2013

Role of NOD2/RIP2 signaling in acute bacterial pneumonia and sepsis

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ROLE OF NOD2/RIP2 SIGNALING IN ACUTE BACTERIAL PNEUMONIA AND SEPSIS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University
and Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Veterinary Medicine
through the Department of Pathobiological Sciences

by
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BVSc, University of Peradeniya, 2007
May 2013

Dedicated to my parents

late Karthigesu Theivanthiran and Sakunthala Theivanthiran

Acknowledgments

This project could not have been undertaken without the commitment of my major adviser, Dr. Samithamby Jeyaseelan, who provided scientific and intellectual guidance, encouragement, and financial support throughout the course of my graduate studies. I am deeply indebted to my committee members: Drs. Ronald L. Thune, Inder Sehgal, Ji-Ming Feng and Antonieta Guerrero-Plata, for their critical advice and guidance during my graduate research. My sincere thanks go to the lung biology laboratory members: Dr. Sanjay Batra and Dr. Shanshan Cai, Dr. Ritwij Kulkarni, Jin Lilang, Arvind Pandey and Kanapathipillai Jeyagowri for their support, sharing skills and friendship.

I am thankful to Marylin Dietrich, Peter Mottram and Dan Chisenhall for their assistance in flow cytometry, confocal microscopy and in proofreading my manuscripts. I wish to express my sincere thanks to Dr. Jaime Lobo and my friends in the Department of Pathobiological Sciences, the Jones family and the Sri Lankan community in Baton Rouge for their friendship and support. I thank my family for their unflagging love and support to successfully complete the Ph.D. degree.

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Commonly Used Abbreviations

ALI	Acute lung injury
ALRTI	Acute lower respiratory tract infections
AM	Alveolar macrophages
AP-1	Activating protein-1
ARDS	Acute respiratory distress syndrome
BALF	Bronchoalveolar lavage fluid
Birc1e	Baculoviral IAP repeat containing 1e
BSA	Bovine serum albumin
CCR2	Chemokine (C-C motif) receptor 2
CFU	Colony forming unit
CLP	Cecal ligation and puncture
CXCR-2	Chemokine (CXC motif) receptor 2
DAMPs	Danger associated molecular patterns
DMEM	Dulbecco's modified Eagle's medium
FACS	Flourescence activated cell sorting
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony stimulating factor
i.p.	Intraperitoneal
i.t.	Intratracheal
ICAM-1	Intracellular cell-adhesion molecule-1
IFN-γ	Interferon gamma
IL-17	Interlukin 17
IL-6	Interlukin 6
IRAKs	Interleukin-1 receptor-associated kinases
IRF	Interferon regulatory factor
JNK	C-Jun N-terminal kinase
KC	Keratinocyte cell-derived chemokines
LIX	Lipopolysaccharide-induced CXC chemokines

LPS	Lipopolysaccharide
LRRs	Leucine rich repeats
LTB4	Leukotriene B4
MAPKs	Mitogen associated protein kinases
MCP-1	Monocyte chemoattractant protein-1
MD-2	Myeloid differentiation protein-2
MIP-2	Macrophage inflammatory protein-2
MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary response gene (88)
NACHT	Neuronal apoptosis inhibitor protein CIITA, HET-E and TP-1
NAIP5	NLR family apoptosis inhibitory protein 5
NALPS	NACHT, LRR and PYD domain-containing proteins
NF- κB	Nuclear Factor kappa B
NLRs	Nod like receptors
NOD1	Nucleotide oligomerisation domain 1
NOD2	Nucleotide oligomerisation domain 2
OD	Optical density
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PMN	Polymorphonuclear leukocytes
PRRs	Pattern recognition receptors
PYD	Pyrin domain
RBC	Red blood cells
RIP2	Receptor interacting protein 2
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STAT	Signal transduction and transcription proteins
STAT3	Signal transduction and transcription protein 3
TIR	Toll-interleukin (IL)-1 receptor homology domain
TIRAP	Toll-interleukin 1 receptor (TIR) domain-containing adapter protein
TLRs	Toll-like receptors
TNF- α	Tumor necrotic factor alpha

TRAM	TRIF related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
VCAM-1	Vascular cell-adhesion molecule-1
WT	Wild type

