MAPKs), including p38, ERK, and JNK, which are important for mediation of inflammatory gene expression (Kawasaki and Kawai, 2014). To determine whether RCAN1 plays a role on MAPK activation, cell lysates from *P. aeruginosa* LPS-activated wild-type and RCAN1-deficient BMMs were subjected to Western blotting to assess the phosphorylation levels of ERK, JNK, and p38 (Fig 3.9A). Interestingly, RCAN1-deficient BMMs displayed significantly increased phosphorylation of ERK but reduced phosphorylation of JNK

compared to wild-type BMMs (Fig 3.9B and C). Moreover, no statistically significant differences in p38 phosphorylation were observed between wild-type and RCAN1-deficient BMMs (Fig 3.9D). These findings suggest that RCAN1 differentially regulates MAPK signaling pathways in our system.

3.2.6 RCAN1-deficient mice display enhanced MyD88-NF-κB-mediated cytokine and chemokine production *in vivo* during *P. aeruginosa* LPS stimulation

To investigate the role of RCAN1 in TLR-MyD88-dependent pathway *in vivo*, we incorporated RCAN1-deficient mice into a model of *P. aeruginosa* LPS-induced acute pneumonia. Wild-type and RCAN1-deficient mice were intranasally administered 1 μg *P. aeruginosa* LPS per gram of body weight for 4 h or 24 h. Lung tissues and BALF were collected to determine the MyD88-NF-κB-mediated production of IL-6 (Fig 3.10A and B), TNF (Fig 3.10C and D) and MIP-2 (Fig 3.10E and F) by ELISA. RCAN1-deficient mice displayed enhanced production of IL-6, TNF and MIP-2 in lungs and BALF compared to wild-type mice, which was consistent with the pattern of *in vitro* cytokine production.

The *in vivo* products of the TRIF-ISRE pathway including IFN-β (Fig 3.11A and B), RANTES (Fig 3.11C and D) and IP-10 (Fig 3.11E and F) were analyzed by ELISA. Compared to the *in vitro* data, RCAN1 deficiency only partially affected these cytokine and chemokine production. In the lungs, there was no significant differences in IFN-β, RANTES, and IP-10 production observed between wild-type and RCAN1-deficient mice following *P. aeruginosa* LPS administration. In the BALF, there was an increased production of IFN-β at 4 h and RANTES at 24 h in RCAN1-deficient mice, and no significant difference in IP-10 between wild-type and RCAN1-deficient mice.

3.2.7 RCAN1-deficient mice display increased NF-κB activity and reduced ISRE binding activity *in vivo* following *P. aeruginosa* LPS challenge

To determine the TLR-mediated activities of transcription factors *in vivo*, nuclear extracts from the lungs of *P. aeruginosa*-challenged wild-type and RCAN1-deficient mice were subjected to EMSA for NF-κB and ISRE activity. *P. aeruginosa* LPS-induced NF-κB activity was significantly enhanced in the lungs of RCAN1-deficient mice at 4 h compared to wild-type mice (Fig 3.12A and B). By contrast, a trend of decreased ISRE binding activity at 4 h in RCAN1-deficient mice was observed. However, this result failed to reach statistical significance (Fig 3.13A and C). Moreover, we also determined the binding specificity of LPS-induced ISRE to IRF3 and IRF7 by performing a supershift assay. Consistent with *in vitro* results, anti-IRF3 antibody and anti-IRF7 antibody reduced LPS-induced ISRE binding activity (Fig 3.13B and D). Additionally, the IRF3 mRNA expression in the lungs of wild-type and RCAN1-deficient mice was not induced in response to *P. aeruginosa* LPS stimulation, whereas a trend of decreased IRF7 mRNA level at 4 h was observed in the lungs of RCAN1-deficient mice compared to the wild-type mice, which did not reach statistical significance (Fig 3.14). These results demonstrate that RCAN1 plays a negative regulatory role in NF-κB activity *in vivo* and RCAN1 deficiency has a limited impact on ISRE binding activity *in vivo*.

3.2.8 RCAN1-deficent mice have increased neutrophil recruitment *in vivo* following *P. aeruginosa* LPS stimulation

TLR signaling is essential for recruitment of neutrophils to the site of injury or bacterial infection (Lebeis et al., 2007; Moles et al., 2014). To examine the impact of RCAN1 deficiency on neutrophil infiltration *in vivo*, the lung and BALF lysates from LPS-

challenged wild-type and RCAN1-deficient mice at 4 h and 24 h were collected to measure the activity of the neutrophil granule-specific enzyme myeloperoxidase (MPO) (Fig 3.15A and B). A significantly increased MPO activity was found in the lungs of RCAN1-deficient mice compared to wild-type mice. In addition, the lung histology data suggest that RCAN1-deficient mice had enhanced neutrophil recruitment in response to *P. aeruginosa* LPS stimulation compared to wild-type counterparts (Fig 3.15C). These results indicate that RCAN1 plays an important role in LPS-induced neutrophil recruitment in the lungs.

3.3 Discussion

TLRs are highly conserved pattern-recognition receptors that are essential for production of proinflammatory cytokines and antimicrobial mediators in innate immunity (Sabroe et al., 2008). Ligand binding to TLRs activates two distinct pathways, MyD88-NF-κB and TRIF-IRF-ISRE, which are tightly controlled in healthy individuals (Takeda and Akira, 2004). Negative regulation of TLR signaling is essential for maintaining proper homeostasis and preventing immune pathology (Kondo et al., 2012). Although many negative regulators have been identified in the past decade, the molecular mechanisms of how these negative regulators govern TLR signaling are incompletely understood. We previously identified a small evolutionary conserved protein, RCAN1, as a central negative regulator of inflammation during *P. aeruginosa* infection (Junkins et al., 2013a). Herein, we utilized *P. aeruginosa* LPS to directly activate TLR4 signaling and revealed a differential role of RCAN1 in regulation of MyD88-NF-κB and the TRIF-IRF-ISRE pathways *in vitro*: RCAN1 downregulates MyD88-NF-κB pathway through inhibition of IκBα phosphorylation, and promotes activation of TRIF-ISRE pathway through regulation of IRF7 activation and expression (Fig 3.16). The *in vivo* results support an inhibitory role

of RCAN1 in the MyD88-NF-κB pathway, and the impact of RCAN1 deficiency on neutrophil recruitment suggests an important role of RCAN1 in host defense against microbial infection.

The RCAN1 gene consists of 7 exons, of which exons 1-4 can be alternatively spliced into different transcript isoforms (Davies et al., 2007). Alternative splicing and differential promoter usage contribute to generation of different RCAN1 isoforms. The two main isoforms, RCAN1-1 and RCAN1-4, contain exons 1, 5, 6, 7 and exons 4, 5, 6, 7, respectively, and have been identified in a variety of tissues. By contrast, RCAN1-2 and RCAN1-3 proteins are not detectable in tissues and their functions are not clear (Fuentes et al., 1997). However, the expression of the isoforms RCAN1-1 and RCAN1-4 is regulated differently. RCAN1-1 is constitutively expressed in most tissues, whereas the transcription of RCAN1-4 is induced by several stimuli, including intracellular Ca^{2+}, vascular endothelial growth factor (VEGF), injury and oxidative stress (Canellada et al., 2008; Holmes et al., 2010; Porta et al., 2007; Sobrado et al., 2012). In this study, we found that *P. aeruginosa* LPS largely induced mRNA expression of RCAN1-4, but not RCAN1-1 in macrophages, suggesting a potential involvement of RCAN1-4 in LPS-activated TLR4 signaling. Additionally, the inhibitory effects of RCAN1-1 on NF-κB activity have also been previously identified (Liu et al., 2015). Thus, it remains possible that the constitutively expressed RCAN1-1 also plays a role in regulation of TLR signaling.

In the MyD88-NF-κB pathway, we discovered an enhanced phosphorylation level of IκBα, but not IKKα and IKKβ, in *P. aeruginosa* LPS-stimulated RCAN1-deficient BMMs. This led to increased NF-κB activation and upregulated IL-6, TNF and MIP-2 production. Previous studies have shown that RCAN1 is able to inhibit NF-κB activation

by affecting IκBα phosphorylation, and the regulation of IκBα by RCAN1 can be achieved through calcineurin-dependent (Alzuherri and Chang, 2003; Frantz et al., 1994; Steffan et al., 1995; Wu et al., 2013) or -independent mechanisms (Kim et al., 2006; Liu et al., 2015). Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase that consists of a catalytic subunit, calcineurin A, and a regulatory subunit, calcineurin B (Rusnak and Mertz, 2000). RCAN1 interacts with calcineurin A and inhibits the calcineurin-dependent phosphatase activity (Fuentes et al., 2000). Calcineurin was previously shown to facilitate NF-κB activation (Alzuherri and Chang, 2003; Frantz et al., 1994; Frischbutter et al., 2011; Palkowitsch et al., 2011; Steffan et al., 1995). A study reported that calcineurin synergizes with protein kinase C-dependent pathway to enhance NF-κB DNA binding activity by inducing phosphorylation and degradation of IκBα in T-cell lines (Steffan et al., 1995). Moreover, constitutively active expression of calcineurin in muscle C2C12 cells is associated with increased phosphorylation level of IκBα (Alzuherri and Chang, 2003). Additionally, calcineurin has been identified to upregulate TCR-induced NF-κB activity through interaction with Carma1-Bcl10-Malt1 complex and dephosphorylation of Bcl10 in T-cells (Frischbutter et al., 2011; Palkowitsch et al., 2011). By contrast, RCAN1 has also been found to inhibit NF-κB activation independent of calcineurin activity (Kim et al., 2006; Liu et al., 2015). A recent study found that the N-terminal domain RCAN1 directly interacts with IκBα and affects the phosphorylation of IκBα at tyrosine 42 in HEK293 cells (Liu et al., 2015). Therefore, it is possible that RCAN1 suppresses *P. aeruginosa* LPS-induced MyD88-NF-κB pathway indirectly through inhibition of calcineurin or directly interacts with IκBα.

RCAN1 inhibits the NFAT pathway by limiting calcineurin activity (Fuentes et al., 2000). Many studies have demonstrated cooperation between NF-κB and NFAT pathways. NF-κB and NFAT can recognize similar DNA binding sites in target gene promoters, and coordination between them mediates maximal production of cytokines and chemokines (McCaffrey et al., 1992; Sica et al., 1997). Physical interactions between NF-κB and NFAT have been found in cardiomyocytes, which promote the gene expression for cardiac hypertrophic responses (Liu et al., 2012). Moreover, we recently identified that inhibition of NFAT reduced NF-κB DNA binding activity and NF-κB inhibition diminished NFAT DNA binding activity during *P. aeruginosa* lung infection (Pang et al., 2017). In light of these facts, it is likely that increased NFAT activity contributes to increased *P. aeruginosa* LPS-induced NF-κB activation in RCAN1-deficient systems.

There is accumulating evidence for RCAN1's role in pathway activation; RCAN1 enhances cAMP-induced CREB phosphorylation and CREB-mediated gene transcription in neuronal PC12 cells (Kim and Seo, 2011), and mediates neuronal apoptosis by activation of caspase-3 and caspase-9 responsible for apoptotic signaling (Sun et al., 2011). Furthermore, the positive role of RCAN1 has previously discovered to depend on post-translational modifications such as phosphorylation and expression level of RCAN1 (Liu et al., 2009; Shin et al., 2011). Our data provide the first evidence that RCAN1 contributes to the activation of the TRIF-IRF7-ISRE pathway. RCAN1 deficiency impedes *P. aeruginosa* LPS-induced ISRE binding activity and TRIF-IRF-ISRE-mediated mRNA and protein expression of IFN-β, RANTES and IP-10 *in vitro*. Impaired IRF7 mRNA and protein expression, but not IRF3, were shown in RCAN1-deficient BMMs, suggesting that RCAN1 facilitates the TRIF-ISRE pathway by targeting IRF7. A study reported that

calcineurin negatively regulates the TRIF pathway-mediated IFN-β production in LPS-activated mouse macrophage cell line RAW 264.7 (Kang et al., 2007). Thus, RCAN1 may promote TRIF-IRF7-ISRE pathway activation by inhibition of calcineurin. However, considering the ability of RCAN1 to modulate protein phosphorylation, it remains formally possible that RCAN1 directly modulates phosphorylation of IRF7 or related proteins in the pathway.

The TRIF-dependent pathway plays a well-characterized role in host antiviral defense by increasing production of type-I IFN production (Vercammen et al., 2008). Moreover, it also defends against bacterial infection by mediating MyD88-independent activation of NF-kB and production of inflammatory mediators (Ullah et al., 2016). However, the mechanisms involved in TRIF-mediated host protection against bacterial pathogens are not fully understood. Recent studies have shown that TRIF deficiency reduces production of proinflammatory cytokines and chemokines, such as IFN-β, TNFα, KC, RANTES and IP-10, and diminished neutrophil recruitment, leading to increased bacterial burden and decreased survival (Cai et al., 2009; Carrigan et al., 2010; Power et al., 2007). In this study, we dissected the differential roles of the RCAN1 in regulating the production of chemoattractants of neutrophils and how this affected neutrophil recruitment triggered by LPS. We found that the LPS-activated RCAN1-deficient BMMs displayed reduced, but not abolished, RANTES and IP-10 production via the TRIF-IRF-ISRE pathway. By contrast, the MyD88-mediated production of chemokine MIP-2 was greatly enhanced in RCAN1-deficient BMMs. Furthermore, neutrophil recruitment in the lungs of RCAN1-deficient mice was not affected by impaired production of RANTES and IP-10 mediated through TRIF pathway by BMMs. These findings suggest that the role of

MyD88-dependent pathway is dominant over TRIF-dependent pathway in bacterial infection. A previous study showed that MyD88-deficient mice manifested a much more remarkable phenotype, including reduced survival and impaired bacterial clearance, compared with TRIF-deficient mice (Cai et al., 2009). Thus, our findings support the established model for a dominant role of the MyD88-dependent pathway in response to bacterial infection.

The *P. aeruginosa*-LPS stimulated alveolar macrophages displayed similar cytokine and chemokine production pattern as BMMs except IL-6, which showed a trend of increase in RCAN1-deficient alveolar macrophages but it did not reach statistical significance (Fig. 3.4A). However, the *in vivo* cytokine data showed that the levels of IL-6 were significantly elevated in RCAN1-deficient mice (Fig. 3.10A and B). This discrepancy may be due to the neutrophils infiltrated from bloodstream became the major immune cell type in the *P. aeruginosa*-infected lungs (Koh et al., 2009), which could be the major source contributing to the *in vivo* level of IL-6 instead of alveolar macrophages.

The *in vivo* pattern of TRIF-ISRE-regulated IFN-β, RANTES and IP-10 production in lungs and BALF following *P. aeruginosa* LPS stimulation was not consistent with the *in vitro* data. Furthermore, the *in vivo* ISRE binding activity was not significantly impaired in RCAN1-deficient mice, whereas RCAN1 deficiency greatly reduced ISRE binding activity in macrophages. This could be explained by the fact that lung tissues consist of different kinds of immune cells and non-immune cells other than macrophages, and RCAN1 may function differently in these other cell types. Previous reports have described diverse roles of RCAN1 in calcineurin activity, which depends on cell types and cellular context (Liu et al., 2009; Ryeom et al., 2003; Sanna et al., 2006; Shin et al., 2011; Vega et

al., 2003). Phosphorylation of RCAN1 by TAK1 at serine 94 and 136, switches RCAN1 from an inhibitor to a facilitator of calcineurin-NFAT signaling in cardiomyocytes (Liu et al., 2009). In cardiac hypertrophic model in mice, RCAN1 promotes calcineurin activity (Sanna et al., 2006). In contrast, in T cells (Ryeom et al., 2003) and many other cell types, RCAN1 inhibits calcineurin activity. Similarly, CpG DNA-induced TLR signaling stimulates IFN-β production in dendritic cells, but not in macrophages (Schroder et al., 2007). Many cell types have the TRIF pathway capacity including dendritic cells, neutrophils, natural killer (NK) cells, T-cells, lung epithelial cells, lung endothelial cells and fibroblasts in bacterial or viral infection (Cassatella et al., 1997; Dorner et al., 2004; Feng et al., 2005; Fukui et al., 2013; Hess et al., 1987; Kallfass et al., 2013; Klimpel et al., 1990; Piqueras et al., 2006; Sundstrom et al., 2001). It is possible that these cell types participate in the *in vivo* model of *P. aeruginosa* infection. However, how RCAN1 and TRIF function in each one of these cell types is not clear. A combination of the possible inhibitory, stimulatory or no effect of RCAN1 on TLR signaling in different cell types *in vivo* likely contributes to the results that we observed in RCAN1-deficient mice *in vivo*. Although the *in vivo* data in RCAN1-deficient mice could not specifically reveal the role of macrophages, they provide the valuable information of how MyD88-dependent and TRIF-dependent cytokine/chemokine profile changes in RCAN1-deficient animals. Future studies should focus on the investigation of RCAN1 deficiency in macrophages *in vivo*.

In this study, we also identified a differential role of RCAN1 in regulation of MAPK activation, whereby RCAN1 deficiency leads to enhanced ERK phosphorylation and reduced JNK phosphorylation in response to *P. aeruginosa* LPS stimulation *in vitro*, supporting the notion that RCAN1 can differentially regulate signal transduction pathways.

The enhanced ERK phosphorylation in *P. aeruginosa* LPS-stimulated RCAN1-deficient macrophages is consistent with our previous observation of ERK hyperphosphorylation in RCAN1-deficient macrophages during *P. aeruginosa* infection (Junkins et al., 2013a). ERK and NF-κB p65 interactions have been previously described (Wang et al., 2005), and enhanced ERK phosphorylation upregulates NF-κB activity (Chen et al., 2016; Dhawan and Richmond, 2002; Jiang et al., 2004). JNK plays a critical role in inducing the expression of pro-apoptotic proteins (Dhanasekaran and Reddy, 2008). Multiple studies have demonstrated crosstalk between NF-κB and JNK activation. NF-κB was found to suppress JNK activation by mediating production of JNK inhibitors (De Smaele et al., 2001; Pinna et al., 2017; Tang et al., 2001). In addition, LPS is able to induce apoptosis in macrophages through autocrine secretion of TNFα (Xaus et al., 2000). Therefore, it is possible that the enhanced NF-κB activation by RCAN1 deficiency would inhibit the *P. aeruginosa* LPS-induced JNK phosphorylation and pro-apoptotic events.

Altogether, our findings demonstrate a novel regulatory mechanism of RCAN1 in TLR signaling, which differentially regulates MyD88-NF-κB and TRIF-IRF7-ISRE signaling pathways. This study broadens our understanding of regulation of TLR signaling in innate immunity and suggests that RCAN1 could be a potential therapeutic target in many inflammatory and autoimmune diseases with dysregulation of TLR signaling.
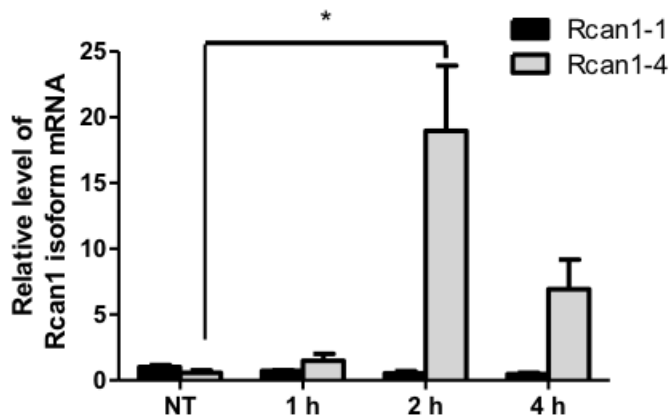
Fig 3.1. Rcan1-4 transcription is induced by *P. aeruginosa* LPS in macrophages. Wild-type (+/+) BMMs were treated with 200 ng/ml *P. aeruginosa* LPS for 1 h, 2 h, 4 h or left untreated (NT). Total RNA isolated from these cells was reverse transcribed to cDNA and subjected to real-time quantitative PCR for *Rcan1-1* and *Rcan1-4*. The *Rcan1-1* and *Rcan1-4* gene expression was normalized to housekeeping control gene *HPRT* (n=3 ± SEM, *p < 0.05).
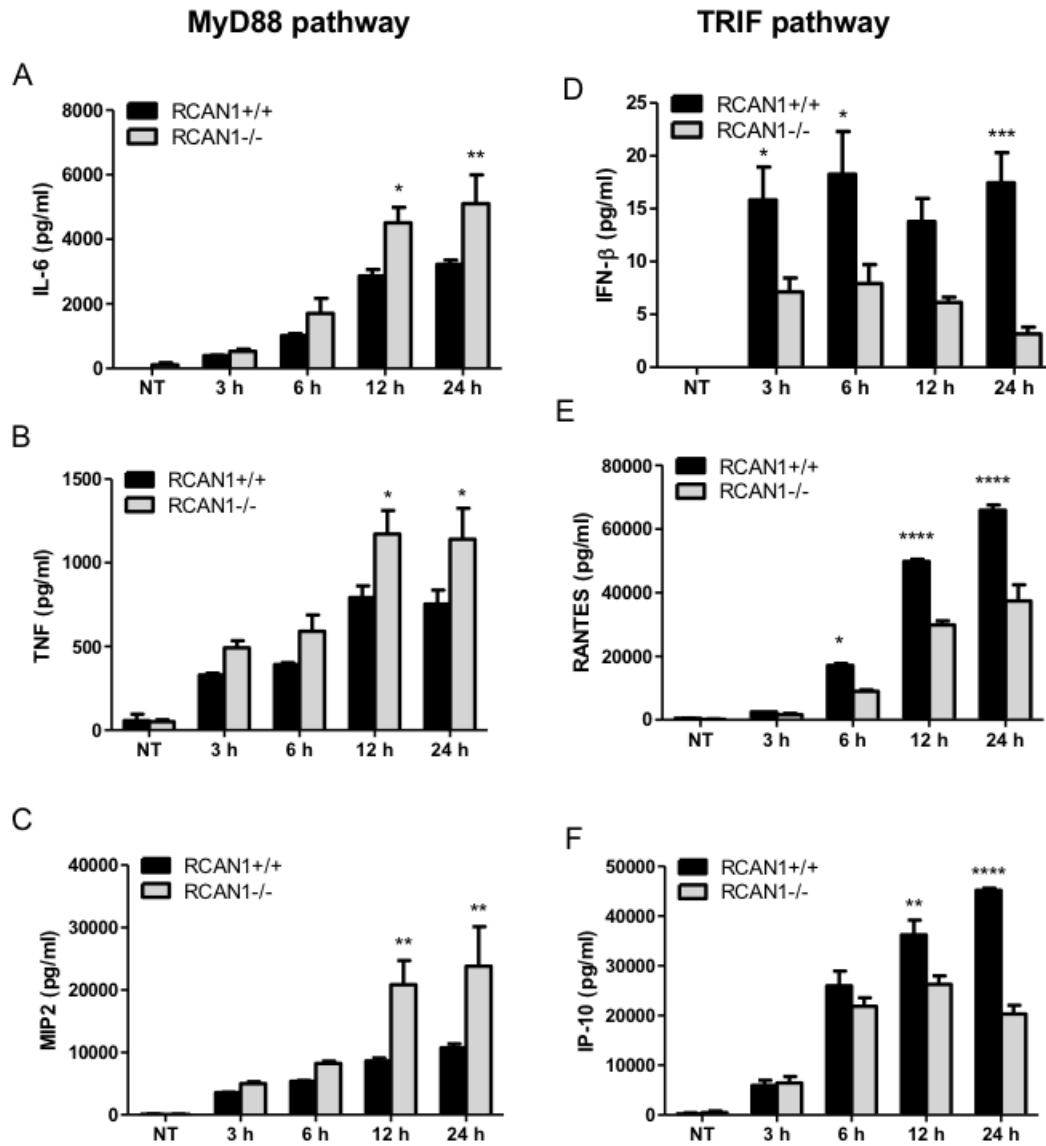
Fig 3.2. RCAN1 deficiency upregulates MyD88-NF-κB-mediated cytokine and chemokine production but downregulates TRIF-IRF-ISRE-mediated cytokine and chemokine production in BMMs during *P. aeruginosa* LPS stimulation. Wild-type (+/+) and RCAN1-deficient (-/-) BMMs were stimulated with 200 ng/ml *P. aeruginosa* LPS for 3 h, 6 h, 12 h, 24 h or left untreated (NT). Cell supernatants were collected for the determination of IL-6 (A), TNF (B), MIP-2 (C), IFN-β (D), RANTES (E) and IP-10 (F) secretion by ELISA (n=3 ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001 ****p < 0.0001).
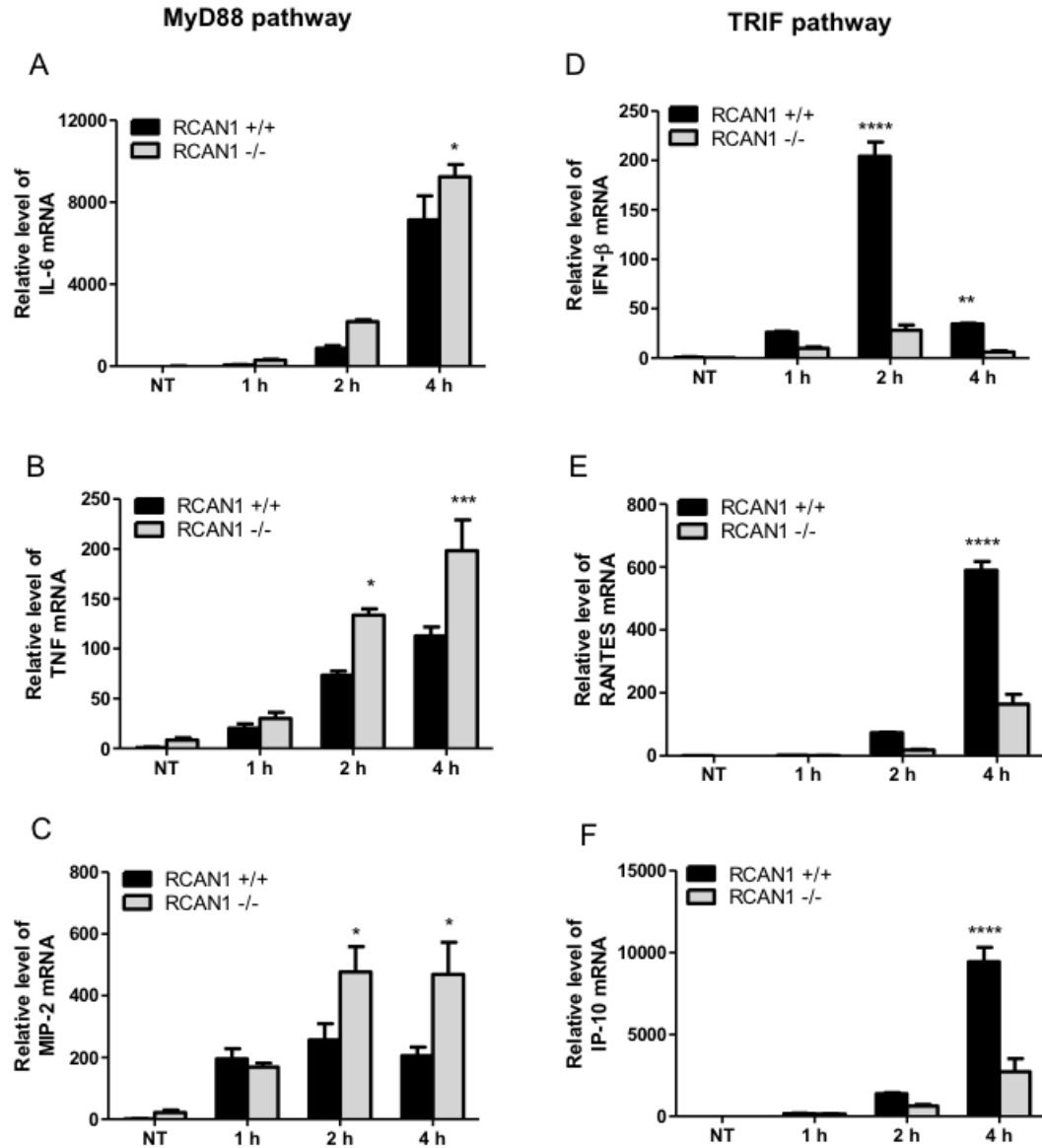
Fig 3.3. RCAN1-deficient BMMs display enhanced cytokine gene expression in MyD88-dependent pathway and reduced cytokine gene expression in TRIF-dependent pathway during *P. aeruginosa* LPS stimulation. Wild type (+/+) and RCAN1-deficient (-/-) BMMs were treated with 200 ng/ml *P. aeruginosa* LPS for 1 h, 2 h, 4 h or left untreated (NT). Total RNA isolated from these cells was reverse transcribed to cDNA and subjected to real-time quantitative PCR for determining *IL-6* (A), *TNF* (B), *MIP-2* (C) *IFN-β* (D), *RANTES* (E) and *IP-10* (F) gene expression. The gene expression was normalized to housekeeping control gene *HPRT* (n=3 ± SEM, *p < 0.05, ***p < 0.001 ****p < 0.0001).
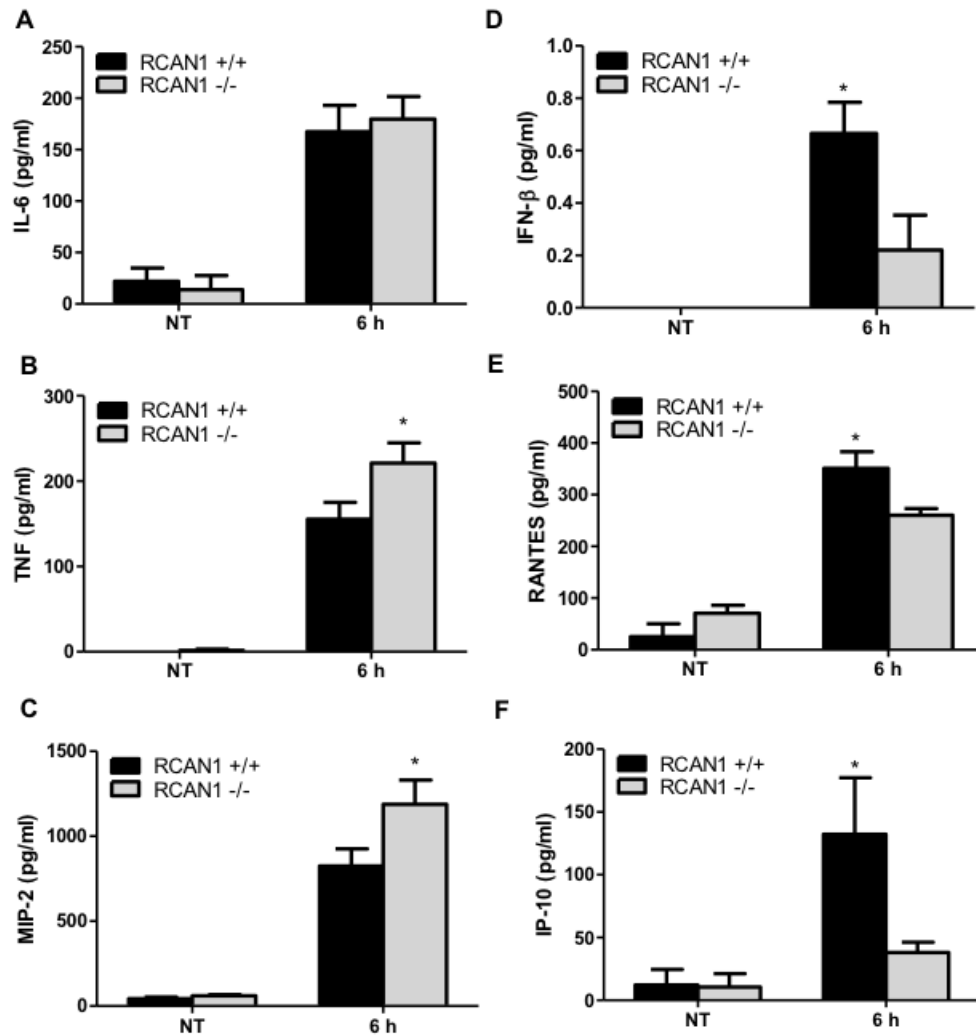
Fig 3.4. RCAN1 deficiency upregulates MyD88-mediated cytokine and chemokine production but downregulates TRIF-IRF-ISRE-mediated cytokine and chemokine production in BALF alveolar macrophages during *P. aeruginosa* LPS stimulation. Wild-type (+/+) and RCAN1-deficient (-/-) alveolar macrophages were stimulated with 200 ng/ml *P. aeruginosa* LPS for 6 h or left untreated (NT). Cell supernatants were collected for the determination of IL-6 (A), TNF (B), MIP-2 (C), IFN-β (D), RANTES (E) and IP-10 (F) secretion by ELISA. (n=3 ± SEM, *p < 0.05).
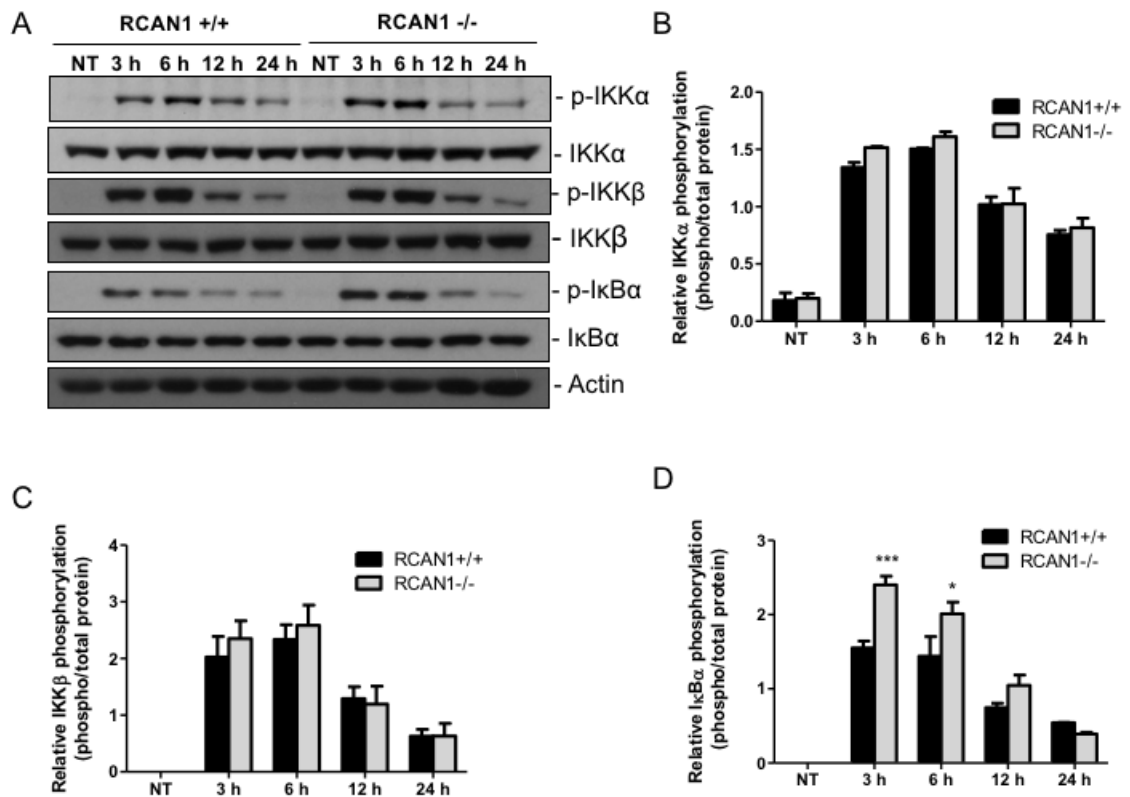
Fig 3.5: RCAN1 deficiency elevates IκBα phosphorylation *in vitro* following *P. aeruginosa* LPS challenge. Wild-type (+/+) and RCAN1-deficient (-/-) BMMs were stimulated with 200 ng/ml *P. aeruginosa* LPS for 3 h, 6 h, 12 h, 24 h or left untreated (NT). Cell lysates were subjected to Western blot analysis for phospho- and total IKKα, IKKβ and IκBα, as well as actin as a loading control. Blots are representative of three independent experiments (A). Densitometry analysis of phosphorylated IKKα (B), IKKβ (C) and IκBα (D) was normalized to their total protein respectively (n=3 ± SEM, *p < 0.05, ***p < 0.001).
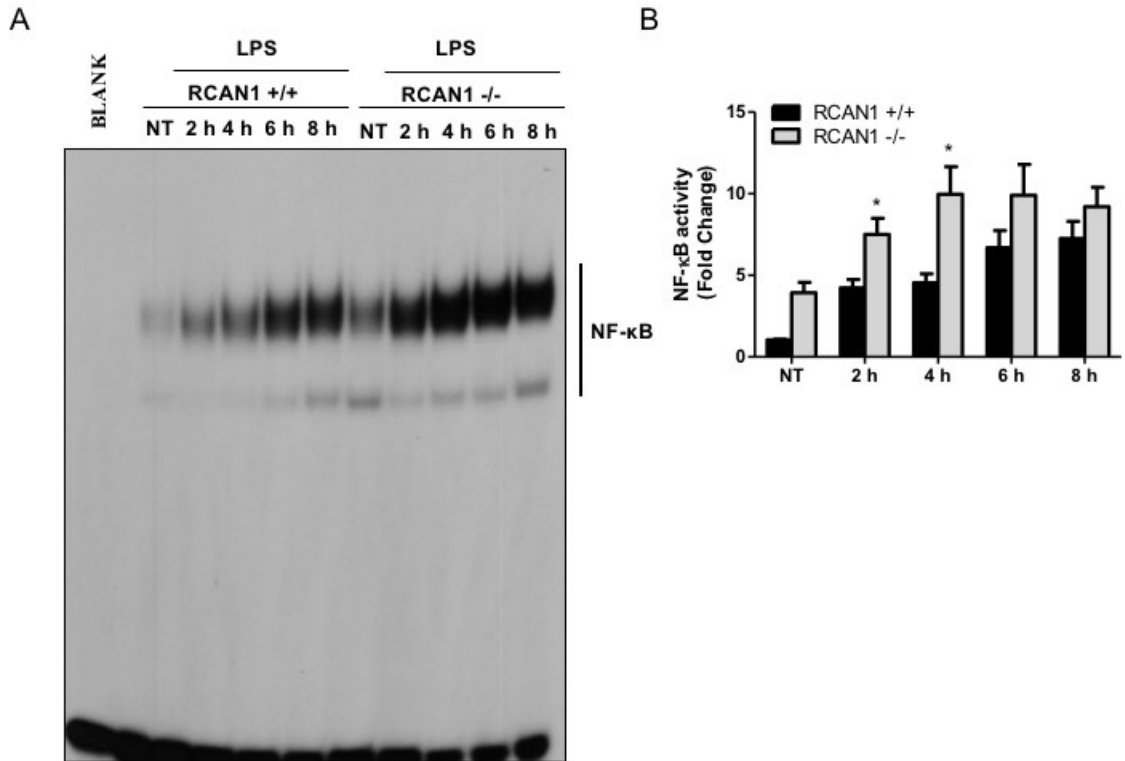
Fig 3.6. RCAN1 deficiency enhances NF-κB activity *in vitro* following *P. aeruginosa* LPS challenge of mouse BMMs. Wild-type (+/+) and RCAN1-deficient (-/-) BMMs were treated with 200 ng/ml *P. aeruginosa* LPS for 2 h, 4 h, 6 h, 8 h or left untreated (NT). Nuclear proteins were extracted and subjected to EMSA by incubation with ^{32}P-labeled NF-κB DNA probe (A). Data are representative of three individual experiments. Scan densitometry was performed for analysis of NF-κB activity (B), and data are expressed as fold change (n = 3 ± SEM, *p < 0.05).
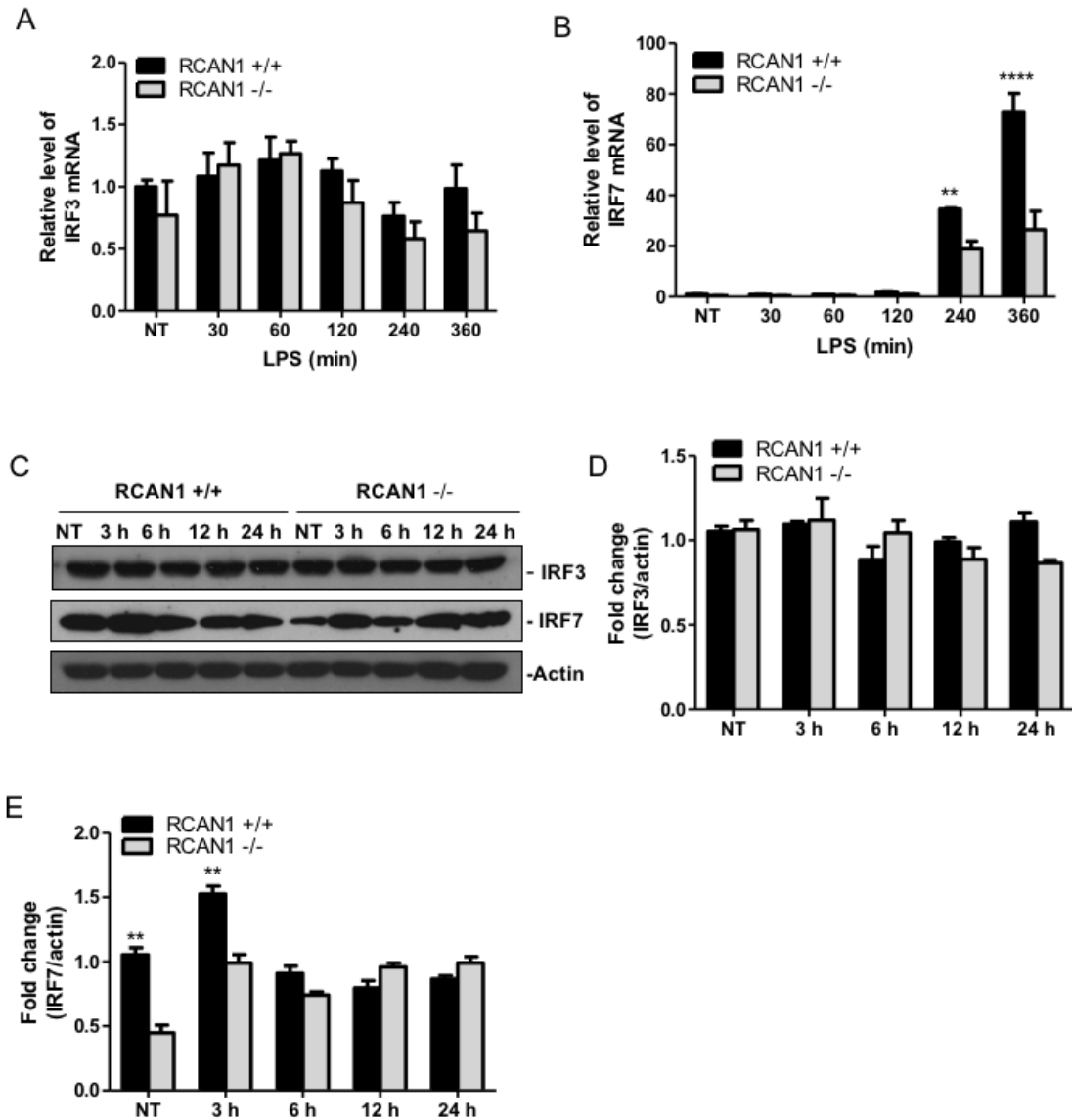
Fig 3.7. RCAN1-deficient BMMs display diminished IRF7 mRNA and protein expression upon *P. aeruginosa* LPS stimulation. Wild type (+/+) and RCAN1-deficient (-/-) BMMs were treated with 200 ng/ml *P. aeruginosa* LPS for various time points or left untreated (NT). The total RNA isolated from these cells was reverse transcribed to cDNA and subjected to real-time quantitative PCR for *IRF3* (A) and *IRF7* (B) gene expression. The gene expression was normalized to housekeeping control gene *HPRT*. Cell lysates were immunoblotted to measure IRF3, IRF7 and actin protein levels. Immunoblots are representative of three independent experiments (C). Densitometry analysis of IRF3 and IRF7 levels was normalized to actin (D, E), and data are presented as fold change (n=3 ± SEM, **p<0.01, ****p<0.0001).
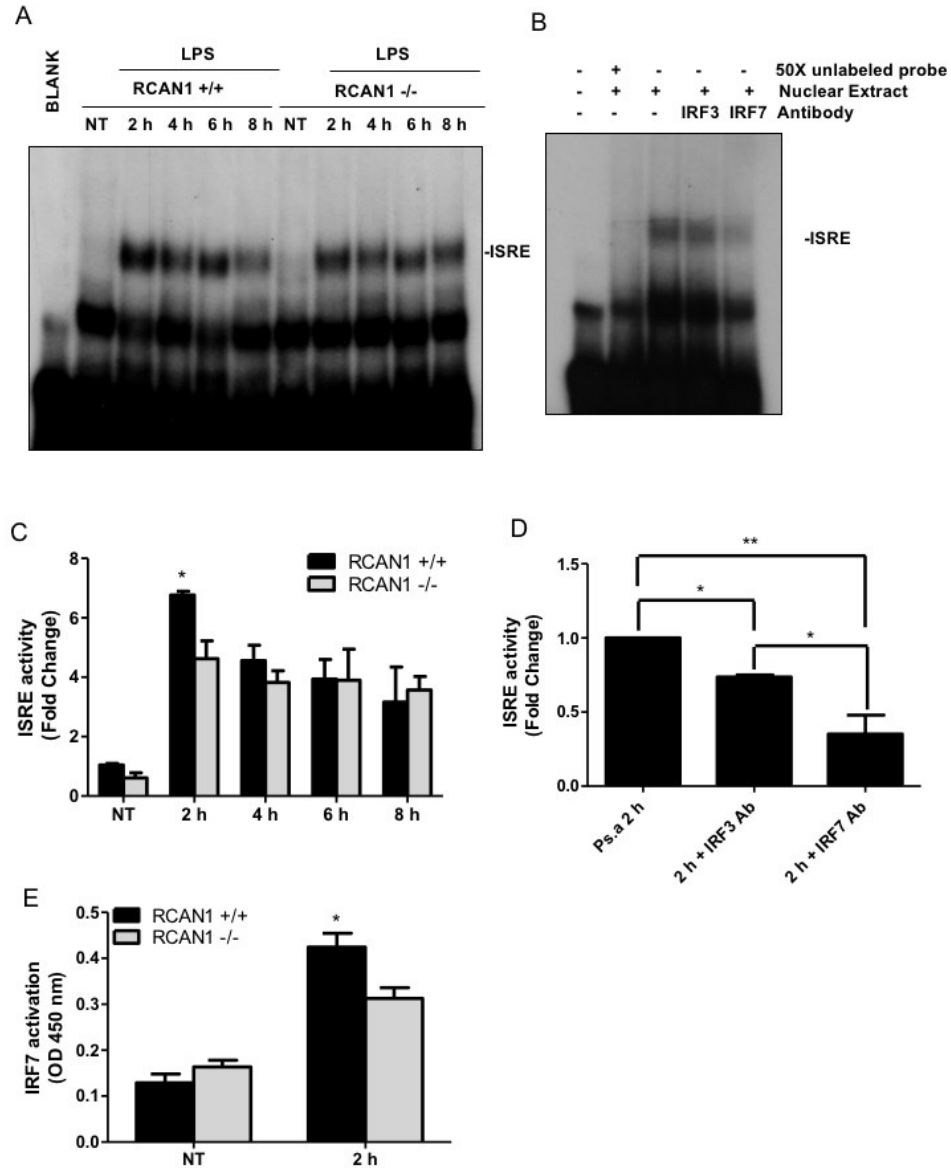
Fig 3.8. RCAN1 deficiency impairs TRIF-IRF-ISRE activity *in vitro* during *P. aeruginosa* LPS stimulation. Wild-type (+/+) and RCAN1-deficient (-/-) BMMs were treated with 200 ng/ml *P. aeruginosa* LPS for 2 h, 4 h, 6 h, 8 h or left untreated (NT). Nuclear proteins were extracted and subjected to EMSA by incubation with ^{32}P-labeled ISRE DNA probe (A). Data are representative of three individual experiments. Nuclear extracts from wild-type (+/+) BMMs treated with 200 ng/ml *P. aeruginosa* LPS for 2 h were incubated with or without specific antibodies to IRF3 and IRF7 for 1 h or 50 X unlabeled ISRE probe for 30 min at room temperature before EMSA experiment using the ^{32}P-labeled ISRE probe (B). Data are representative of three individual experiments. Scan densitometry was performed for analysis of ISRE activity (C, D), and data are expressed as fold change. Cell nuclear extracts from NT and LPS 2 h stimulated wild-type and RCAN1-deficient BMMs were subjected to transcription factor ELISA for determining IRF7 activity (E). (n = 3 ± SEM *p < 0.05, **p < 0.01).