Abstract

Bacterial pneumonia and sepsis are two important causes of mortality in the world. Emergence of multidrug resistant bacteria has necessitated the development of new treatment and/or prevention strategies to augment host immune defense. In this context, the innate host defense is critical in clearing pathogenic bacteria from the host. Early neutrophil recruitment is a critical step in a multistep requence leading to bacterial clearance. Pattern recognition receptors (PRRs) play a critical role in the innate immune system. Receptor interacting protein 2 (RIP-2) is an adaptor for the nod-like receptors (NLR) NOD1 and NOD2. Nucleotide oligomerisation domain 2 (NOD2) is an intracellular PRR that is shown to be important for host defense against intracellular bacterial pathogens. However, the role of NOD2 and RIP-2 during Gram-negative bacterial pneumonia and polymicrobial sepsis has not been explored. Thus, we hypothesize that the NOD2/RIP-2 axis is critical for host defense during bacterial pneumonia and sepsis/septic peritonitis. To test this hypothesis, we infected NOD2(NOD2$^{-/-}$), RIP-2(RIP-2$^{-/-}$) deficient mice intratracheally (i.t) with *E. coli* (10^6 CFUs/mouse) and *Klebsiella pneumoniae* (10^3 CFUs/mouse). We observed that NOD2/RIP2 signaling is critical for the host defense during gram-negative pneumonia and poly microbial sepsis. The NOD2/RIP2 axis regulates neutrophil recruitment via IL-17A production. We also found that NOD2/RIP-2 signaling is essential for the production of IL-6 and activation of STAT3. We demonstrated that RIP-2 regulates inflammasome activity that is independent of NOD2 signaling. Taken together, these data demonstrate that the NOD2/RIP-2 axis plays a critical role in neutrophil-mediated host defense through IL-17A production and by inflammasome activation.

In cecal ligation puncture (CLP) induced sepsis, RIP2$^{-/-}$ mice show increased mortality with higher bacterial burden in the peritoneum and systemic organs compared to WT controls. We found reduced neutrophil influx IL-17A and IL-1β levels in the peritoneum of RIP2$^{-/-}$ mice after CLP. Furthermore, we also observed increased systemic inflammation accompanied by vital organ damage in the knockout mice. As a whole our data suggest a critical role of RIP2 in neutrophil recruitment, along with IL-17A and IL-1β during sepsis.

Chapter 1: Introduction

Bacterial pneumonia and sepsis are major causes of mortality worldwide [1, 2]. Bacterial lung infections can result in sepsis and vice versa. Despite recent medical advancements, there has been little to no improvement in death rates associated with these diseases. As the current treatment of choice for any bacterial infection including bacterial pneumonia is antibiotics; widespread antibiotic usage has resulted in the emergence of multidrug resistant bacterial strains [3, 4]. Growing populations of immunocompromised individuals also make the treatment of bacterial pneumonia difficult. Therefore, using immunotherapy to modulate the immune response could be an important therapeutic strategy [5, 6].

The immune response against various pathogens involves complex interactions of both the innate and adaptive responses. The innate immune response is the first line of defense against pathogens, is non-specific, and initiates quickly upon exposure to a pathogen. On the contrary, the adaptive response is pathogen-specific, takes several days to develop and has a memory response. During acute infections, the innate host defense plays a vital role in eliminating the pathogen [7, 8]. Early neutrophil recruitment is an essential step to contain the infection. Pathogen Associated Molecular Patterns (PAMPs) trigger Pattern Recognition Receptors (PRRs) and downstream signaling effectors to induce expression of pro-inflammatory cytokines and neutrophil recruitment during infection.

Receptor Interacting Protein 2 (RIP-2) is an adaptor for cytosolic NOD-like receptors (NLRs),NOD2 (CARD (define CARD) 15) is an NLR involved in detection of muramyl dipeptide (MDP), which is a conserved glycoprotein present in bacterial cell walls [9, 10]. NOD2 interacts with MDP through a C-terminal leucine rich repeat (LRR)[9, 10]. NOD2 is expressed mainly in leukocytes, dendritic cells and epithelial cells[11, 12]. RIP-2 is highly up-regulated in multiple tissues during bacterial infections in humans and in animal models. Previous reports show that NOD2 and RIP-2 play a critical role in host immunity to intracellular pulmonary pathogens, such as *Listeria monocytogenes* [13]*, Legionella pneumophila* [14]*, Chlamydophila pneumoniae*[15] and *Mycobacterium tuberculosis* [16]. In

addition, during *Staphylococcus aureus* infection, $NOD2^{-/-}$ mice showed enhanced mortality due to defective neutrophil phagocytosis. However, the role of NLRs, especially NOD2/RIP-2 signaling in extracellular Gram-negative infection of the lung and sepsis is not known.