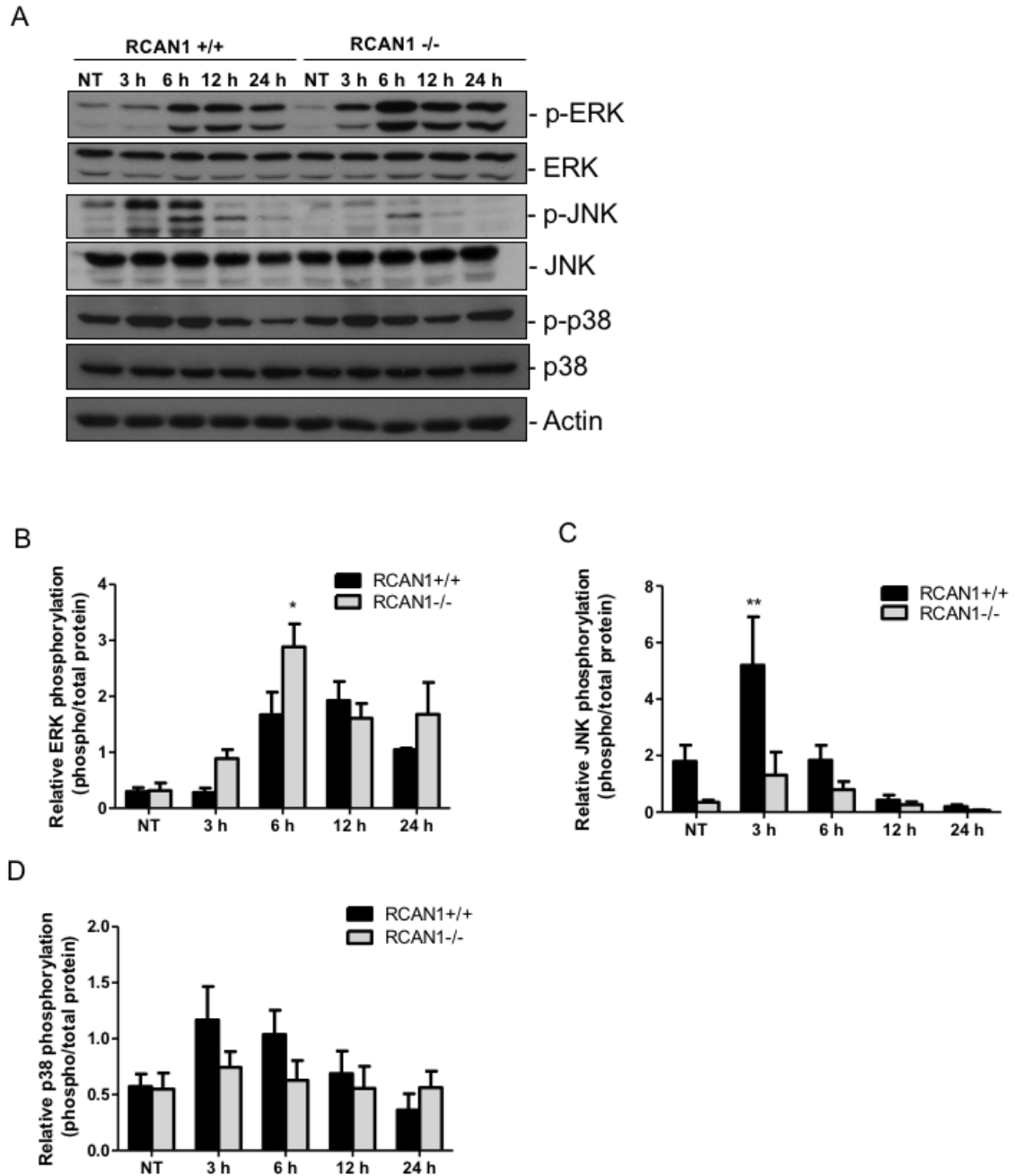
Fig 3.9. RCAN1 differentially regulates MAPK kinase activation *in vitro* in response to *P. aeruginosa* LPS challenge. Wild-type (+/+) and RCAN1-deficient (-/-) BMMs were challenged with 200 ng/ml *P. aeruginosa* LPS for 3 h, 6 h, 12 h, 24 h or left untreated (NT). Cell lysates were subjected to Western blot analysis for phospho- and total ERK, JNK and p38, as well as actin as loading control. Blots are representative of three independent experiments (A). Densitometry analysis of phosphorylated ERK (B), JNK (C) and p38 (D) was normalized to their total protein respectively (n=3 ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$).
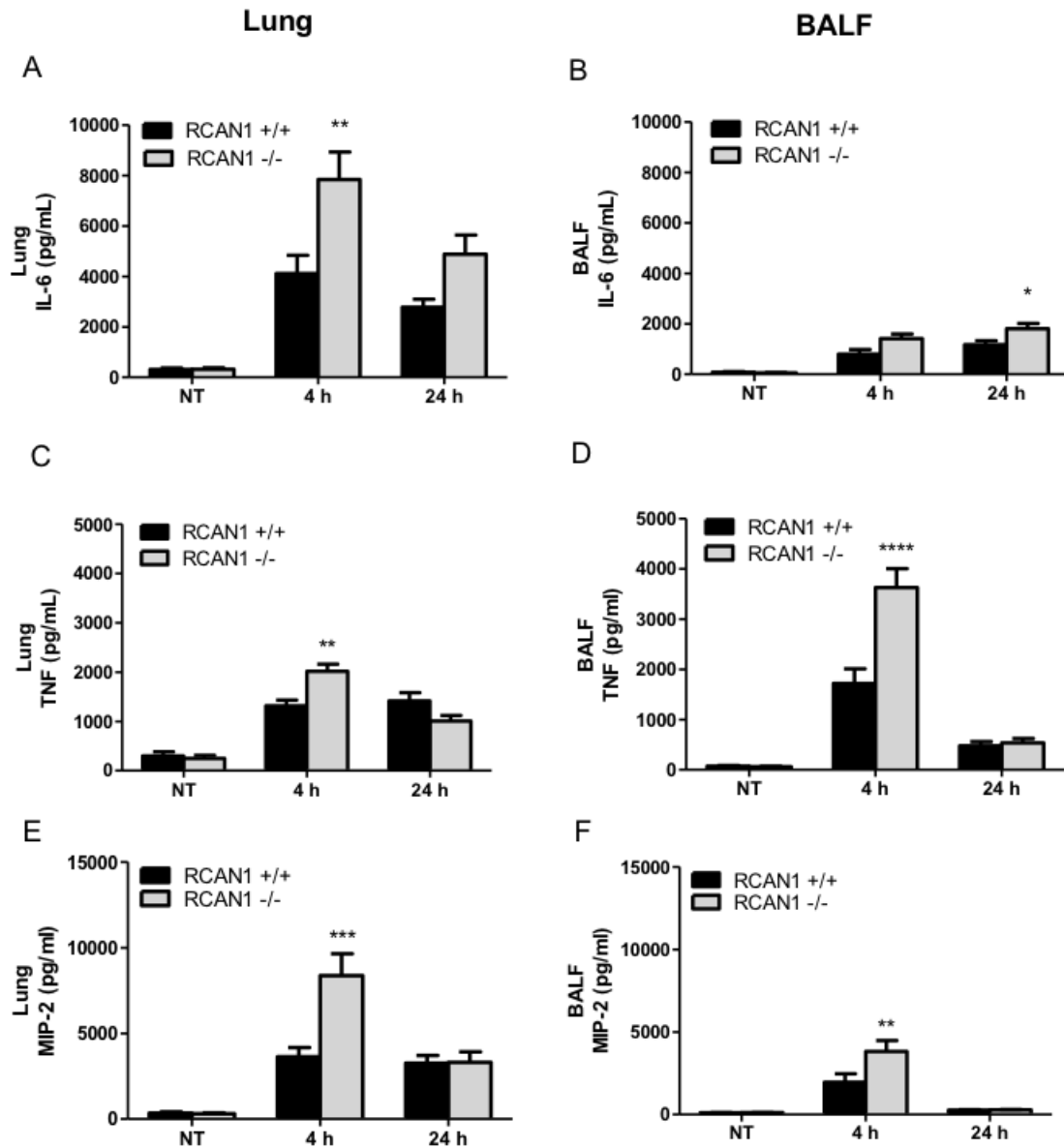
Fig 3.10. RCAN1-deficient mice display enhanced MyD88 pathway-mediated proinflammatory cytokine and chemokine production in response to *P. aeruginosa* LPS stimulation *in vivo*. Wild-type (+/+) and RCAN1-deficient (-/-) mice were treated intranasally with 1 μg *P. aeruginosa* LPS per gram of body weight, 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 determination of IL-6 (A, B) and TNF (C, D), MIP-2 (E, F) production by ELISA. (n=9 ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).
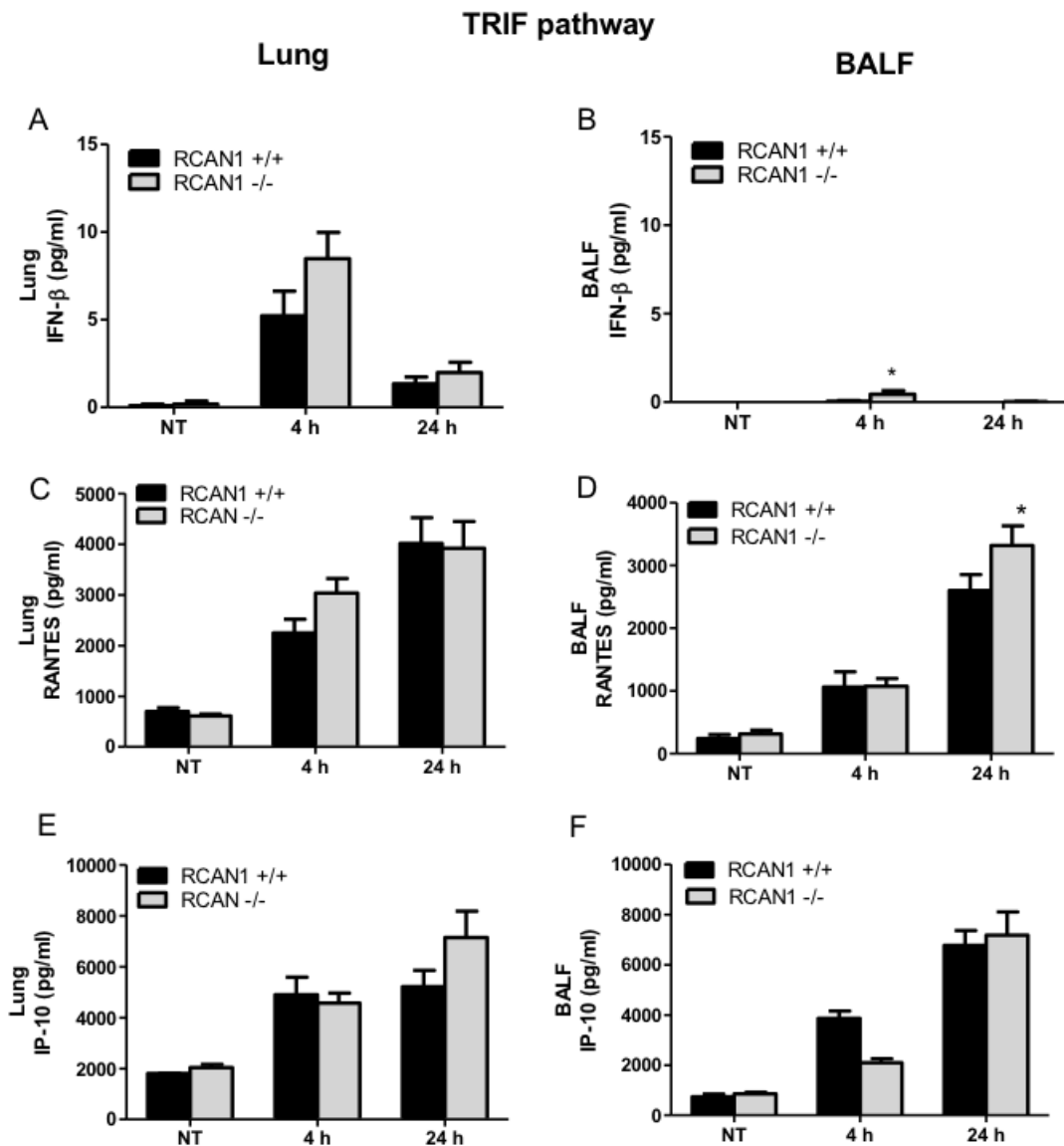
Fig 3.11. RCAN1 deficiency has minor effects on TRIF-IRF-ISRE-regulated cytokine and chemokine production following *P. aeruginosa* LPS stimulation in lung. Wild-type (+/+) and RCAN1-deficient (-/-) mice were administered intranasally with 1 μg *P. aeruginosa* LPS per gram of body weight, or an equivalent 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 IFN-β (A, B), RANTES (C, D) and IP-10 (E, F) production by ELISA (n=9 ± SEM, *p < 0.05).
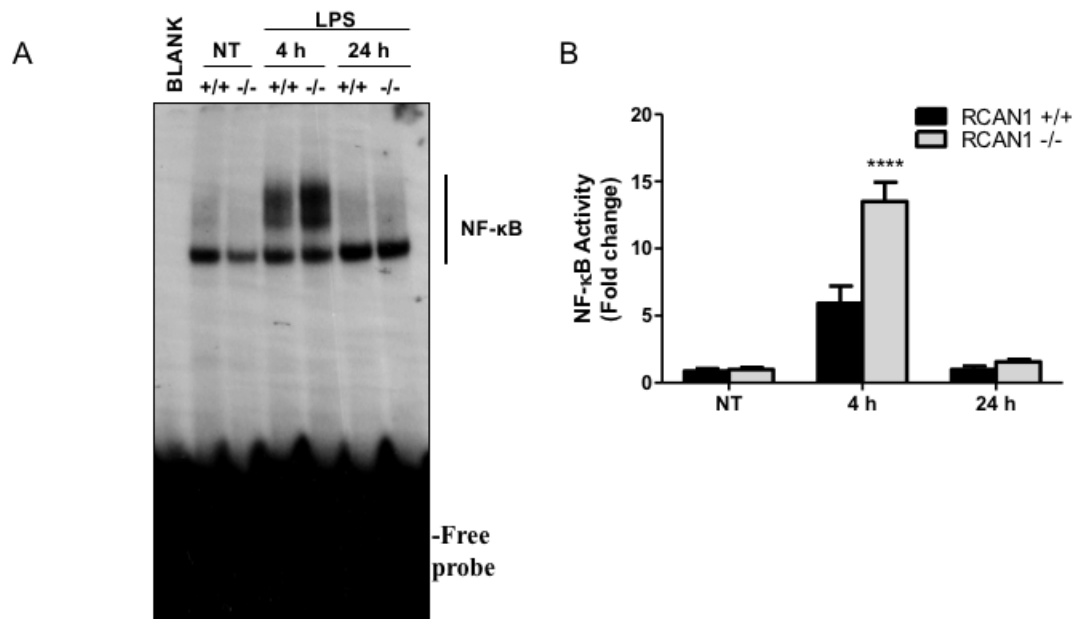
Fig 3.12. RCAN1-deficient mice show increased activity of transcription factor NF-κB *in vivo* **following** *P. aeruginosa* **LPS challenge.** Wild-type (+/+) and RCAN1-deficient (-/-) mice were challenged intranasally with 1 µg *P. aeruginosa* LPS per gram of body weight, or an equivalent volume of saline as a control (NT) for 4 h or 24 h. Nuclear proteins were extracted from lung tissues and subjected to EMSA by incubation with ^{32}P-labeled NF-κB DNA probe (A). Data are representative of six individual experiments. Scan densitometry was performed for analysis of NF-κB activation (B), and data are expressed as fold change versus wild-type untreated lungs (n=6 ± SEM, ****p < 0.0001).
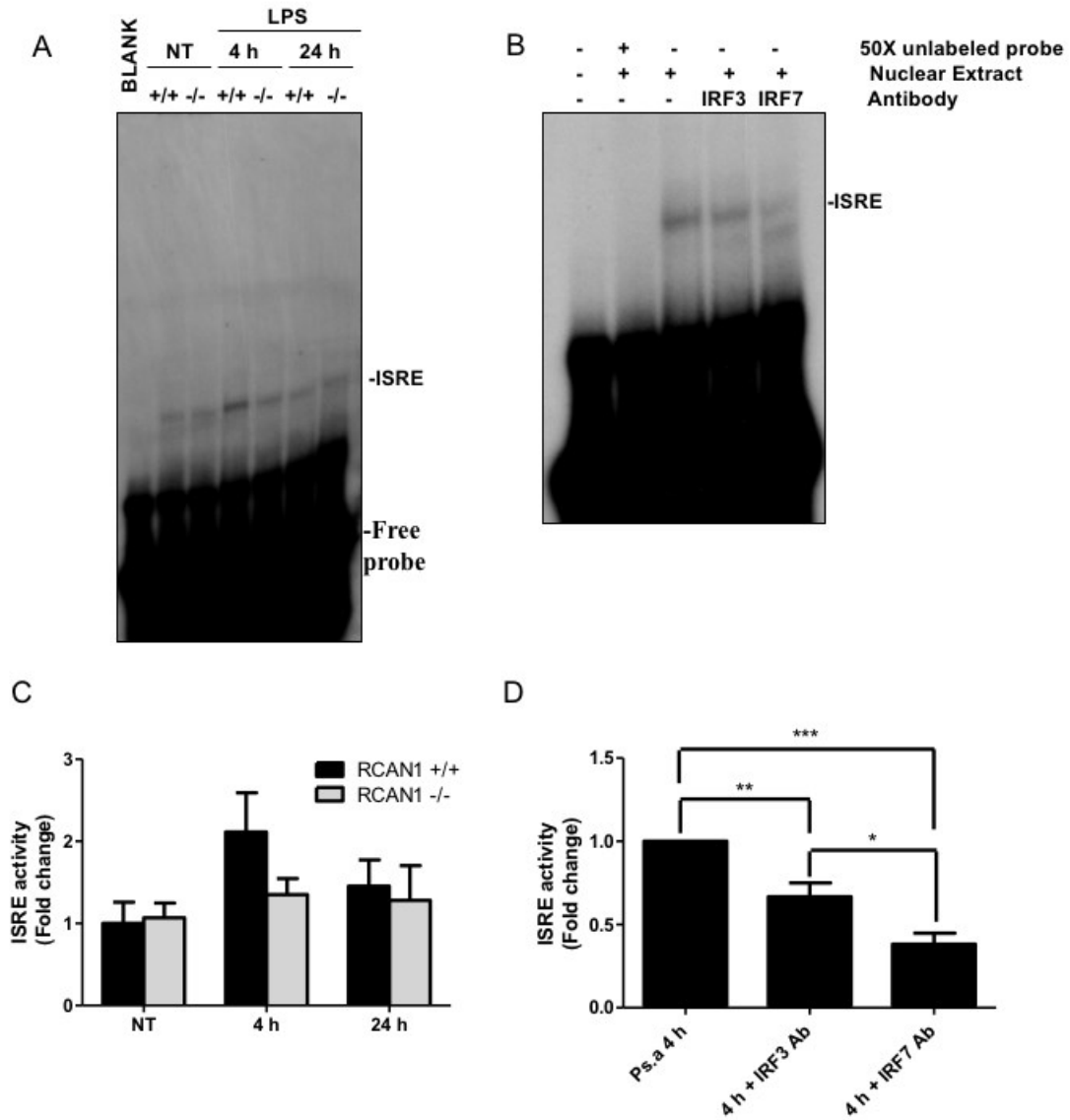
Fig 3.13: RCAN1 deficiency does not significantly affect TRIF-IRF-ISRE activity in lung following *P. aeruginosa* LPS challenge. Wild-type (+/+) and RCAN1-deficient (-/-) mice were challenged intranasally with 1 μg *P. aeruginosa* LPS per gram of body weight, or an equivalent volume of saline as a control (NT) for 4 h or 24 h. Nuclear proteins were extracted from lung tissues and subjected to EMSA by incubation with ^{32}P-labeled ISRE DNA probe (A). Data are representative of six individual experiments. Nuclear proteins from the lungs of wild-type (+/+) mice treated with *P. aeruginosa* LPS for 4 h were incubated with or without specific antibodies to IRF3 and IRF7 for 1 h or 50 X unlabeled ISRE probe for 30 min at room temperature before EMSA experiment using the ^{32}P-labeled ISRE probe (B). Data are representative of five individual experiments. Scan densitometry was performed for analysis of ISRE activity (C, D), and data are expressed as fold change. n = 6 ± SEM (C). n = 5 ± SEM *p < 0.05, **p < 0.01, ***p < 0.001 (D).
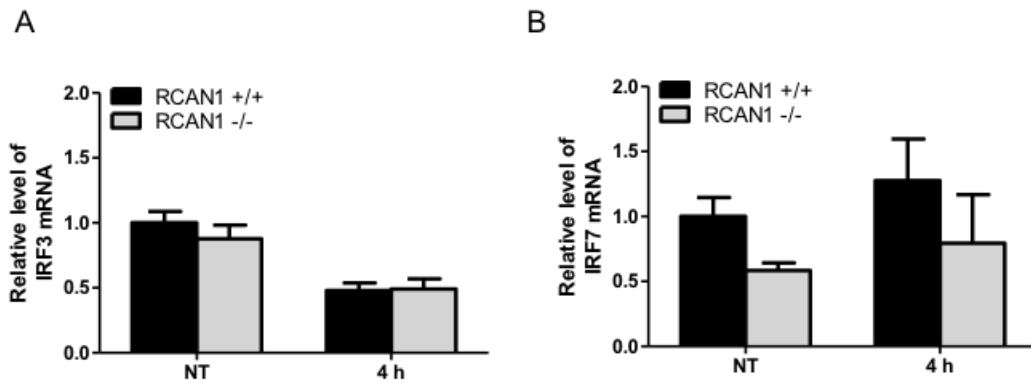
Fig 3.14. RCAN1 deficiency does not significantly impair IRF7 mRNA expression in lung in response to *P. aeruginosa* LPS stimulation. Wild-type (+/+) and RCAN1-deficient (-/-) mice were administered intranasally with 1 μg *P. aeruginosa* LPS per gram of body weight, or an equivalent volume of saline as a control (NT) for 4 h. The total RNA extracted from lungs was reverse transcribed to cDNA and subjected to real-time quantitative PCR for *IRF3* (A) and *IRF7* (B) gene expression. The gene expression was normalized to housekeeping control gene *HPRT* (n = 3 ± SEM).
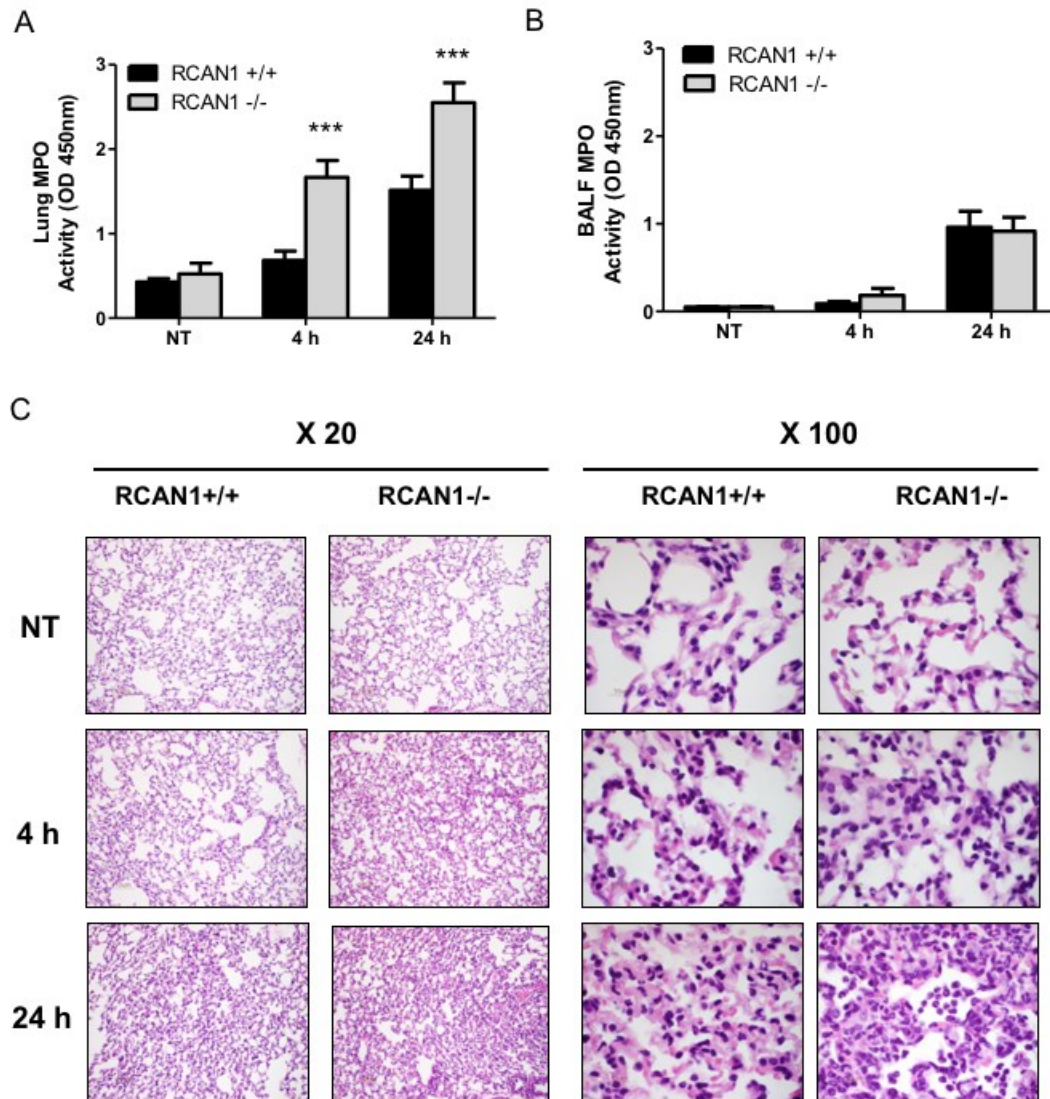
Fig 3.15. RCAN1-deficient mice display enhanced neutrophil infiltration in lung following *P. aeruginosa* LPS stimulation. Wild-type (+/+) and RCAN1-deficient (-/-) mice were stimulated intranasally with 1 μg *P. aeruginosa* LPS per gram of body weight, or an equivalent volume of saline as a control (NT) for 4 h or 24 h. Lungs and BALF were collected after 4 h or 24 h. MPO activities were measured in the Lung (A) and BALF (B) lysate (n=9 ± SEM, ***p < 0.001). The upper lobe of the left lung was collected for H&E staining (original magnification X 20 or X 100) (C). Pictures are representative of 6 mice.
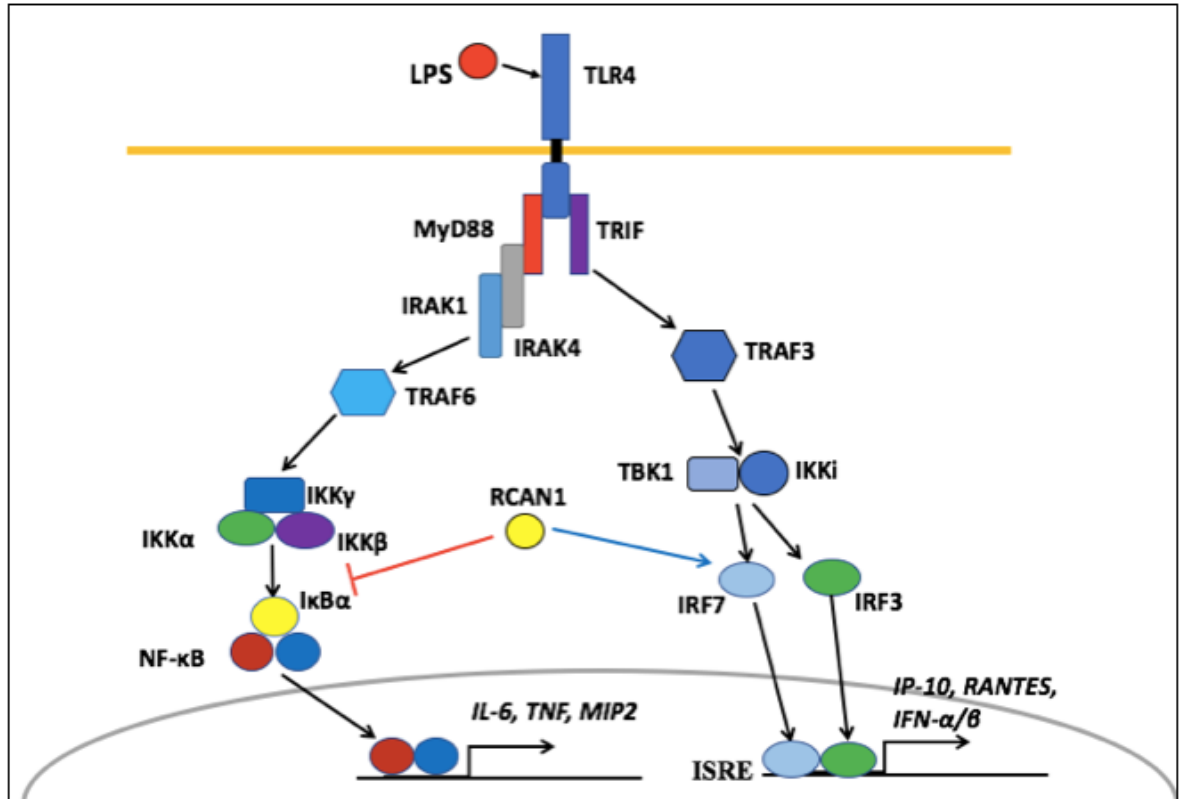
Fig 3.16. Schematic representation of RCAN1-regulated MyD88- and TRIF-dependent signaling pathways. Binding of *P. aeruginosa* LPS to TLR4 activates MyD88- and TRIF-dependent signaling pathways. RCAN1 downregulates MyD88-NF-κB pathway through inhibition of IκBα phosphorylation, and promotes activation of TRIF-ISRE pathway through regulation of IRF7 activation and expression.

Chapter Four – Early growth response 1 deficiency protects host against *Pseudomonas aeruginosa* lung infection

4.1 Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that causes significant morbidity and mortality in cystic fibrosis patients and immunocompromised individuals (Pang et al., 2019). Normally, efficient clearance of pulmonary *P. aeruginosa* infections requires proinflammatory cytokines and chemokines that direct immune cell recruitment to the site of infection (Lavoie et al., 2011). However, excessive and sustained production of proinflammatory cytokines can cause systemic inflammation, severe tissue damage and death (Chen et al., 2018; Jaffer et al., 2010). Systemic inflammation in response to *P. aeruginosa* infection has been shown in humans and other mammals (Kurahashi et al., 1999; Li et al., 2013; Marra et al., 2006). A tightly controlled inflammation level ensures effective host defense to bacterial infection and maintenance of tissue homeostasis (Liu and Cao, 2016). However, the molecular mechanisms controlling host immune responses to *P. aeruginosa* infection remain incompletely defined.

Egr-1, also known as NGFI-A, Krox24, Tis8, Zif268 or ZENK (DeLigio and Zorio, 2009), is a zinc-finger transcription factor that binds to a GC-rich consensus promoter sequence, GCG(G/T)GGGCG, and transactivates genes that regulate cell growth, migration, differentiation and apoptosis (Dinkel et al., 1998; Min et al., 2008; Pignatelli et al., 2003; Thiel and Cibelli, 2002). Egr-1 is broadly expressed in different cell types (Tsai et al., 2000a), and, as its name suggests, is rapidly induced by a wide range of stimuli, including growth factors, cytokines, stress and injury (Dieckgraefe and Weems, 1999; Hoffmann et al., 2008; Lim et al., 1998; Liu et al., 2000). Egr-1 can function either as a

transcriptional activator or a repressor (Gashler et al., 1993; Thiel and Cibelli, 2002). Binding of transcriptional co-repressors NAB1 and NAB2 to the inhibitory domain of Egr-1 causes repression of Egr-1-mediated gene transcription (Qu et al., 1998; Russo et al., 1995). Egr-1 can also bind and modulate the activity of NF-κB and NFAT transcription factors (Decker et al., 2003; Ma et al., 2009). Elevated Egr-1 expression has been linked to production of inflammatory mediators in pulmonary diseases (Cho et al., 2006; Chu et al., 2013; Reynolds et al., 2006). However, the role of Egr-1 in host defense against *P. aeruginosa* lung infection has not been elucidated.

In this study, we used a mouse model of bacterial pneumonia to examine the biological implications of Egr-1 during *P. aeruginosa* infection. We found that Egr-1 expression was rapidly and transiently induced by *P. aeruginosa* in both mouse lung tissues and macrophages. Furthermore, Egr-1 deficiency resulted in less mortality and enhanced bacterial clearance without affecting neutrophil recruitment but was associated with elevated nitric oxide level during *P. aeruginosa* lung infection. The levels of proinflammatory cytokines, IL-1β, IL-6 and TNF, were significantly decreased in infected Egr-1-deficient mice. Interestingly, Egr-1 deficiency had differential impact on chemokine production *in vivo*, which resulted in impaired production of MIP-2 (CXCL2) and KC (CXCL1) but upregulated production of LIX (CXCL5). *In vitro* studies revealed that Egr-1-deficient neutrophils and macrophages had enhanced intracellular bacterial killing ability, which correlated with upregulated nitric oxide production. Further study revealed a physical interaction between Egr-1 and NF-κB p65 in *P. aeruginosa*-infected macrophages. These findings suggest that Egr-1 deficiency protects host against *P. aeruginosa* by

reducing the risk of systemic inflammation and upregulating nitric oxide production for bacterial clearance.

4.2 Results

4.2.1 Egr-1 deficiency decreases mortality and enhances bacterial clearance but has no effect on neutrophil recruitment during *P. aeruginosa* lung infection

Aberrant Egr-1 expression has been implicated in pulmonary inflammatory diseases (Cho et al., 2006; Chu et al., 2013; Reynolds et al., 2006). We first identified an increase of Egr-1 mRNA level in lung at 4 h following *P. aeruginosa* infection, which suggests that Egr-1 may be involved in regulation of *P. aeruginosa*-induced inflammatory responses *in vivo* (Fig 4.1A). Moreover, the levels of Egr-1 mRNA and protein were highly upregulated in macrophages in response to *P. aeruginosa* infection *in vitro* (Fig 4.1B-D). To determine the biological implications of Egr-1 induction during *P. aeruginosa* lung infection, we assessed mortality and bacterial clearance using a mouse model of acute bacterial pneumonia. Wild-type and Egr-1-deficient mice were intranasally infected with *P. aeruginosa* 8821 and monitored for 10 days post-infection. No mortality was observed in Egr-1-deficient mice whereas 30% mortality of wild-type mice was observed by 2 days post-infection (Fig 4.2A). However, the difference did not reach statistical significance using the log-rank test. Furthermore, Egr-1-deficient mice displayed significantly decreased disease scores from day 2 to day 4 post-infection compared to wild-type mice (Fig 4.2B). No mortality was observed after day 2 of post-infection due to recovery from infection in mice, which was associated with reduced disease scores in subsequent post-infection days. These findings suggest that Egr-1 has deleterious effects to host during *P. aeruginosa* lung infection. To further characterize the effect of Egr-1 on bacterial clearance

during *P. aeruginosa* lung infection, we assessed the bacterial burden in the lungs and BALF of wild-type and Egr-1-deficient mice at 24 h post-infection by CFU counting. Egr-1-deficient mice had less bacterial burden in lungs (Fig 4.2C) and BALF (Fig 4.2D) compared to wild-type mice, suggesting that Egr-1 deficiency promotes bacterial clearance of *P. aeruginosa*. Neutrophils and macrophages are major phagocytic cells that are important for combating *P. aeruginosa* infection in the lung (Lovewell et al., 2014). To examine whether Egr-1 influences pulmonary infiltration of neutrophils and macrophages, we extracted the cells from *P. aeruginosa*-infected lungs and BALF, and analyzed them by flow cytometry analysis. We found that the number of neutrophils was increased whereas the number of macrophages was decreased in the lung and BALF cell populations of wild-type and Egr-1-deficient mice upon *P. aeruginosa* infection (Fig 4.2E-H). However, the numbers of neutrophils and macrophages in the lung and BALF showed no significant difference between wild-type and Egr-1-deficient mice. These findings suggest that Egr-1 does not affect the recruitment of neutrophils and macrophages in response to *P. aeruginosa* infection, and the enhanced bacterial clearance is mediated through other mechanisms.

4.2.2 Egr-1 deficiency impairs proinflammatory cytokine production but has differential effects on chemokine production *in vivo* during *P. aeruginosa* lung infection

Egr-1 has previously been shown to contribute to the production of inflammatory mediators (Pawlinski et al., 2003). To determine whether the decreased mortality of Egr-1-deficient mice was linked to the alleviated inflammation level, we examined the *in vivo* production of proinflammatory cytokines, IL-1β, IL-6 and TNF, which are highly correlated with the intensity of systemic inflammatory response (Hernandez-Rodriguez et

al., 2004). Egr-1-decifient mice showed reduced levels of IL-1β (Fig 4.3A and B), IL-6 (Fig 4.3C and D) and TNF (Fig 4.3E and F) in lungs or BALF compared to wild-type mice during *P. aeruginosa* lung infection. The IL-1β production was significantly decreased in the BALF but not in the lungs of Egr-1-deficient mice, which had a trend of decrease but did not reach statistical significance. Furthermore, the *P. aeruginosa*-induced mRNA expression of IL-1β, IL-6 and TNF in lungs strongly correlated with protein levels (Fig 4.4A-C). These finding suggest that Egr-1 contributes to inflammation in the *P. aeruginosa*-infected lung. To assess whether Egr-1 plays a role in chemokine production, we tested the *in vivo* levels of chemokines, MIP-2, KC, and LIX, which share the same chemokine receptor CXCR2 and are essential for neutrophil recruitment in response to *P. aeruginosa* lung infection (Tsai et al., 2000b). Interestingly, Egr-1-deficient mice displayed impaired production of MIP-2 (Fig 4.5A and B) and KC (Fig 4.5C and D) but upregulated production of LIX (Fig 4.5E and F) in lungs and BALF. Moreover, the mRNA expression of MIP-2, KC and LIX in lungs had similar patterns as their protein levels (Fig 4.4D-F). These results suggest a differential regulatory role of Egr-1 in chemokine production, and the upregulated LIX production may compensate the effect caused by reduced MIP-2 and KC production, which could explain the limited effect of Egr-1 deficiency on neutrophil infiltration *in vivo*.

4.2.3 Egr-1 deficiency diminishes proinflammatory cytokine production *in vitro* but differentially regulates chemokine production in macrophages and dendritic cells following *P. aeruginosa* infection

Macrophages are abundant within the alveolar space where they play important roles in host defense against *P. aeruginosa* (Kooguchi et al., 1998). Egr-1 expression was

previously shown to be induced by LPS in human monocytes through MEK-ERK-Elk-1 pathway (Guha et al., 2001). To test whether *P. aeruginosa* induces Egr-1 expression in macrophages, wild-type BMMs were infected with *P. aeruginosa* 8821 at a MOI 10 for 30 min, 1 h, 2 h, 4 h or left untreated. We found that Egr-1 mRNA and protein expression were rapidly and transiently induced upon *P. aeruginosa* infection (Figure 4.1B-D). To examine the impact of Egr-1 on production of cytokines and chemokines *in vitro*, wild-type and Egr-1-deficient BMMs were infected with *P. aeruginosa* 8821 at a MOI of 10 for 3 h, 6 h and 12 h. Egr-1-deficient BMMs showed diminished levels of IL-1β, IL-6, TNF, MIP-2, KC and LIX compared to wild-type BMMs (Fig 4.6). Similarly, the cytokine and chemokine mRNA levels were decreased in Egr-1-deficient BMMs compared to wild-type BMMs (Fig 4.7). The reduced LIX production in Egr-1-deficient BMMs, in contrast to the *in vivo* data, that showed increased LIX in Egr-1-deficient mice, suggesting that other immune cells may contribute to the increased *in vivo* LIX production (Kooguchi et al., 1998).

Dendritic cells contribute to host defenses against pulmonary *P. aeruginosa* infection (Peluso et al., 2010; Worgall et al., 2001). Similar to our findings in BMMs, we observed that Egr-1-deficient bone marrow-dendritic cells (BMDCs) produced less IL-1β, IL-6, TNF and MIP-2 production than wild-type BMDCs during *P. aeruginosa* infection (Fig 4.8A-D), which is consistent with macrophage and *in vivo* results. Interestingly, no significant difference was observed in KC production (Fig 4.8E), and LIX production was markedly increased in Egr-1-deficient BMDCs compared to wild-type BMDCs (Fig 4.8F), suggesting Egr-1 regulates chemokine production differently in different cell types, and dendritic cells may be a major source contributing to the *in vivo* LIX levels.

4.2.4 Egr-1 deficiency impairs NF-κB activity both *in vivo* and *in vitro* in response to *P. aeruginosa* infection

We previously determined that transcription factors NF-κB and NFAT are essential for mediating *P. aeruginosa*-activated inflammatory responses (Pang et al., 2017; Power et al., 2004). To determine whether Egr-1 impacts NF-κB and NFAT activities *in vivo*, nuclear extracts from the lungs of *P. aeruginosa*-infected wild-type and Egr-1-deficient mice were subjected to EMSA for NF-κB and NFAT DNA-binding activities. The *P. aeruginosa*-induced lung NF-κB and NFAT activities markedly peaked by 4 h post-infection. High NF-κB activity was sustained at 24 h in *P. aeruginosa*-infected wild-type mice, but in Egr-1-deficient mice NF-κB activity returned to near basal levels (Fig 4.9A and B), suggesting Egr-1 plays a role in stabilization of NF-κB DNA binding activity during *P. aeruginosa* infection *in vivo*. No significant difference of NFAT activity was observed between wild-type and Egr-1-deficient mice (Fig 4.9C and D). This suggests that Egr-1 had little influence on *P. aeruginosa*-induced NFAT activation *in vivo*. To further analyze NF-κB and NFAT activities *in vitro*, nuclear extracts from *P. aeruginosa*-infected or mock-infected wild-type and Egr-1-deficient BMMs were processed for EMSA analysis. The Egr-1-deficient BMMs had significantly reduced NF-κB and NFAT activities compared to wild-type BMMs during *P. aeruginosa* infection (Fig 4.10), suggesting that Egr-1 affects NF-κB and NFAT activation *in vitro*.

4.2.5 Egr-1 has no effect on IκBα phosphorylation but physically interacts with NF-κB in macrophages in response to *P. aeruginosa* infection

In unstimulated cells, NF-κB is sequestered and inactivated by IκBα in cytoplasm. Phosphorylation of IκBα leads to dissociation of NF-κB from IκBα, and the liberated NF-

κB translocates to the nucleus where it transactivates genes that regulate immunity, inflammation and cell fate (Kawai and Akira, 2007). No significant difference of IκBα phosphorylation level and total IκBα was observed between wild-type and Egr-1-deficient BMMs during *P. aeruginosa* infection (Fig 4.11), suggesting that IκBα is unlikely to be a direct target of Egr-1. Since mitogen-activated protein kinase (MAPK) pathways regulate inflammatory responses to *P. aeruginosa* infection (Pang et al., 2018), we tested the *P. aeruginosa*-activated phosphorylation levels of p38, ERK and JNK in macrophages. However, no significant difference was found between wild-type and Egr-1-deficient BMMs (Fig 4.12). NF-κB p65 subunit plays a critical role in regulation of inflammatory responses by binding to the promoter of inflammatory genes (Oeckinghaus and Ghosh, 2009). To further assess whether Egr-1 directly affects NF-κB activation, the cell lysates from *P. aeruginosa*-infected or untreated wild-type and Egr-1-deficient BMMs were subjected to immunoprecipitation using anti-Egr-1 or anti-NF-κB p65 antibody followed by western blot analysis. NF-κB p65 subunit was detected in the 1 h *P. aeruginosa*-infected cell lysates immunoprecipitated with anti-Egr-1 antibody (Fig 4.13A). Likewise, Egr-1 was detected in the 1 h *P. aeruginosa*-infected cell lysates in the reciprocal immunoprecipitation experiment using anti-NF-κB p65 antibody (Fig 4.13B). These results suggest that Egr-1 physically interacts with the NF-κB p65 subunit without affecting IκBα phosphorylation upon *P. aeruginosa* infection.

4.2.6 Egr-1 deficiency elevates nitric oxide production both *in vivo* and *in vitro* through enhancing expression of inducible nitric oxide synthase

Nitric oxide is a free radical produced by many types of immune cells including macrophages and neutrophils, and it functions as a toxic defense molecule against

infectious organisms (Coleman, 2001). Importantly, nitric oxide mediates intracellular killing of *P. aeruginosa* in human bronchial epithelial cells (Darling and Evans, 2003). To analyze nitric oxide levels in lung tissues, we measured the concentration of nitrite, an oxidative product of nitric oxide, in the lysates and supernatants of lungs and BALF from wild-type and Egr-1-deficient mice. The nitrite levels in lungs and BALF were significantly increased in Egr-1-deficient mice (Fig 4.14A-D). Nitric oxide production is mediated by inducible nitric oxide synthase (iNOS) in response to inflammatory stimuli (Tripathi et al., 2007). Egr-1-deficient mice had upregulated iNOS mRNA expression in lung at 4 h post-*P. aeruginosa* infection compared to wild-type mice (Fig 4.14E), suggesting that upregulated iNOS expression in Egr-1-deficient mice is responsible for the increased nitric oxide production. To further assess the role of Egr-1 in nitric oxide production and iNOS expression *in vitro*, wild-type and Egr-1-deficient BMMs or neutrophils were infected with *P. aeruginosa* 8821 at an MOI of 10 for various time points. Egr-1-deficient BMMs showed significantly upregulated iNOS protein expression at 6 h compared to wild-type BMMs during *P. aeruginosa* infection (Fig 4.15). Moreover, the *P. aeruginosa*-induced nitric oxide production was enhanced in both Egr-1-deficient neutrophils (Fig 16A and B) and BMMs (Fig 4.16E and F) compared to wild-type neutrophils and BMMs, which is consistent with the *in vivo* results. These findings suggest that Egr-1 deficiency increases nitric oxide production through upregulation of iNOS expression.

4.2.7 Egr-1 deficiency leads to enhanced intracellular killing ability in neutrophils and macrophages

   To determine whether Egr-1 deficiency affects phagocytic activity and intracellular killing ability *in vitro*, wild-type and Egr-1-deficient neutrophils or BMMs were infected

with *P. aeruginosa* 8821 for 1 h or 3 h. Egr-1-deficient neutrophils had enhanced phagocytic activity and intracellular killing ability compared to wild-type neutrophils (Fig 4.16C and D). Furthermore, no significant difference of phagocytic activity was observed between wild-type and Egr-1-deficient BMMs but the intracellular killing ability of Egr-1-deficient BMMs was significantly increased compared to wild-type BMMs (Fig 4.16G and H). These findings demonstrate that Egr-1 has a negative impact on intracellular killing ability in neutrophils and macrophages.

4.3 Discussion

Host inflammatory responses to *P. aeruginosa* infection are regulated by multiple signaling pathways, including MyD88-NF-κB, NFAT and MAPK pathways (Pang et al., 2017; Power et al., 2004; Zhang et al., 2007). These inflammatory responses aid bacterial clearance, but excessive inflammation leads to persistent *P. aeruginosa* infection and lung damage (Lin and Kazmierczak, 2017). The molecular mechanisms involved in regulation of inflammation during *P. aeruginosa* infection are not fully understood. The Egr-1 transcription factor governs many cellular processes and inflammation (Dinkel et al., 1998; Min et al., 2008; Pawlinski et al., 2003; Pignatelli et al., 2003; Thiel and Cibelli, 2002). Aberrant Egr-1 expression has been implicated in pulmonary inflammation (Wu et al., 2009). In this study, we identified upregulated Egr-1 expression in response to *P. aeruginosa* infection, and characterized a detrimental role of Egr-1 in host defense against this bacteria by promoting systemic inflammation and negatively regulating nitric oxide production (Fig 4.17).

Production of proinflammatory cytokines, IL-1β, IL-6 and TNF, highly correlates with the intensity of systemic inflammatory response (Hernandez-Rodriguez et al., 2004).

Although robust inflammatory responses aid bacterial clearance, persistent *P. aeruginosa* infection-caused systemic inflammatory response and organ failure can be life-threatening (Kurahashi et al., 1999). Previous studies have shown that Egr-1 contributed to production of inflammatory mediators in response to stimulation by bacterial cell wall components, such as LPS and peptidoglycan (Pawlinski et al., 2003; Xu et al., 2001). Our data show that Egr-1-deficient mice had reduced but not abolished levels of IL-1β, IL-6 and TNF compared to wild-type mice during *P. aeruginosa* lung infection, which was associated with decreased mortality. The production of IL-1β, IL-6 and TNF is primarily mediated through MyD88-NF-κB pathway during *P. aeruginosa* infection (Power et al., 2004). Activation of MyD88-NF-κB pathway leads to IκBα phosphorylation and NF-κB activation (Kawai and Akira, 2007). Egr-1 deficiency had no effect on IκBα phosphorylation but significantly impairs NF-κB activation following *P. aeruginosa* infection. This finding suggests that Egr-1 directly affects NF-κB activity but not its upstream signaling. Previous studies have shown that Egr-1 interacted with other transcription factors and cooperatively regulated transcription of proinflammatory cytokine genes (Decker et al., 2003; Ma et al., 2009). The interaction between Egr-1 and the other transcription factors results in formation of stable heterodimeric protein complexes, which promotes DNA binding and transcription activities. Herein, we provided strong evidence showing that Egr-1 physically interacted with NF-κB p65 upon *P. aeruginosa* infection, leading to a prolonged NF-κB activation. Additionally, we previously identified that NFAT mediated inflammatory gene expression through crosstalk with NF-κB during *P. aeruginosa* infection (Pang et al., 2017). However, our data showed that Egr-1 deficiency had a limited impact on NFAT activation *in vivo*.

A strong inflammatory response usually associates with enhanced bacterial clearance. However, our findings demonstrate that Egr-1 deficiency caused enhanced bacterial clearance without affecting neutrophil and macrophage infiltration, suggesting the bacterial clearance may be mediated through other mechanisms. Nitric oxide is a potent antimicrobial agent that is produced from L-arginine by NOS enzymes: iNOS, neuronal NOS (nNOS), and endothelial NOS (eNOS) (Schairer et al., 2012). The isoforms nNOS and eNOS are constitutively expressed and produce low level of nitric oxide whereas iNOS is induced in many types of cells by inflammatory stimuli and produces large amount of nitric oxide (Forstermann and Sessa, 2012). Importantly, nitric oxide synthesized from iNOS is important for combating *P. aeruginosa* infection (Darling and Evans, 2003). Our results revealed that Egr-1 deficiency led to upregulated iNOS expression and nitric oxide production, associated with enhanced intracellular killing ability in neutrophils and macrophages. These results could explain the enhanced bacterial clearance in Egr-1-deficienct mice even though there was no difference in the numbers of recruited neutrophils or macrophages. Egr-1 is able to positively or negatively regulate target gene expression (Gashler et al., 1993). Consistent with our findings, a previous study demonstrated that P2X7-dependent Egr activation decreased iNOS expression and nitric oxide production whereas it increased TNF-α production, and the Egr DNA binding sites were identified in the iNOS promoter (Friedle et al., 2011). We also identified an elevated phagocytic activity in Egr-1-deficienct neutrophils, which may also contribute to bacterial clearance. A previous study reported that suppression of Egr-1 expression and nuclear translocation by β-defensins 2 and 3 enhanced expression of phagocytic receptors and promotes phagocytosis of *P. aeruginosa* (Wu et al., 2018b). Moreover, Egr-1 was previously

reported to promote autophagy, which is able to suppress phagocytic activity (Chen et al., 2008b; Zhu et al., 2018). Hubbard, *et al.* found that the alveolar macrophages from bone-marrow transplanted mice displayed diminished (~30% of normal) phagocytic activity in response to *P. aeruginosa* infection, which correlated with ~3.5 times higher bacterial burden in the lungs compared to wild-type mice (Hubbard et al., 2011). Similarly, our data showed that Egr-1-deficient neutrophils had increased phagocytic activity (~30% more) (Fig. 4.16C) and Egr-1-deficient mice displayed a ~4.5-fold decrease of pulmonary bacterial burden compared to wild-type mice (Fig. 4.2C).

MIP-2, KC and LIX chemokines aid neutrophil recruitment during *P. aeruginosa* lung infection (Carrigan et al., 2010; Tsai et al., 2000b). Indeed, our data showed that Egr-1 influenced production of these chemokines by macrophages and dendritic cells. Notably, the Egr-1-deficient macrophages had reduced LIX production whereas the Egr-1-deficient dendritic cells had increased LIX production. However, by comparing the peak value (12 h) of LIX concentrations between these two types of cells, 1.5×10^6 Egr-1-deficient dendritic cells produced approximately 5-fold higher LIX concentration than that was produced by the same number of Egr-1-deficient macrophages, suggesting that dendritic cells may be the major source of LIX *in vivo*, and the increased LIX level may compensate the effects caused by reduced MIP-2 and KC production. Thus, Egr-1 deficiency had no effect on neutrophil recruitment, which is consistent to a previous study showing that Egr-1 deficiency had no impact on leukocyte recruitment to lung in a mouse endotoxemia model (Pawlinski et al., 2003). The differential chemokine production pattern between macrophages and dendritic cells can be explained by the fact that macrophages and dendritic cells respond differently to the same stimuli due to their differentially expressed

receptors and distinct signaling transduction (Sallusto et al., 1999; Schroder et al., 2007; Werling et al., 2004; Xu et al., 2015). A previous study by Werling et al. demonstrated that macrophages and dendritic cells had differential cytokine production after exposure to the same TLR ligands or bacterial stimuli (Werling et al., 2004). Furthermore, Sallusto et al. showed that macrophages did not produce chemokine TARC (CCL17) and ELC (CCL19), but these two chemokines were produced by dendritic cells in response to LPS or bacterial stimulation (Sallusto et al., 1999).

Altogether, our findings demonstrate a novel regulatory mechanism of Egr-1 during *P. aeruginosa* infection, which promotes inflammatory responses by physically interacting with NF-κB and negatively regulating nitric oxide production by suppressing iNOS expression. Our studies suggest that molecules that inhibit Egr-1 may increase clearance of *P. aeruginosa* and lower the risk of pathogenic systemic inflammation. Several Egr-1 inhibitors have already been described, including the anti-tumor drug mithramycin A, which inhibits radiation-induced apoptosis by preventing Egr-1 binding to target promoters (Zhao et al., 2015), and metformin, an anti-diabetic drug, which suppresses Egr-1 expression in mesangial cells through inhibition of miR-34a (Wu et al., 2018a). Egr-1 has been found to be highly expressed in diabetic human and mammal models (Ao et al., 2019; Hu et al., 2018; Trinh et al., 2016). Moreover, the diabetic patients were susceptible to respiratory tract infections with *P. aeruginosa* due to increased airway glucose (Gill et al., 2016), and have increased risk of septic shock caused by bacterial infections (Koh et al., 2012). Therefore, we speculate that metformin could be an attractive candidate to treat *P. aeruginosa* lung infection, and this drug has ease of administration and excellent safety profile. However, the use of Egr-1 inhibitors in clinical practice remains a work-in-progress.
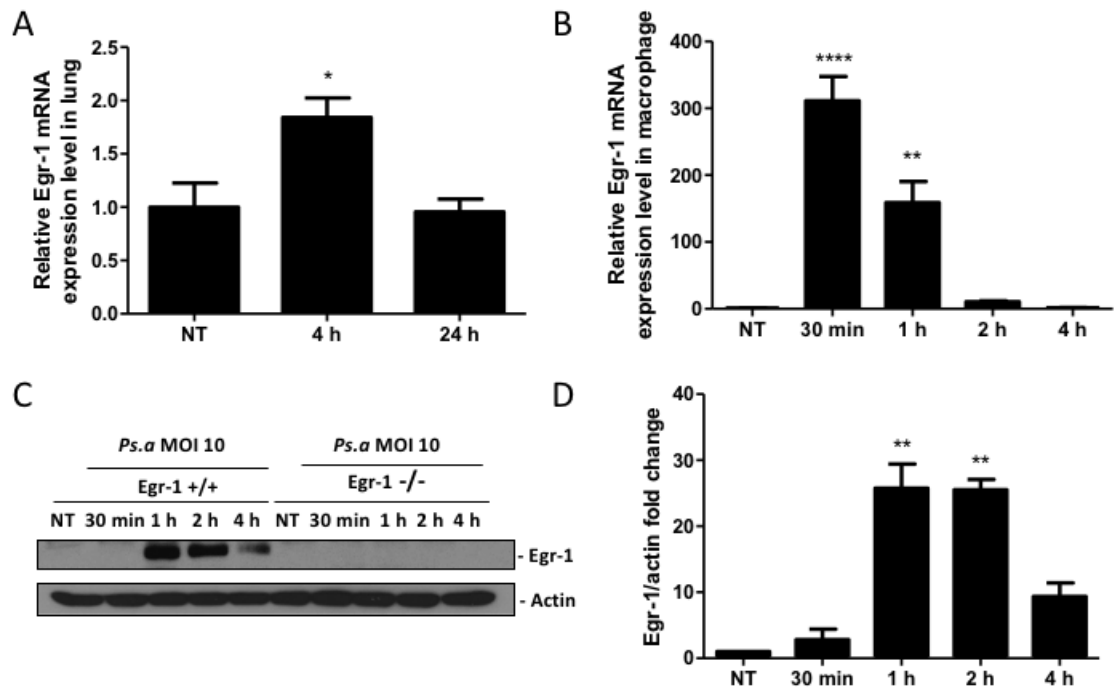
Fig 4.1. Egr-1 expression is induced in response to *P. aeruginosa* infection both *in vivo* and *in vitro*. Wild-type (+/+) and Egr-1-deficient (-/-) mice were intranasally infected with 1×10^9 CFU/mouse of *P. aeruginosa* 8821 for 4 h, 24 h or an equivalent volume of saline as a control (NT). The total RNA extracted from lungs was reverse transcribed to cDNA and subjected to real-time quantitative PCR for Egr-1 gene expression. The gene expression was normalized to housekeeping control gene HPRT (A) (n = 3 ± SEM, *p < 0.05). BMMs were infected with *P. aeruginosa* strain 8821 at a MOI of 10 for 30 min, 1 h, 2 h, 4 h or left untreated (NT). Total RNA isolated from these cells was reverse transcribed to cDNA and subjected to real-time quantitative PCR for Egr-1 gene expression. The Egr-1 mRNA levels were normalized to endogenous control HPRT (B) (n=3 ± SEM, **p < 0.01, ****p < 0.0001). Cell lysates were subjected to Western blot analysis for Egr-1 protein expression and actin as a loading control. Blots are representative of three independent experiments (C). Densitometry analysis of Egr-1 protein levels was normalized to actin, and data are presented as fold change (D) (n=3 ± SEM, **p < 0.01).
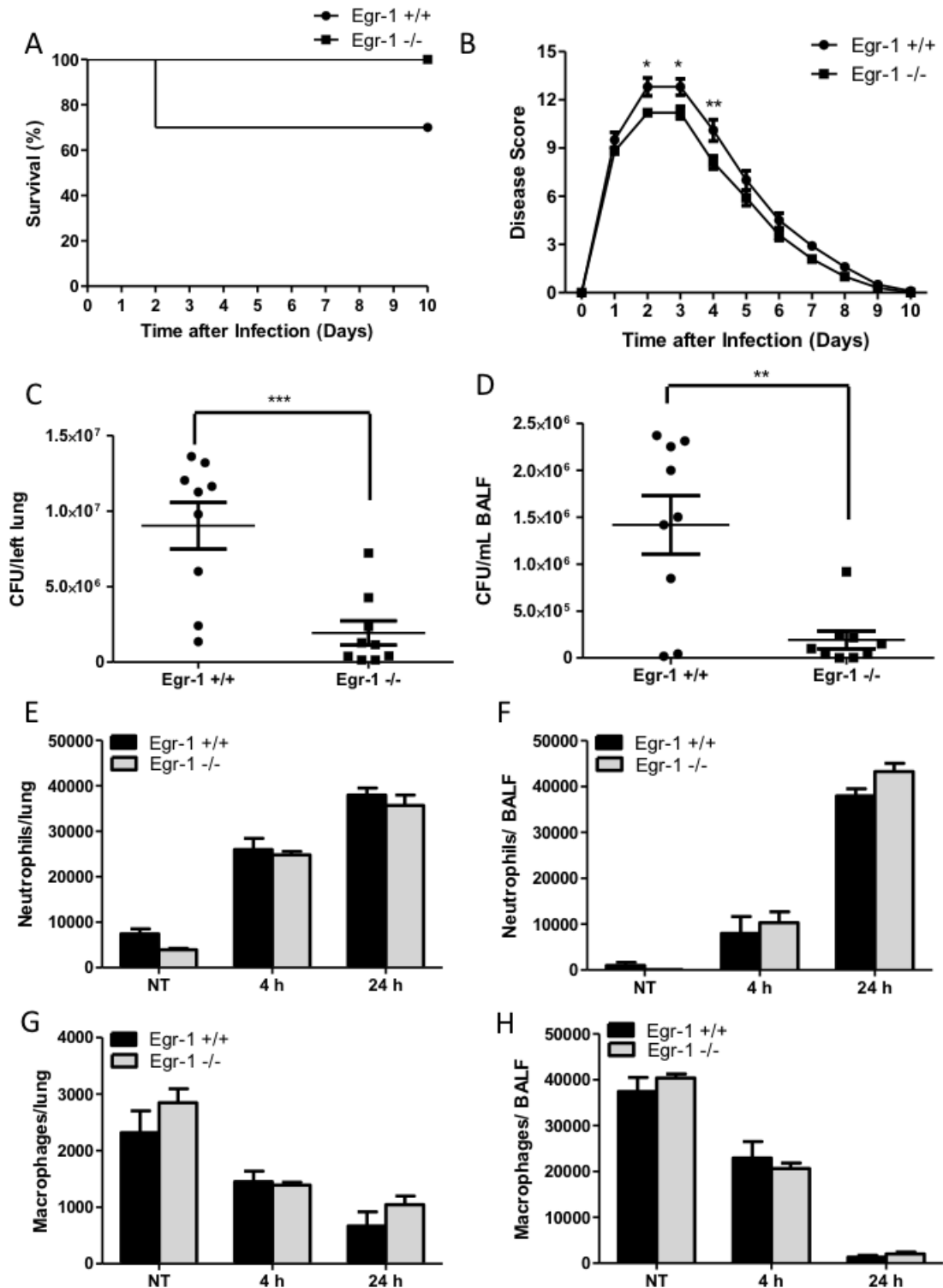
Fig 4.2. Egr-1 deficiency decreases mortality, enhances bacterial clearance but has no effect on neutrophil recruitment during *P. aeruginosa* infection *in vivo*. Wild-type (+/+) and Egr-1-deficient (-/-) mice were intranasally infected with 1×10^9 CFU/mouse of *P. aeruginosa* 8821 or an equivalent volume of saline as a control (NT). For survival study,

mice were monitored daily up to 10 days (A), and the disease scores were calculated daily (B) (n = 10 ± SEM, $*p < 0.05$, $**p < 0.01$). To determine bacterial clearance, the bacterial burden in the lung (C) and BALF (D) was assessed at 24 h (n = 9, $**p < 0.01$, $***p < 0.001$). For immune cell recruitment studies, lung tissues and BALF were collected at 4 h or 24 h post-infection. The number of neutrophils and macrophages infiltrated to the lung (E, G) and BALF (F, H) were determined by flow cytometry analysis at 4 h and 24 h. A total 5×10^4 cells from one lung or BALF sample was analyzed on a flow cytometer, and data were presented as cell numbers (n = 5).
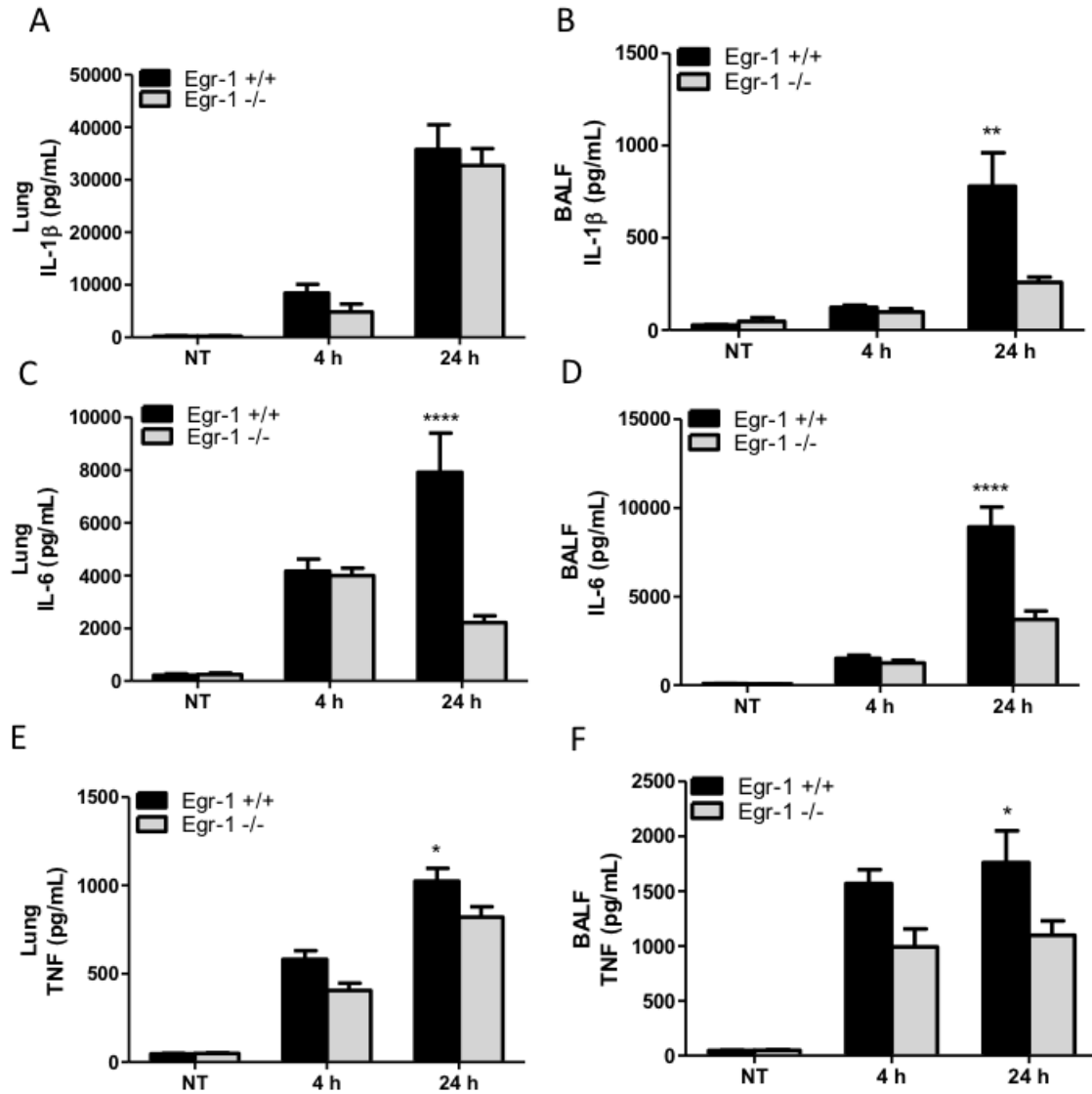
Fig 4.3. Egr-1-deficient mice displayed impaired proinflammatory cytokine production following *P. aeruginosa* lung infection. Wild-type (+/+) and Egr-1-deficient (-/-) mice were intranasally infected with 1×10^9 CFU/mouse of *P. aeruginosa* 8821 for 4 h, 24 h or an equivalent volume of saline as a control (NT). Mice are sacrificed after infection time points. Lung tissues and BALF were collected for detection of IL-1β (A, B), IL-6 (C, D) and TNF (E, F) (n = 7-9 ± SEM, *p < 0.05, **p < 0.01, ****p < 0.0001).
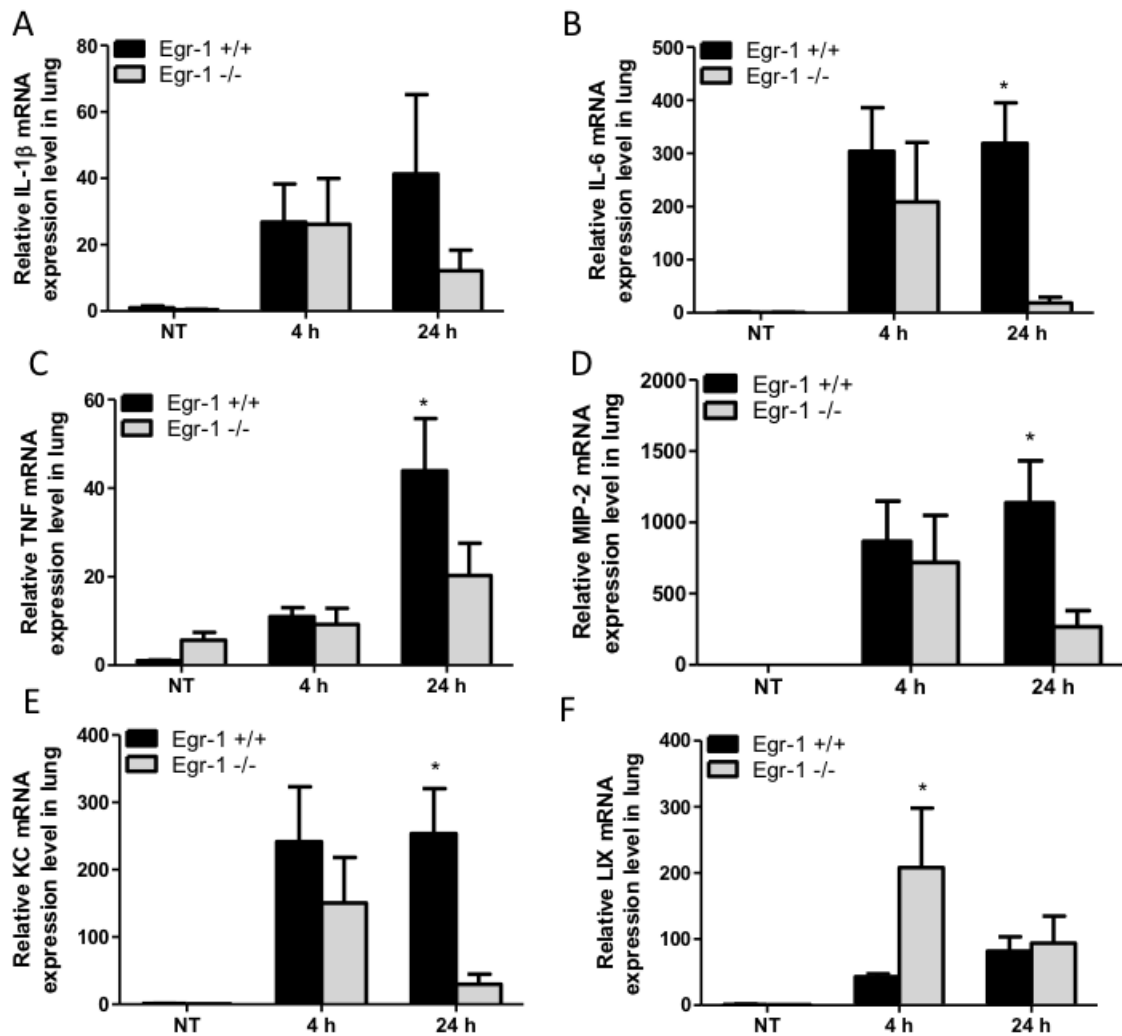
Fig 4.4. Egr-1 deficiency impairs proinflammatory cytokine mRNA transcription but has differential effects on chemokine mRNA transcription during *P. aeruginosa* lung infection. Wild-type (+/+) and Egr-1-deficient (-/-) mice were intranasally infected with 1 X 10^9 CFU/mouse of *P. aeruginosa* 8821 for 4 h, 24 h or an equivalent volume of saline as a control (NT). The total RNA extracted from lungs was reverse transcribed to cDNA and subjected to real-time quantitative PCR for *IL-1β* (A), *IL-6* (B), *TNF* (C), *MIP-2* (D), *KC* (E) and *LIX* (F) gene expression. The gene expression was normalized to housekeeping control gene HPRT (n = 3 ± SEM, *p < 0.05).
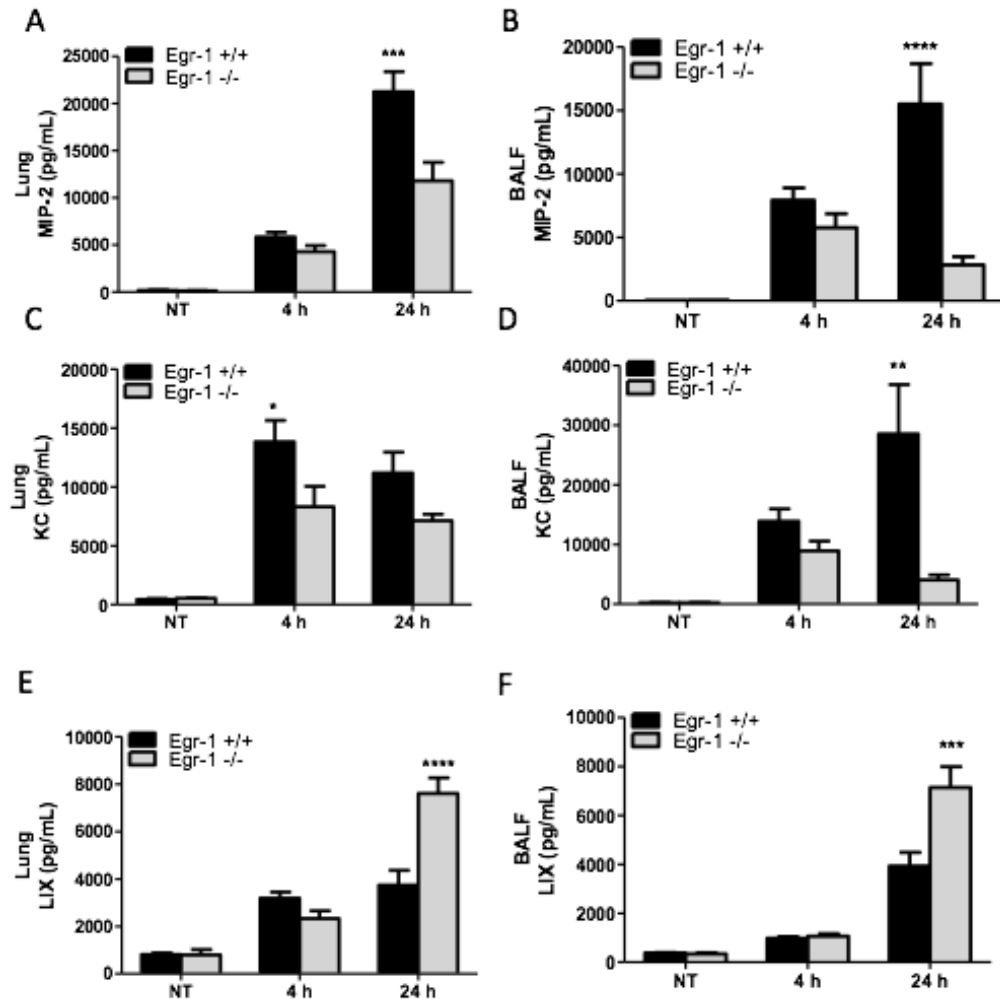
Fig 4.5: Egr-1 differentially regulates chemokine production following *P. aeruginosa* infection *in vivo*. Wild-type (+/+) and Egr-1-deficient (-/-) mice were intranasally infected with 1 X 10^9 CFU/mouse of *P. aeruginosa* 8821 for 4 h, 24 h or an equivalent volume of saline as a control (NT). Mice are sacrificed after infection time points. Lung tissues and BALF were collected for detection of MIP2 (A, B), KC (C, D) and LIX (E, F) (n= 7-9 ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001 ****p < 0.0001).
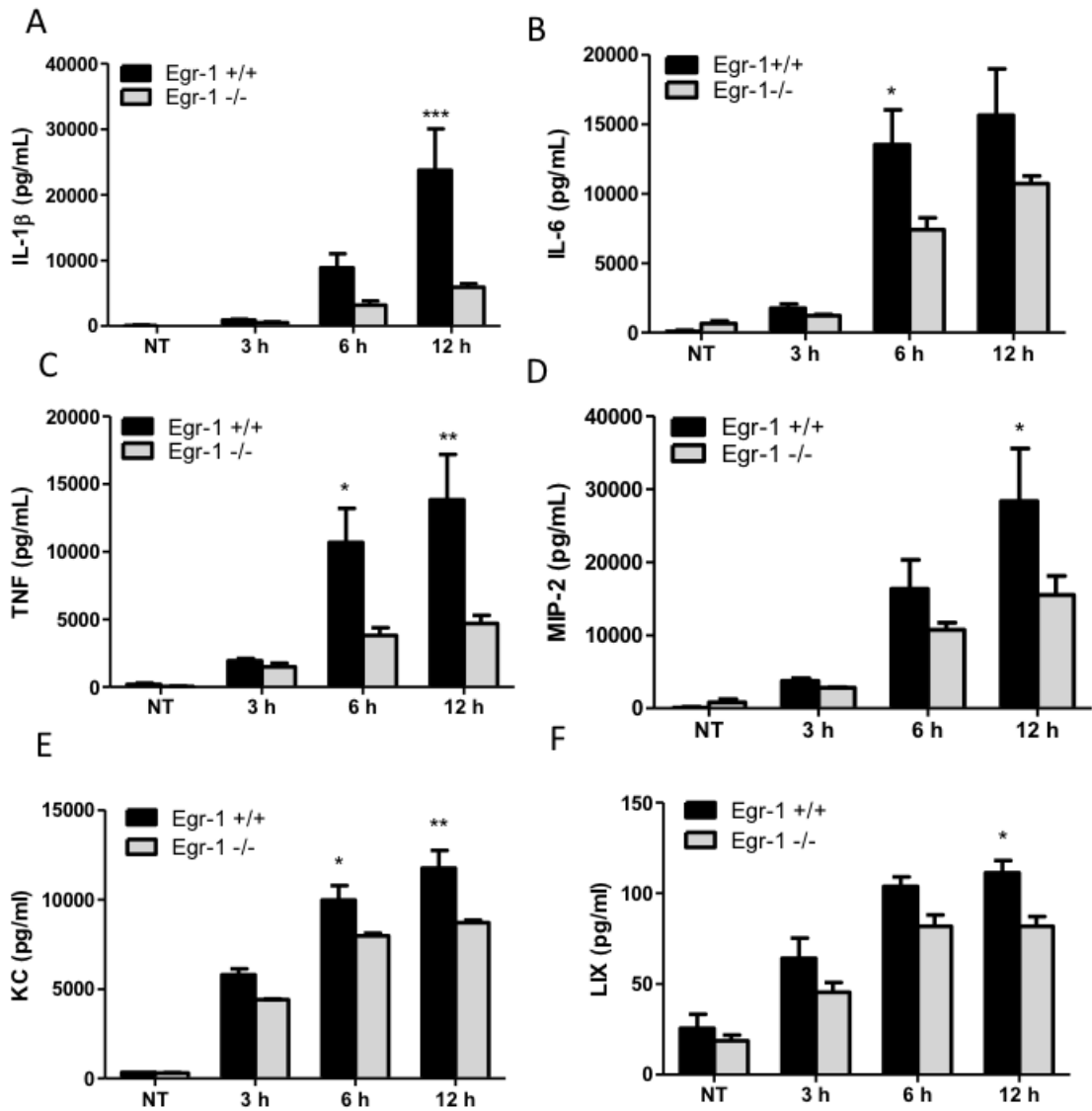
Fig 4.6. Egr-1-deficient BMMs display impaired proinflammatory cytokine and chemokine protein production following *P. aeruginosa* infection. Wild-type (+/+) and Egr-1-deficient (-/-) BMMs were infected with *P. aeruginosa* 8821 at a MOI of 10 for 3 h, 6 h, 12 h or left untreated (NT). Cell supernatants were collected for the determination of IL-1β (A), IL-6 (B), TNF (C), MIP-2 (D), KC (E) and LIX (F) secretion by ELISA. (n = 3 ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001).
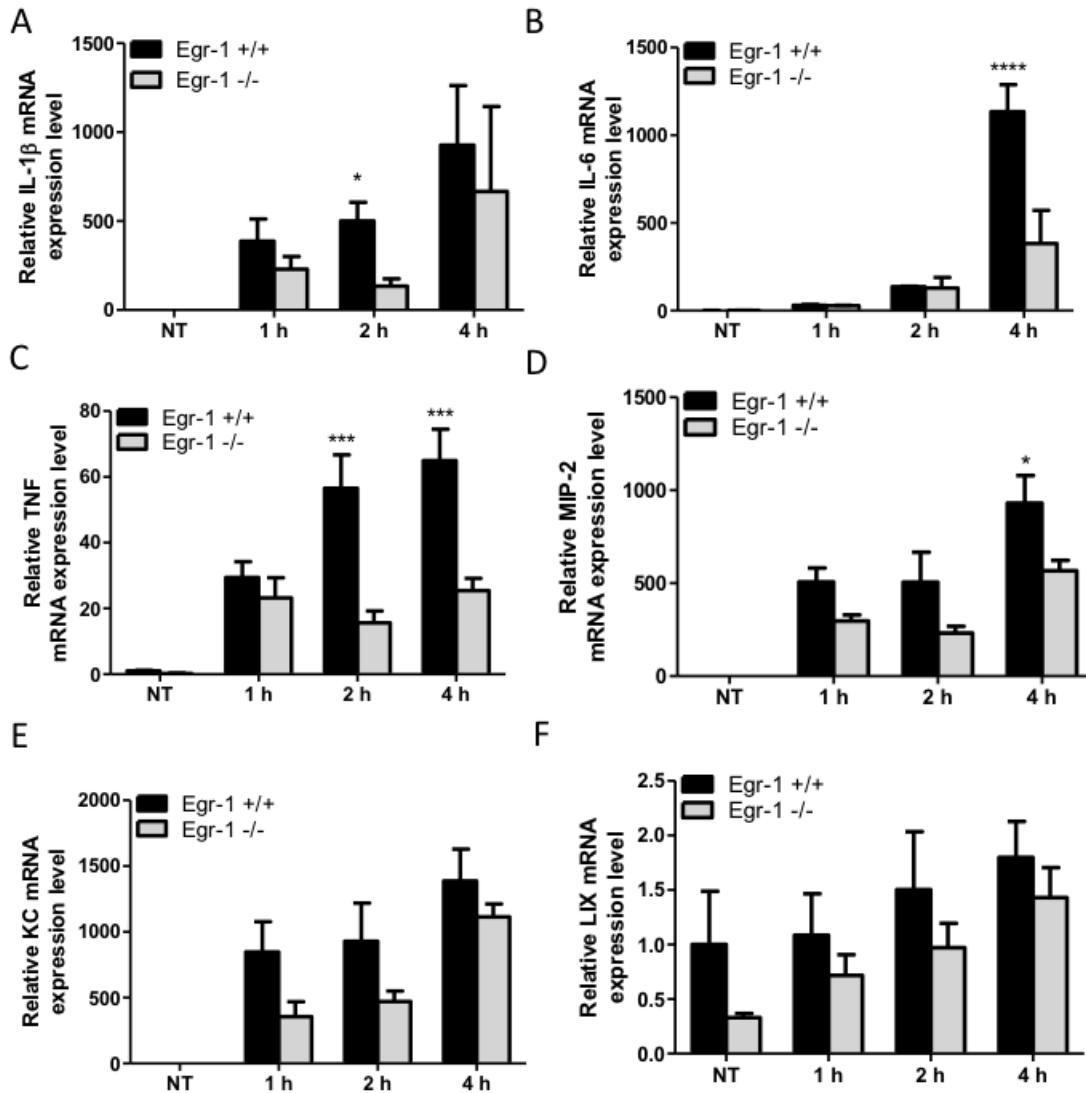
Fig 4.7. Egr-1-deficient BMMs display impaired proinflammatory cytokine and chemokine mRNA transcription following *P. aeruginosa* infection. Wild-type (+/+) and Egr-1-deficent (-/-) BMMs were infected with *P. aeruginosa* 8821 at a MOI of 10 for 1 h, 2 h, 4 h or left untreated (NT). Total RNA isolated from these cells was reverse transcribed to cDNA and subjected to real-time quantitative PCR for *IL-1β* (A), *IL-6* (B), *TNF* (C), *MIP-2* (D), *KC* (E) and *LIX* (F) gene expression. The gene expression was normalized to housekeeping control gene HPRT (A) (n = 3 ± SEM, *p < 0.05, ***p < 0.001, ****p < 0.0001).
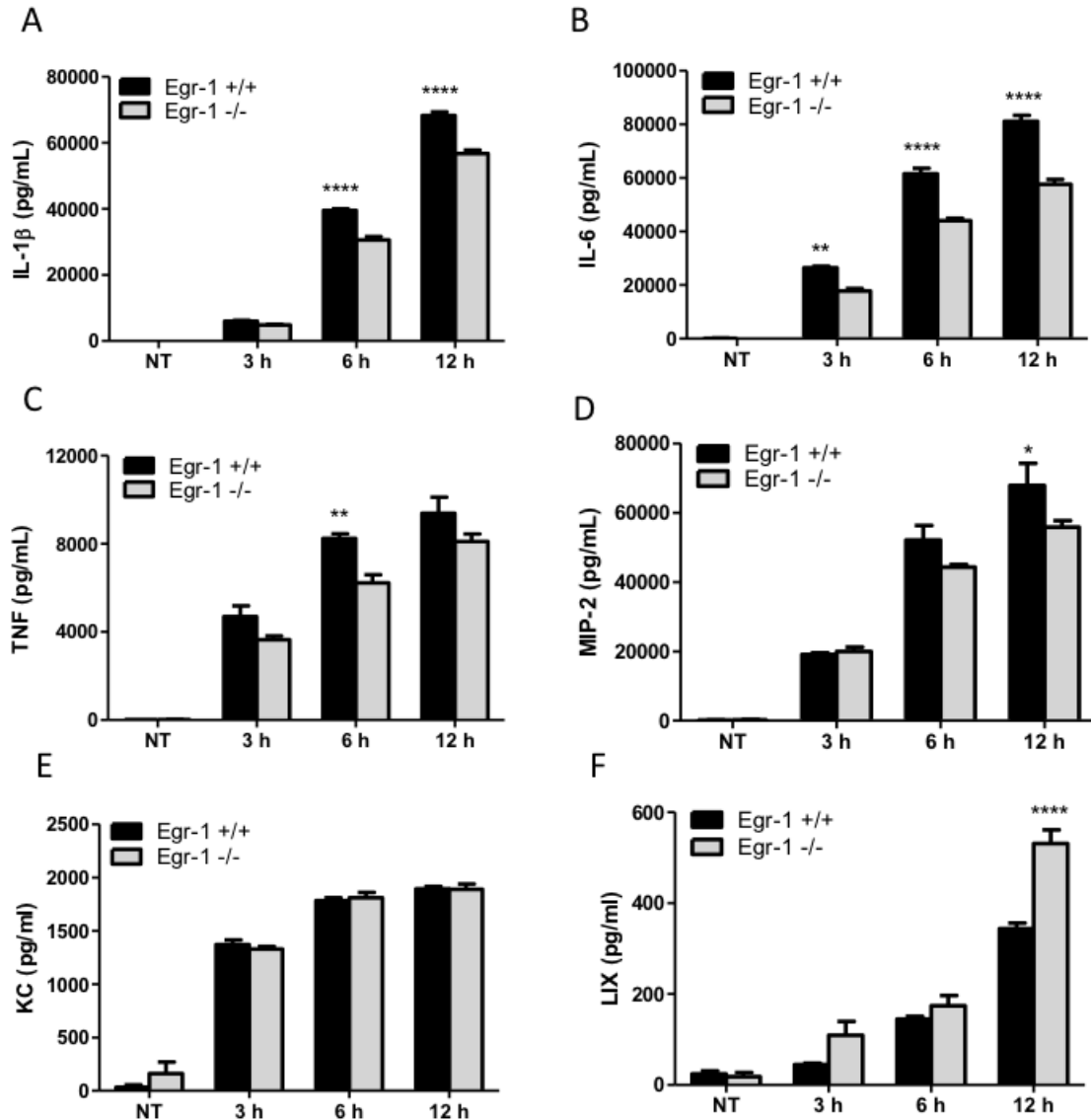
Fig 4.8. Egr-1-deficient BMDCs have increased LIX production following *P. aeruginosa* infection. Wild-type (+/+) and Egr-1-deficient (-/-) BMDCs were infected with *P. aeruginosa* strain 8821 at a MOI of 10 for 3 h, 6 h, 12 h or left untreated (NT). Cell supernatants were collected for the determination of IL-1β (A), IL-6 (B), TNF (C), MIP-2 (D), KC (E) and LIX (F) secretion by ELISA. (n = 3 ± SEM, *p < 0.05, **p < 0.01, ****p < 0.0001).
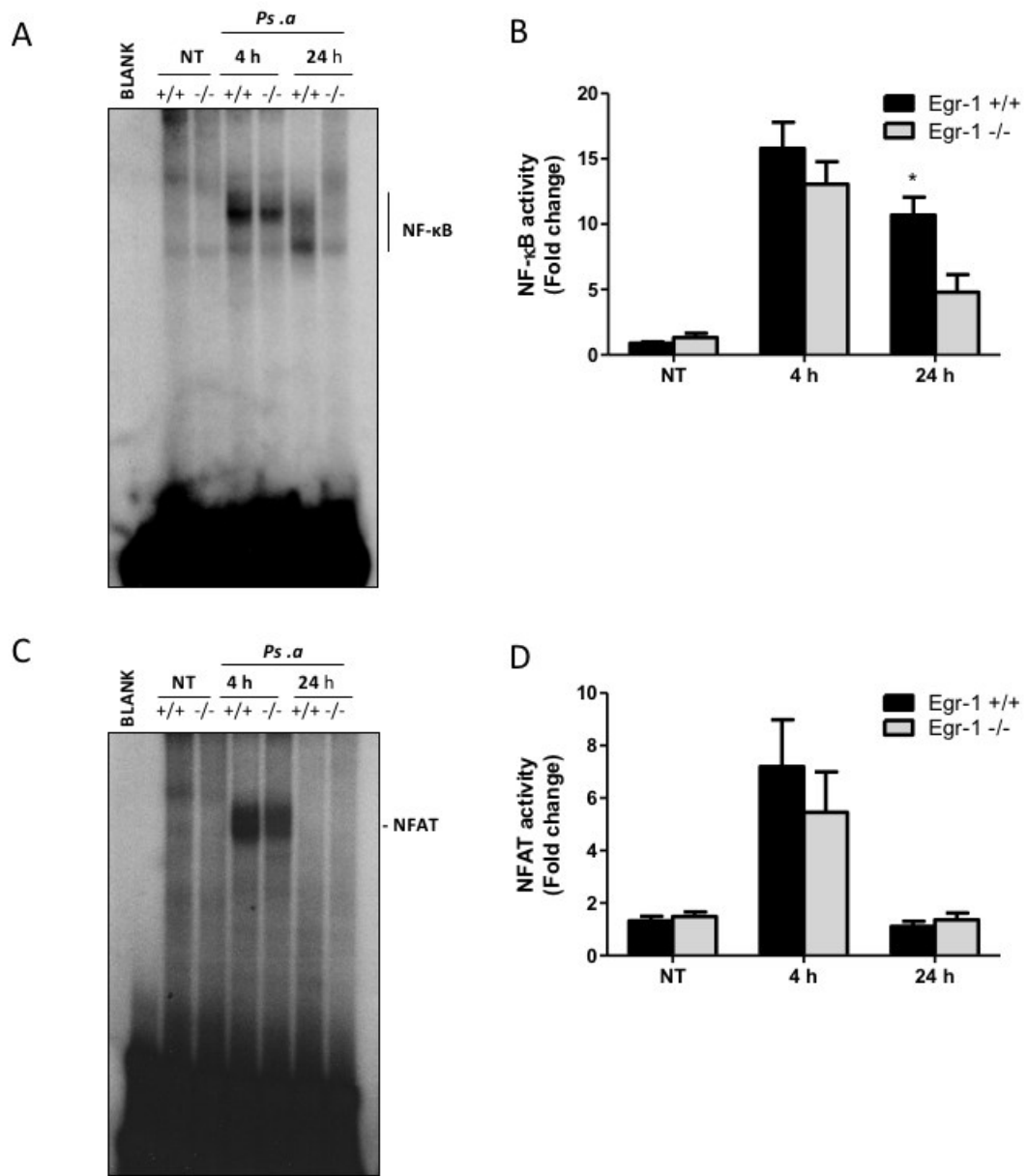
Fig 4.9. Egr-1 deficiency displays impaired NF-κB activation following *P. aeruginosa* infection *in vivo*. Wild-type (+/+) and Egr-1-deficient (-/-) mice were intranasally infected with 1×10^9 CFU/mouse of *P. aeruginosa* 8821 for 4 h, 24 h or an equivalent volume of saline as a control (NT). Mice are sacrificed after infection time points. Nuclear proteins were extracted and subjected to EMSA by incubation with ^{32}P-labeled NF-κB (A) and NFAT (C) DNA probes. Scan densitometry was performed for analysis of NF-κB (B) and NFAT (D) activation, and data are expressed as fold change versus wild-type untreated lung (n= 6 ± SEM, *p < 0.05).
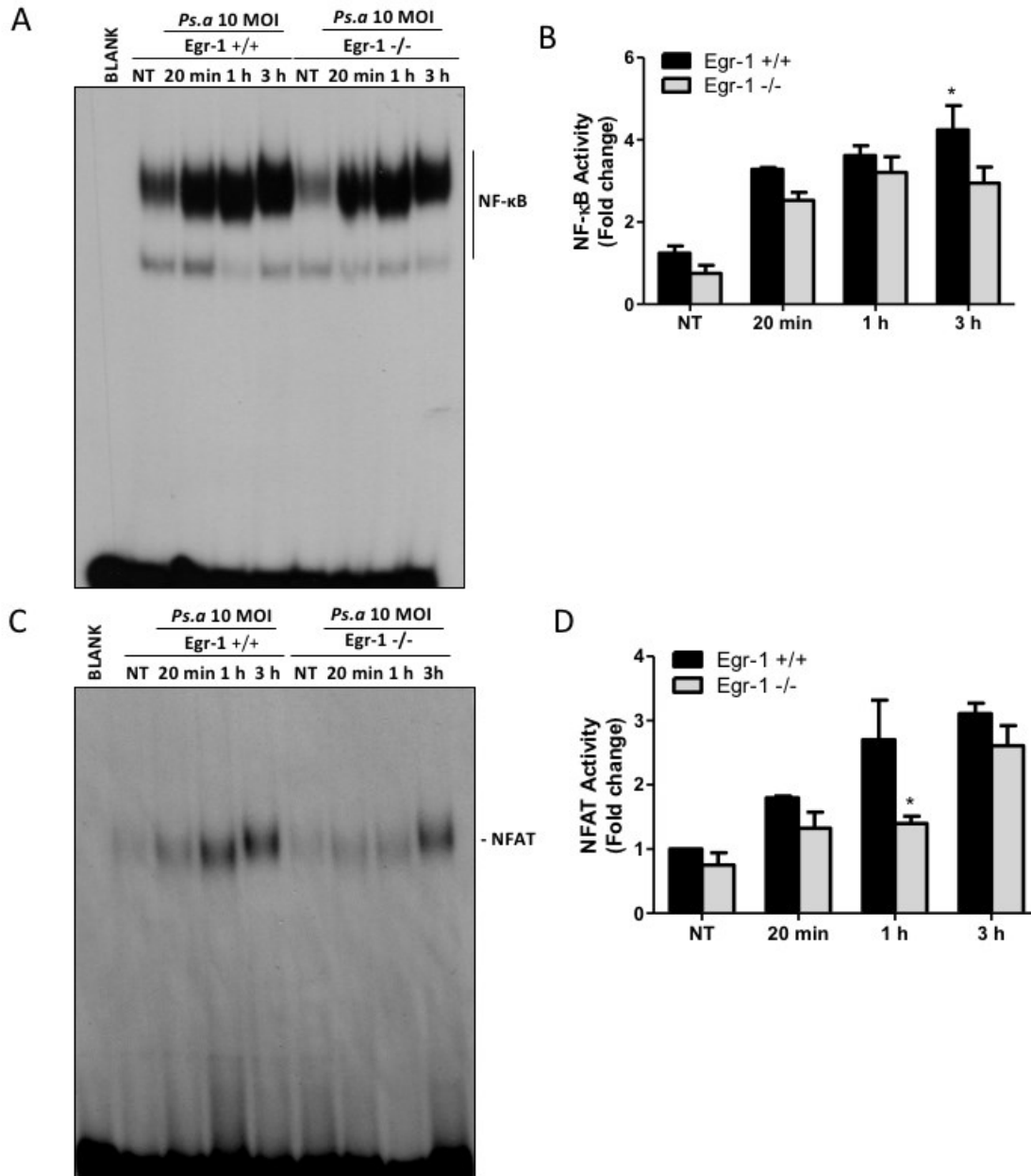
Fig 4.10. Egr-1-deficient BMMs display diminished NF-κB and NFAT activation during *P. aeruginosa* infection. Wild-type (+/+) and Egr-1-deficient (-/-) BMMs were infected with *P. aeruginosa* 8821 at a MOI of 10 for 20 min, 1 h, 3 h or left untreated (NT). Nuclear proteins were extracted and subjected to EMSA by incubation with ^{32}P-labeled NF-κB (A) and NFAT (C) DNA probes. Data are representative of three individual experiments. Densitometry analysis was performed for NF-κB (B) and NFAT (D) activities, and data are expressed as fold change (n = ± 3 SEM, *p < 0.05).
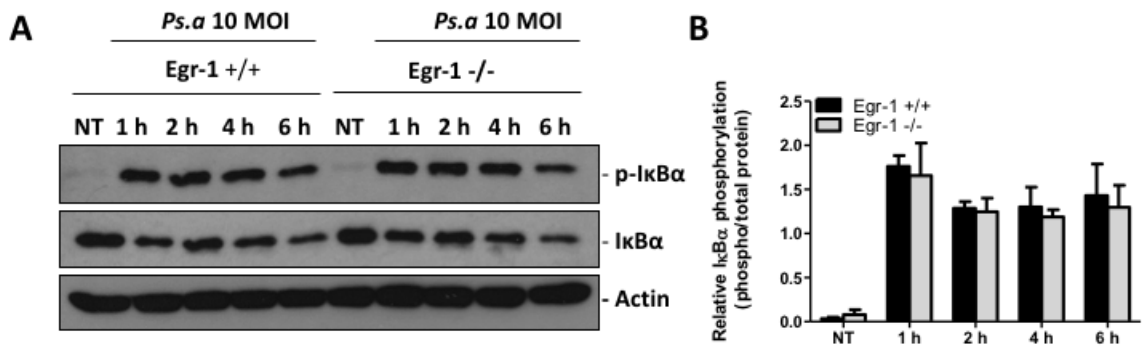
Fig 4.11. Egr-1 has no effect on IκBα phosphorylation in macrophages in response to *P. aeruginosa* **infection.** Wild-type (+/+) and Egr-1-deficient (-/-) BMMs were infected with *P. aeruginosa* 8821 at a MOI of 10 for 1 h, 2 h, 4 h, 6 h or left untreated (NT). Cell lysates were subjected to Western blot analysis for determining phosphorylated and total IκBα, as well as actin as a loading control. Blots are representative of three independent experiments (A). Densitometry analysis of phosphorylated IκBα was normalized to total IκBα (B) (n = 3 ± SEM).
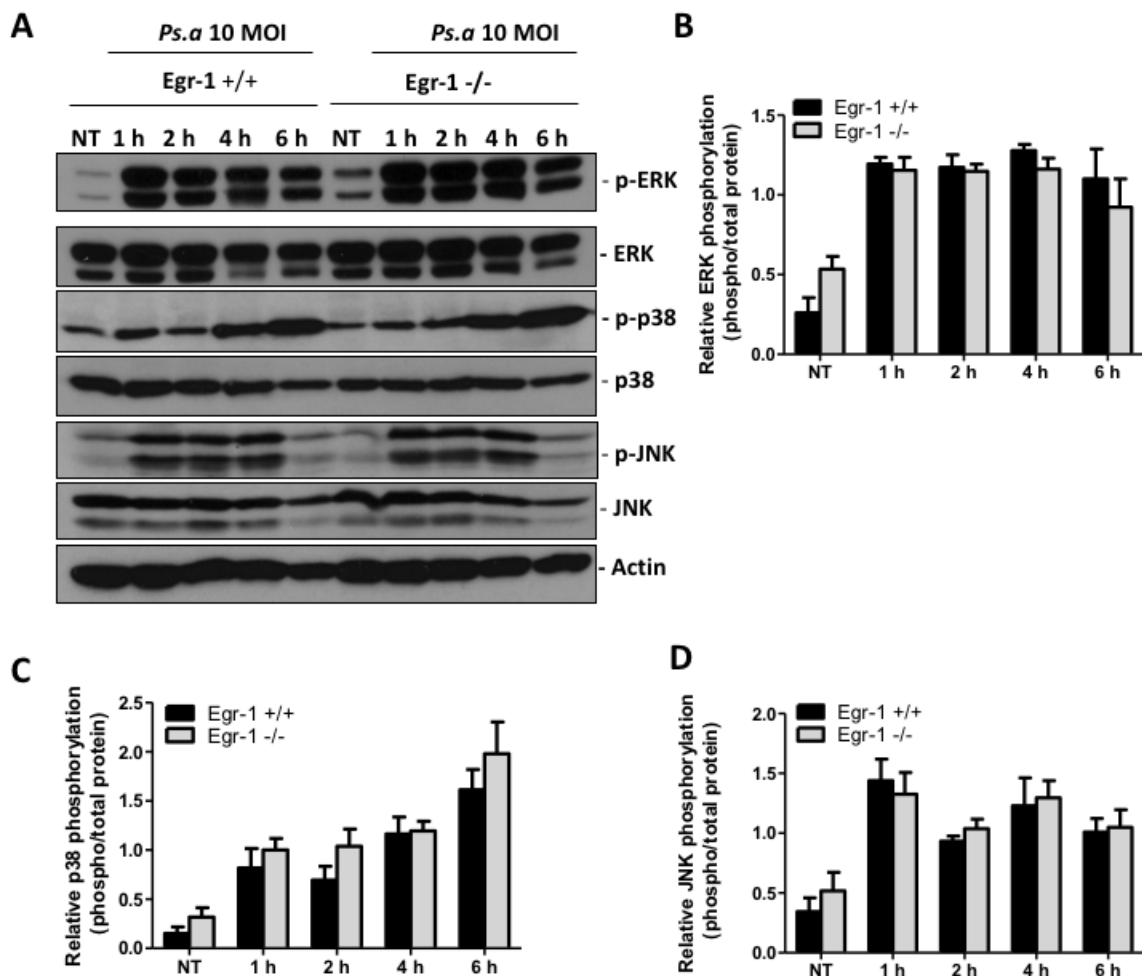
Fig 4.12. Egr-1 deficiency has no effect on MAPK activation in macrophages during *P. aeruginosa* infection. Wild-type (+/+) and Egr-1-deficient (-/-) BMMs were infected with *P. aeruginosa* 8821 at a MOI of 10 for 1 h, 2 h, 4 h, 6 h or left untreated (NT). Cell lysates were subjected to Western blot analysis for determining phospho- and total ERK, p38 and JNK, as well as actin as a loading control. Blots are representative of three independent experiments (A). Densitometry analysis of phosphorylated ERK (B), p38 (C) and JNK (D) was normalized to their total protein respectively (n = 3 ± SEM).
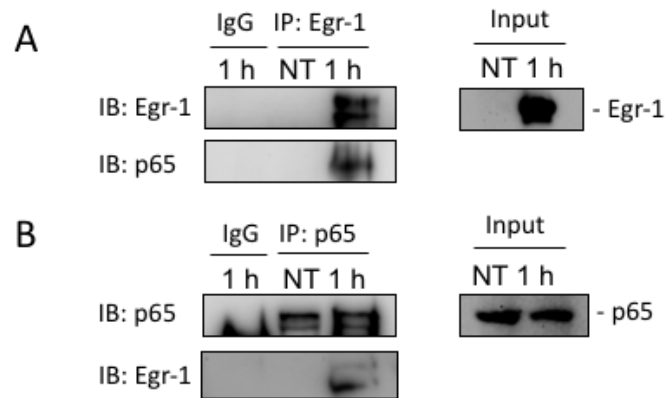
Fig 4.13. Egr-1 physically interacts with NF-κB p65 in macrophages upon *P. aeruginosa* infection. Wild-type (+/+) and Egr-1-deficient (-/-) BMMs were infected with *P. aeruginosa* 8821 at a MOI of 10 for 1 h or left untreated (NT). Cell lysates were subjected to immunoprecipitation using anti-Egr-1 (A) or anti-p65 (B) antibody followed by Western blotting for Egr-1 or p65. Mouse or rabbit IgG was used as a control. Blots are representative of three independent experiments (n = 3).
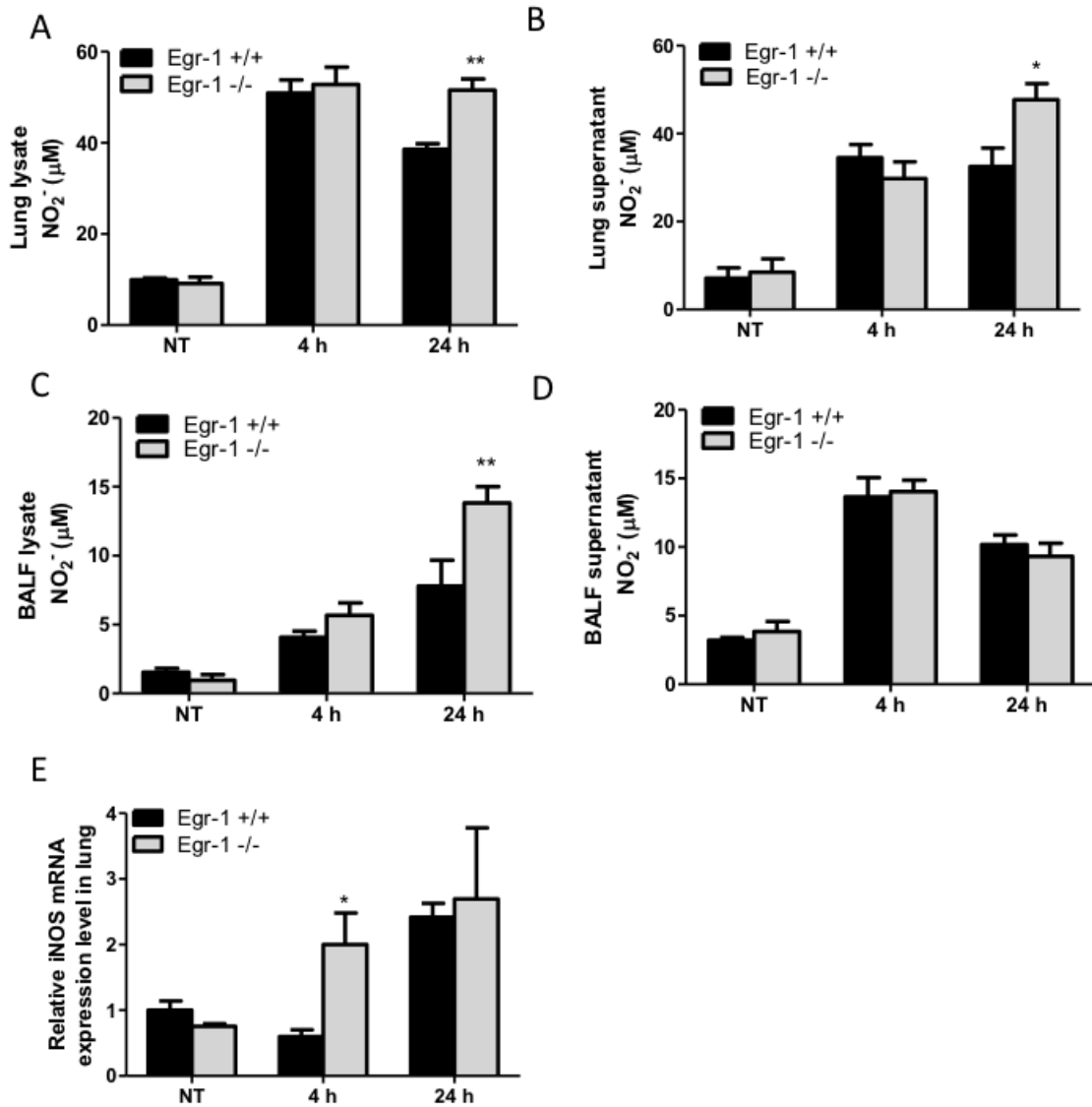
Fig 4.14. Egr-1-deficient mice show enhanced nitric oxide production and upregulated iNOS mRNA expression following *P. aeruginosa* lung infection. Wild-type (+/+) and Egr-1-deficient (-/-) mice were intranasally infected with 1×10^9 CFU/mouse of *P. aeruginosa* 8821 for 4 h, 24 h or an equivalent volume of saline as a control (NT). Mice are sacrificed after infection time points. The NO_2^- levels in lung lysates (A), lung supernatants (B), BALF lysates (C) and BALF supernatants (D) were assessed using a Griess Reagent Kit (n = 7-9 ± SEM, *p<0.05, **p<0.01). The total RNA extracted from lungs was reverse transcribed to cDNA and subjected to real-time quantitative PCR for iNOS gene expression. The gene expression was normalized to housekeeping control gene HPRT (D) (n = 3 ± SEM, *p < 0.05).
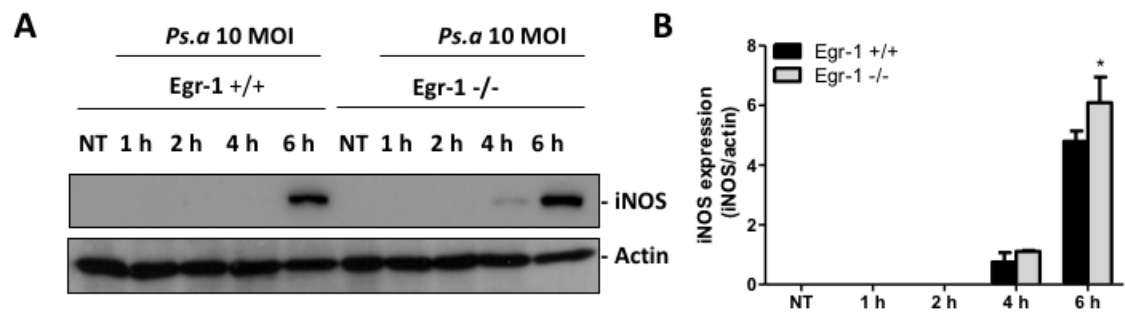
Fig 4.15. Egr-1-deficient BMMs display upregulated iNOS protein expression during *P. aeruginosa* infection. Wild-type (+/+) and Egr-1-deficient (-/-) BMMs were infected with *P. aeruginosa* 8821 at a MOI of 10 for 1 h, 2 h, 4 h, 6 h or left untreated (NT). Cell lysates were subjected to Western blot analysis for determining the protein levels of iNOS and actin as a loading control. Blots are representative of three independent experiments (A). Densitometry analysis of iNOS expression levels was normalized to actin (B) (n = 3 ± SEM, *p < 0.05).
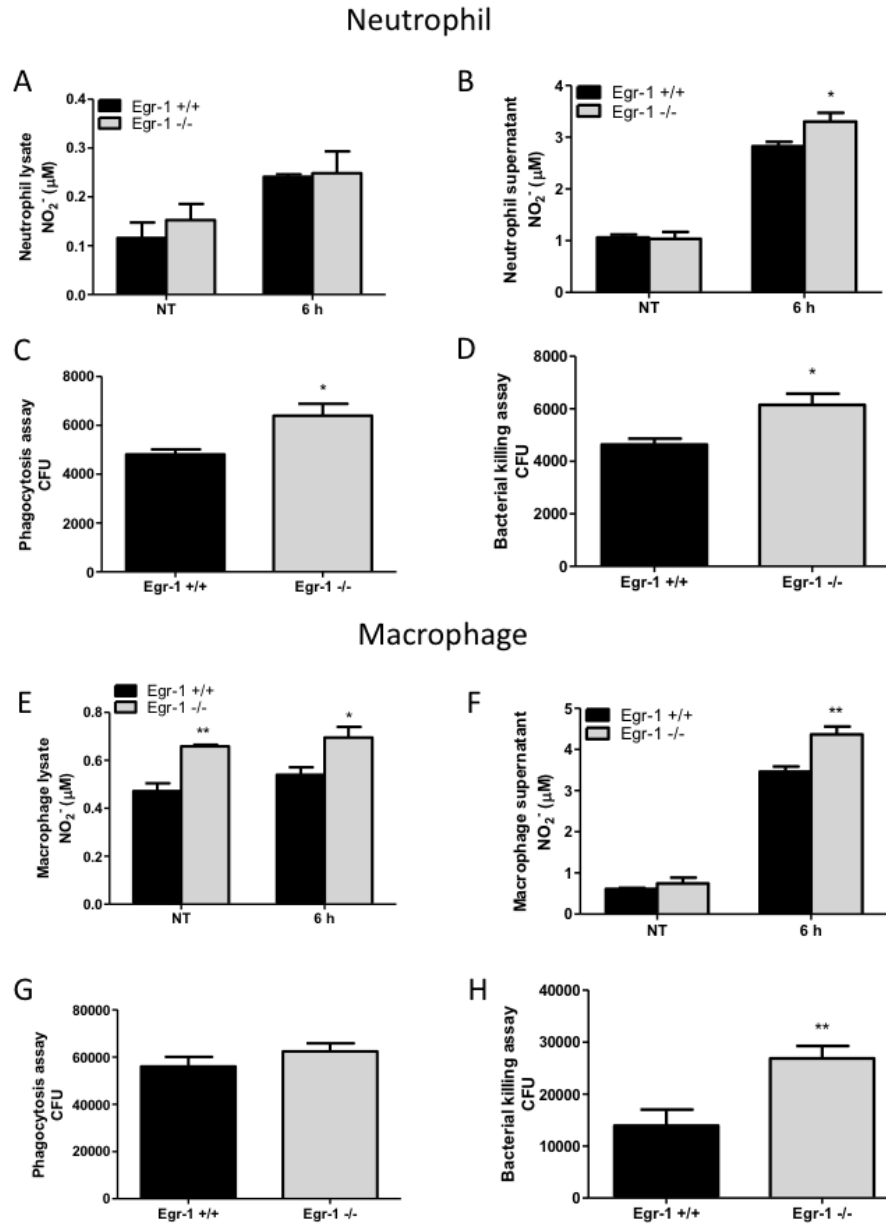
Fig 4.16. Egr-1 deficiency leads to increased nitric oxide production and enhanced bacterial killing ability in neutrophils and macrophages in response to *P. aeruginosa* infection. Wild-type (+/+) and Egr-1-deficient (-/-) neutrophils and BMMs were infected with *P. aeruginosa* 8821 for various time points. The NO_2^- levels were tested in cell lysates (A, E) and supernatants (B, F) at 6 h. The 1 h *P. aeruginosa*-infected neutrophils or macrophages were lysed for phagocytosis assay (C, G). The CFU data represent the number of internalized bacteria within 1 h (n=6 ± SEM, *p < 0.05). The 3 h *P. aeruginosa*-infected neutrophils or macrophages were lysed for bacterial killing assay (D, H). The intracellular killing efficiency was calculated as the number of CFU after 1 hour subtracted by the number of CFU after 3 h infection (1 h CFU – 3 h CFU) (n=6 ± SEM, *p < 0.05, **p < 0.01).
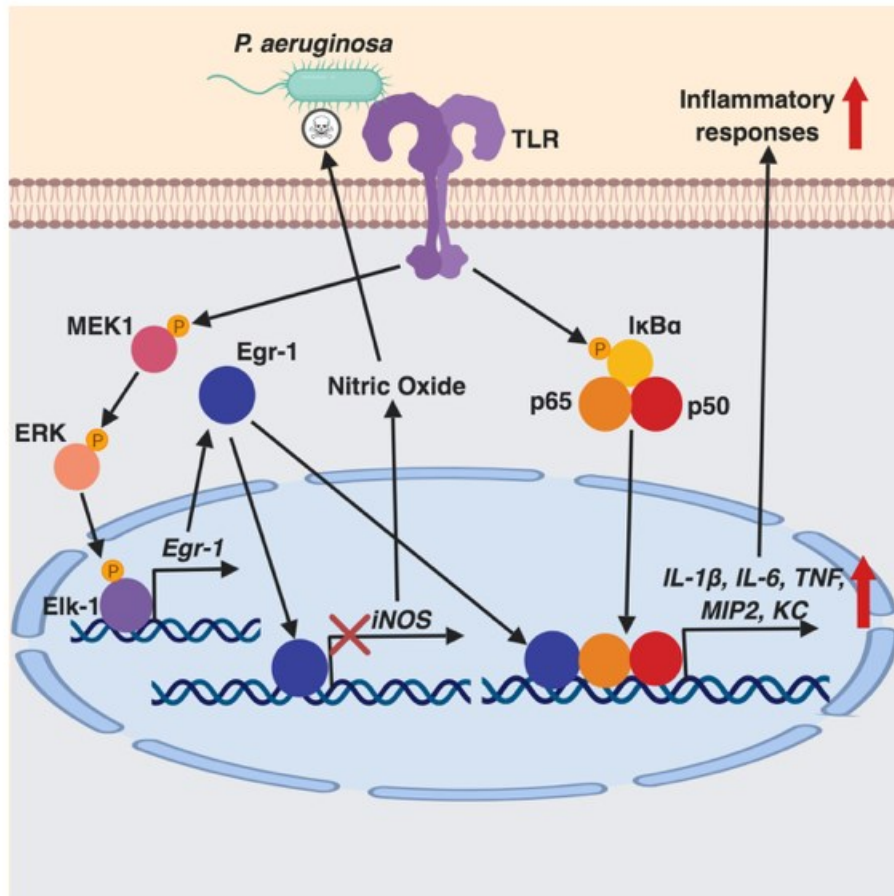
Fig 4.17. Schematic representation of Egr-1-regulated host defense against *P. aeruginosa* infection. Egr-1 negatively regulates nitric oxide production by suppressing iNOS gene expression, and promotes inflammatory cytokine production by physically interact with NF-κB p65 during *P. aeruginosa* infection.

Chapter Five – Conclusion

5.1 Summary and discussion

Pseudomonas aeruginosa has been recognized as a superbug for its remarkable capacity to resist to most currently available antibiotics (Breidenstein et al., 2011). Over the past few decades, scientists have worked on development of the therapeutic approaches from bacteriological aspects, which directly kills *P. aeruginosa* or limits its growth. There has been progress regarding the development of new antibiotics with novel modes of action, resistance to modification by bacterial enzymes, and improvements of drug delivery efficiency. However, *P. aeruginosa* has a remarkable capacity to develop or acquire new resistance mechanisms to these new antibiotics. Moreover, several non-antibiotic therapeutic strategies, such as inhibition of quorum sensing and bacterial lectins, use of iron chelation, phage therapy, nanoparticles, antimicrobial peptides and electrochemical scaffolds, have been reported for treatment of *P. aeruginosa* infections. Although some of the therapeutic strategies have shown significant antimicrobial effects against antibiotic-resistant strains of *P. aeruginosa in vitro* or in animal models, few of them have proceeded to clinical practice due to high cost, side effects and safety concerns (Pang et al., 2019). The therapeutic approaches from immunological aspects that modulate host immunity have drawn a lot of research attention over the past decade, and may shed the light on treatment of *P. aeruginosa* infections.

In healthy individuals, the tightly regulated inflammation effectively clear *P. aeruginosa* from host, whereas the immunocompromised patients with impaired or weakened immune system are not able to generate proper inflammatory responses, and are susceptible to the infections (Dropulic and Lederman, 2016). However, inflammation is a

double-edged sword. Although it is necessary for *P. aeruginosa* clearance, the excessive inflammatory response causes severe lung damage or septic shock, which is associated with high mortality (Lin and Kazmierczak, 2017). Pharmacological modulation of the molecular mechanisms directly toward boosting the host immune response and/or limiting excessive inflammation could be an important strategy to improve the host outcome in *P. aeruginosa* lung infections, which enhances bacterial clearance without causing tissue damage to the host. In this work, I examined the role of two inflammatory regulators RCAN1 and Egr-1 in *P. aeruginosa* LPS-activated TLR signaling and host response to *P. aeruginosa* lung infection, respectively.

5.1.1 Major findings

RCAN1 was previously identified to be a negative regulator of inflammation during *P. aeruginosa* lung infection (Junkins et al., 2013a). In order to gain a deeper insight into the molecular mechanisms of RCAN1-regualted TLR signaling without other confounding effects from complex cellular responses induced by *P. aeruginosa* infection, I used *P. aeruginosa* LPS to directly activate TLR4 signaling both *in vitro* and *in vivo*. RCAN1 deficiency increased the MyD88-NF-κB-mediated cytokine production (IL-6, TNF and MIP-2), whereas the ISRE-mediated cytokine production (IFN-β, RANTES and IP-10) was suppressed. RCAN1 deficiency caused increased IκBα phosphorylation and NF-κB activity in the MyD88-dependent pathway, but impaired ISRE activation and reduced IRF7 expression in the TRIF-dependent pathway. However, the positive regulatory role of RCAN1 in TRIF-IRF7 pathway was not reflected in the *in vivo* study. It suggests that this novel role of RCAN1 may be specific for macrophages. Furthermore, RCAN1 deficiency led to significantly increased *in vivo* levels of IL-6, TNF and MIP-2, and enhanced

pulmonary infiltration of neutrophils in response to *P. aeruginosa* LPS stimulation, suggesting a predominant role of RCAN1 in suppression of MyD88-NF-κB-mediated inflammatory response *in vivo*. Together, these data demonstrate that RCAN1 downregulates the MyD88-NF-κB pathway through inhibition of IκBα phosphorylation, and promotes activation of TRIF-ISRE pathway through regulation of IRF7 activation and expression (Chapter Three).

A mouse model of bacterial pneumonia was applied to examine the biological implications of Egr-1 during *P. aeruginosa* infection. Egr-1-deficient mice displayed decreased mortality, reduced levels of proinflammatory cytokines (IL-1β, IL-6 and TNF) and enhanced *P. aeruginosa* clearance from the lung. Egr-1 deficiency caused diminished NF-κB activation in *P. aeruginosa*-infected cells in a manner that was unrelated to IκBα phosphorylation. A physical interaction between Egr-1 and NF-κB p65 was found in *P. aeruginosa*-infected macrophages, suggesting that Egr-1 could be required for assembly of heterodimeric transcription factors that direct synthesis of inflammatory mediators. While neutrophils are the key immune cells to eradicate *P. aeruginosa*, interestingly, Egr-1 deficiency had no impact on neutrophil recruitment *in vivo* despite differential effects on chemokine production that included diminished accumulation of MIP2 and KC, and increased accumulation of LIX. However, I identified significant increases in nitric oxide production and bacterial killing ability in Egr-1-deficient macrophages and neutrophils that correlated with the enhanced bacterial clearance in Egr-1-deficient mice. Together, these findings suggest that Egr-1 plays a detrimental role in host defense against *P. aeruginosa* lung infection by promoting the systemic inflammation and negatively regulating nitric oxide production for bacterial clearance (Chapter Four).

5.1.2 The function of RCAN1 depends on phosphorylation state

The Chapter Three introduced a dual function of RCAN1 on regulation of MyD88- and TRIF-dependent pathways. RCAN1 was previously found to modulate protein activity either as an activator or inhibitor, and the function of RCAN1 primarily depended on its phosphorylation state, which was mediated by a variety of kinases, such as dual specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), protein kinase A (PKA), mitogen-activated protein kinase 1 (BMK1), TAK1, and leucine-rich repeat kinase 2 (LRRK2) (Abbasi et al., 2006; Han et al., 2017; Jung et al., 2011; Kim et al., 2015; Liu et al., 2009). The phosphorylation of RCAN1 by DYRK1A at Thr192 and PKA at Ser93 enhanced inhibition of calcineurin phosphatase activity (Jung et al., 2011; Kim et al., 2015). By contrast, the phosphorylation of RCAN1 by TAK1 at serine 94 and 136 switched RCAN1 from an inhibitor to a facilitator of calcineurin (Liu et al., 2009), and the phosphorylation of RCAN1 by BMK1 at Ser108 and 112 resulted in dissociation of RCAN1 from calcineurin, leading to NFAT activation (Abbasi et al., 2006). In addition, the phosphorylated RCAN1 has also been identified to promote activation of NF-κB pathway. Han et al. demonstrated that LRRK2-mediated phosphorylation of RCAN1 increased the association between RCAN1 and Toll interacting protein (Tollip), an inhibitory adaptor protein of TLR2 and TLR4, leading to dissociation of Tollip from IRAK1, and upregulation of the TLR-mediated NF-κB activation. However, the authors did not define the phosphorylation sites on RCAN1 modulated by LRRK2 (Han et al., 2017). Therefore, characterization of RCAN1 phosphorylation state in different cellular context will help us to identify the role of RCAN1 in a specific signaling pathway.

5.1.3 The negative feedback loops in *P. aeruginosa*-induced inflammation

Negative feedback is a control mechanism of inflammatory response that switches off the stimulus-induced signaling, important for maintaining homeostasis (Perrimon and McMahon, 1999). In this work, I identified an induction of RCAN1-4 mRNA expression by *P. aeruginosa* LPS at a relatively late time point (2 h) compared to that of inflammatory cytokine genes (1 h) (Fig 3.1 and 3.3), suggesting RCAN1-4 gene is not an immediate early gene, and involved in the late regulatory events. Moreover, Zheng et al indicated that NF-κB was able to mediate RCAN1 gene transcription independent of calcineurin-NFAT signaling (Zheng et al., 2014). The newly synthesized RCAN1 subsequently stabilized IκBα by inhibition of phosphorylation. Furthermore, NF-κB was also found to mediate IκBα mRNA transcription (Cheng et al., 1994). The newly synthesized IκBα protein translocated to nucleus and transported NF-κB back to the cytoplasm by forming a protein complex (Arenzana-Seisdedos et al., 1997; Huang et al., 2000). This negative feedback loop NF-κB-RCAN1-IκB is sufficient to prevent excessive production of inflammatory mediators.

The Chapter Four describes a positive regulatory role of Egr-1 and an interaction between Egr-1 and NF-κB p65 during *P. aeruginosa* lung infection. A study by Yang et al. identified an Egr-1 binding sequence in the *RCAN1* promoter (Yang et al., 2009b). It is possible that Egr-1 induces RCAN1 expression, and RCAN1 suppresses the Egr-1-mediated inflammatory response by disrupting the Egr-1/NF-κB complex through inhibition of NF-κB activation.

5.1.4 Regulation of NF-κB activity by Egr-1

Previous studies have shown that Egr-1 is able to modulate the transcriptional activity of other transcription factors through physical interactions (Chapman and Perkins, 2000; Decker et al., 2003; Ma et al., 2009; Nakashima et al., 2003). In particular, Egr-1 can either promote or suppress NF-κB transcriptional activity, and the differential regulation of NF-κB activity probably depends on the different Egr-1 binding sites on the NF-κB function domains (Chapman and Perkins, 2000; Huang et al., 2017; Ma et al., 2009). In the work in Chapter Four, I identified that Egr-1 promoted the production of NF-κB-mediated inflammatory cytokines IL-1β, TNF and IL-6 and chemokines MIP-2 and KC through physical interaction with NF-κB p65 during *P. aeruginosa* infection. Consistently, a previous study by Ma et al. showed that Egr-1 and NF-κB formed a heterodimer, and promoted IL-8 expression through binding to the NF-κB site within the IL-8 promoter (Ma et al., 2009). Moreover, Huang et al. found that elevated Egr-1 expression was able to promote NF-κB p65 nuclear translocation (Huang et al., 2017). It suggests that the interaction between Egr-1 and NF-κB p65 may initiate in cytoplasm. However, the mechanisms involved in this positive regulation by Egr-1 and the interaction sites within Egr-1 and NF-κB are poorly characterized. By contrast, Chapman et al. demonstrated that the zinc finger DNA binding domain of Egr-1 interacted with the Rel homology domain of NF-κB p65, which led to inhibition of NF-κB p65 transcriptional activity by blocking its DNA binding activity to target promoters (Chapman and Perkins, 2000). Therefore, modulation of NF-κB activity by Egr-1 depends on the interaction sites on NF-κB. The binding of Egr-1 on the transactivation domain of NF-κB may promote NF-κB transcriptional activity, whereas binding of Egr-1 on the nuclear localization signal or DNA

binding domain of NF-κB may inhibit NF-κB nuclear translocation or DNA binding activity, respectively. Creation of point mutations on the transactivation or Rel homology domains of NF-κB will help us to characterize the interaction sites between Egr-1 and NF-κB and identify the role of Egr-1 in different cellular contexts. Inhibition or modification of the interaction between Egr-1 and NF-κB p65 may provide a new avenue to modulate inflammatory response.

5.1.5 Egr-1 suppresses phagocytosis

In Chapter Four, I reported a novel role of Egr-1 in reducing the phagocytic activity of neutrophils during *P. aeruginosa* infection (Fig. 4.16C), and the increased phagocytosis caused by Egr-1 deficiency could be an important factor contributing to clearance of *P. aeruginosa*. Egr-1 may downregulate phagocytosis by reducing the expression of phagocytic receptors or inducing autophagy. The phagocytic receptors of immune cells include Fc receptors, complement receptors, integrins, mannose receptor (CD206), Dectin-1 and scavenger receptors (Gordon, 2016). A recent study by Wu et al. found that knockdown of Egr-1 enhanced expression of mannose and scavenger receptors, leading to increased phagocytosis of *P. aeruginosa* by macrophages (Wu et al., 2018b).

The conserved degradation process autophagy has been found to inhibit phagocytic activity in macrophages (Bonilla et al., 2013; O'Keeffe et al., 2015; Zhu et al., 2018). Furthermore, Egr-1 was previously reported to promote autophagy by upregulating the expression of autophagic proteins LC3B and Atg4B, which are important for autophagosome biogenesis (Chen et al., 2008b; Peng et al., 2017). Given the ability of *P. aeruginosa* to induce autophagy in diverse immune cells (Itoh et al., 2015; Junkins et al.,

2013b; Yuan et al., 2012), it is possible that Egr-1 indirectly inhibits phagocytosis through activation of autophagy.

5.2 Limitations

5.2.1 Limitations of *P. aeruginosa* lung infection model

Pseudomonas aeruginosa can be efficiently cleared from the lungs of healthy human and mice, and *P. aeruginosa* infections are usually hospital-acquired when the patients have defective innate immune system. Because the spontaneous lung infection caused by *P. aeruginosa* does not occur in mice, we used an acute lung infection model by intranasally infecting mice with *P. aeruginosa*. In our study, the number of *P. aeruginosa* 8821 (1 X 10^9 CFU/mouse) used in the lung infection model far exceeded the number of this bacterium normally encountered in the environment, but was necessary to cause acute inflammatory responses in early infection time points, and the infected mice started to recover after day 2. Likewise, the *P. aeruginosa* LPS concentration (1 µg per gram of body weight) used in the mouse model of acute pneumonia was also likely to be much higher than that was exposed in the environment. However, the high dose of *P. aeruginosa* LPS efficiently was necessary to trigger activation of TLR4 signaling and production of proinflammatory cytokine in mouse lung tissues at the early time point 4 h.

Another drawback of this model is that the lung structure of mouse is different from human. The mouse has the four lobes, cranial, middle, caudal and accessory lobes, in right lung and only one lobe in the left lung. In contrast, human have three lobes, upper, middle, and lower lobes, in the right lung and two lobes, upper and lower lobes, in the left lung (Meyerholz, 2018). The different lung structure may manifest different immune responses in human and mouse. Other animal models such as ferret, pig and non-human primates

having the more similar lung structure as human are more reliable and can be applied in future studies.

5.2.2 Limitations of *P. aeruginosa* mucoid strain

This study used a mucoid *P. aeruginosa* strain 8821 that is a clinical isolate from CF patients. The strain of *P. aeruginosa* 8821 is not highly pathogenic, which only caused 30% mortality in C57BL/6 mice even though the mice were infected with a high dose of this bacterial strain (1×10^9 CFU/mouse). *P. aeruginosa* mucoid strains are generally less virulent than *P. aeruginosa* non-mucoid strains, probably due to the downregulated expression of many virulent factors (Ryall et al., 2014; Wu et al., 2004), which have been shown to cause cell death and disrupt cellular functions (Manago et al., 2015; Maurice et al., 2019; Schultz et al., 2000; Wood et al., 2015). Therefore, a non-mucoid *P. aeruginosa* such as PAO1 and PA14 could be applied in survival study and we expect to observe more obvious differences in survival rates between the wild-type and Egr-1 deficient mice. In addition, *P. aeruginosa* 8821 does not express flagella and has modified LPS. The inflammatory responses-induced by *P. aeruginosa* 8821 and non-mucoid strains may be different. After this thesis was formatted, we tested the PAO1-induced proinflammatory cytokine and chemokine production in macrophages and found that PAO1 induces similar cytokine pattern as it was induced by *P. aeruginosa* 8821 but in a lower level. The high cytotoxicity of PAO1 may result in insufficiently induction of host inflammatory responses, despite of the presence of highly inflammatory elicitors, including flagellin and LPS.

5.2.3 Limitations of primary cells

Mouse BMMs were used to study the molecular mechanisms in response to *P. aeruginosa* infection. A drawback of this *in vitro* model is that the growth factor M-CSF

in the media for macrophage differentiation from bone marrow cells may potentially activate the MAPK pathway prior to *P. aeruginosa* infection. The activated MAPK may have crosstalk with other signaling pathways and affect the outcome produced by *P. aeruginosa* infection. Another drawback of using primary cells is that they are not ideal for co-immunoprecipitation study. The low abundant proteins or the weak protein-protein interaction is difficult to be detected in immunoprecipitated primary cell lysates by western blot. Moreover, the primary cells are not well-suited for transfection with plasmid overexpressing a specific gene. From my experience, approximately 50% BMMs were dead after transfection.

5.3 Future investigations

The findings of this work suggest some directions for future investigations. Chapter Three presents that RCAN1 inhibits IκBα phosphorylation and facilitates IRF7 activation. The interaction between RCAN1 and IκBα or IRF7 could be further characterized by co-immunoprecipitation and two-yeast hybrid system. Because co-immunoprecipitation is difficult to accomplish with primary cells due to the low levels of target protein expression and weak interactions, macrophage cell lines such as RAW 264.7 and NR8383 will be used and transfected with *RCAN1*, *IκBα* or *IRF7* containing plasmids in the future study. However, co-immunoprecipitation is not able to analyze direct protein-protein interactions, and RCAN1 may potentially interact with IκBα or IRF7 indirectly through calcinuerin or scaffold proteins. The two-yeast hybrid system allows us to analyze whether the interactions are direct or indirect. It is noteworthy that this technique is only able to test protein-protein interaction in the unstimulated condition. The combination of both techniques would be helpful to address this question.

As mentioned earlier, the different phosphorylation sites render different functions of RCAN1. It is interesting to characterize the phosphorylation sites on RCAN1 following *P. aeruginosa* LPS stimulation. To determine the phosphorylation sites of RCAN1 responsible for regulation of IκBα and IRF7 activities, multiple RCAN1 mutants will be created by substituting the serine or threonine resides with alanine.

The positive regulatory role of RCAN1 in TRIF-IRF7 pathway may be macrophage-specific. It will be interesting to confirm that by creating a transgenic mice with RCAN1-specific knockout macrophages using Cre/loxP system. The method were previously described by Shi et al. (Shi et al., 2018). Briefly, two loxP sites at distant introns will be knocked in RCAN1 gene. The mice with homozygous loxP-flanked *RCAN1* will be crossed with F4/80-Cre mice. The mice from F1 generation will be heterozygous loxP-flanked *RCAN1* and F4/80-Cre. To obtain the homozygous loxP-flanked *RCAN1* with Cre activity, the heterozygous mice will be crossed back to the homozygous loxP-flanked mice. In the F2 generation, the RCAN1 gene will be specifically knocked out by Cre in the F4/80 expressing cells. Once the transgenic mice are created, they can be intranasally administered with LPS as demonstrated in Chapter Three to observe whether the novel role of RCAN1 in TRIF-IRF7 pathway could be displayed *in vivo*.

Chapter Four shows a negative regulatory role of Egr-1 on nitrogen oxide production both *in vivo* and *in vitro* (Fig 4.14 and 4.16), which had an impact on bacterial clearance. To test whether the upregulated nitrogen oxide levels directly link to the enhanced bacterial clearance, the Egr-1-deificient cells or mice will be pre-treated with the iNOS inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME) before *P. aeruginosa* infection. Subsequently, the number of CFU from the L-NAME pretreated Egr-1-deficient

cells or mice will be compared to that of non-treated cells and mice. If the bacterial clearance is decreased in the L-NAME pretreated cells or mice, it suggests that the upregulated nitrogen oxide levels directly linked to the enhanced bacterial clearance.

Inhibition of Egr-1 in host defense against *P. aeruginosa* infection may have potential therapeutic implications, which increases bacterial clearance and lowers the risk of systemic inflammation. Two Egr-1 potential inhibitors mithramycin A and metformin were introduced at the end of the Discussion section in Chapter Four. Another future direction is to test the biological impact of mithramycin A or metformin-mediated Egr-1 inhibition in the mouse model of *P. aeruginosa* pneumonia. Because the strain of *P. aeruginosa* 8821 is not highly pathogenic, which only caused 30% mortality in wild-type mice, the more pathogenic *P. aeruginosa* strains such as PAO1 and PA14 will be applied in the survival study. The mice will be pretreated with the Egr-1 inhibitors before intranasal inoculation of *P. aeruginosa*. The survival rate of Egr-1 inhibitor-pretreated mice will be compared to the non-pretreated group.

Finally, in the two studies, we identified that Egr-1 or RCAN1 deficiencies affected the phosphorylation on many kinds of proteins. As is known to all, phosphorylation is important for regulation of protein functions, including cellular localization, degradation and activation. It is interesting to study the *P. aeruginosa*-induced protein phosphorylation events in a more global and systematic scale rather than just focusing on a single protein using two-dimensional gel electrophoresis or mass spectrometry. It would increase our understanding of global dynamics of phosphorylation network.

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