We hypothesized that the NOD2/RIP-2 axis is essential for local and systemic neutrophil-mediated immune responses during acute bacterial pneumonia and sepsis. Therefore, the major goals of this dissertation are 1)to determine the role of RIP-2 signaling in neutrophil recruitment and chemokine regulation during *Escherichia coli* pneumonia, 2) to determine the effectors upstream of RIP-2 and their role in neutrophil recruitment following Gram-negative bacterial infection, 3) to determine the function of RIP-2 in local and systemic neutrophil mediated host defense during acute bacterial pneumonia and 4) to study the role of RIP-2 during polymicrobial sepsis induced by cecal ligation and puncture. To achieve these goals we intratracheally inoculated NOD2, RIP-2 and NOD2/RIP-2 gene-deficient mice and their wild type controls (C57BL/6) with *E. coli* ($1X10^6$ colony forming unit(CFUs /animal) or *Klebsiella pneumoniae* ($1X10^3$ CFUs/animal). Alternatively we induced sepsis using cecal ligation and puncture(CLP).

The objectives of the first research avenue were A) to determine the expression pattern of RIP-2, cellular adhesion molecules and the activation of STAT3(Signal transducer and activator of transcription 3) NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and MAPKs (Mitogen-activated protein kinases) in lungs during Gram-negative bacterial pneumonia using immunblots; B) determination of the bacterial counts in lungs after bacterial infection; C) to assess neutrophil numbers in BALF(Bronchoalveolar lavage fluid) and in the lung with a hemocytometer and myeloperoxidase assay, D) measure the expression of pro-inflammatory cytokines and chemokines in the lungs of mice after i.t. infection and E) to determine blood neutrophil counts by flow cytometry and neutrophil cytokine expression profiles.

The objectives of the second research avenue were A) to use bacterial culturing, a hemocytometer and the myeloperoxidase assay to determine bacterial CFUs, neutrophil numbers in the BALF and lung tissue after bacterial infection, B) to determine the

expression of cellular adhesion molecules and activation of NF-κB, MAPKs and components of the NADPH (Nicotinamide Adenine Dinucleotide Phosphate-oxidase)oxidase complex, using an immunoblot analysis C) to use bacterial killing assay and phagocytosis assay to determine the ability of neutrophils to clear the bacteria and D) determine the levels of pro-inflammatory cytokines and chemokines in lungs during Gram-negative bacterial pneumonia.

The objectives of the third research avenue were A) to show the expression of RIP-2 and inflammasome activation after infection with Gram-negative bacteria, using an immunoblot B) Measure cytokines and chemokines after Gram-negative infection, C) to assess neutrophil numbers in the circulation of RIP-2 gene deficient mice post-bacterial infection by using flow cytometry and a neutrophil specific antibody (Gr-1/Ly6G), D) to use in-vitro co-culture system of CD4/CD11c cells to determine the role of RIP-2 in interlukin-17A (IL-17A) production and D) to determine whether administration of exogenous IL-6 and IL-17A can improve host defense in mice after Gram negative bacterial infection by determining the bacterial burden in the lungs, spleen, liver and blood.

For the fourth research goal, the following main objectives were achieved: A) we determined the cellular counts, presence of neutrophils and cytokine and chemokine levels in the peritoneal lavage fluid and serum after CLP, B) using flow cytometry with a neutrophil specific antibody we determined neutrophil numbers in the circulation and C) using bacterial culturing we determined the CFUs in the peritoneum and vital organs after CLP induced sepsis.

This dissertation is organized and presented in seven chapters. This general introduction is followed by a review of the literature describing the role of TLRs and NLRs in neutrophil mediated host defense during acute bacterial pneumonia (chapter 2). The third, fourth, fifth and sixth chapters detail the methods and results of the original research performed over the course of my graduate program. Each of these chapters is presented in the format required by the scientific journal in which it has been published or to which it is going to be submitted. The format of chapter 2 conforms to that of *the Innate Immunity* in which the original article was published. The format of chapters 3 and 4 conforms to that of

Infection and Immunity, in which the original research was published. The format of chapters 5 and 6 conforms to that of *Journal of Immunology*, to which the original research is going to be submitted. The seventh or last chapter is devoted to the major conclusions and future directions.

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Chapter 2: Literature Review*

Bacterial infections in the lung represent an important cause of morbidity, mortality and health care expenditure. In fact, bacterial pneumonia is the third leading cause of mortality worldwide. Pneumonia is the result of overwhelming infection of microorganisms such as virus, bacteria, fungi and parasites. Neutrophil recruitment is the pathological hallmark of pneumonia caused by bacteria. Although neutrophils play an important role in the pulmonary host defence during infection excessive neutrophil accumulation leads to acute lung lnjury (ALI)/Acute Respiratory Distress Syndrome (ARDS)[1, 2]. Therefore, the development of drugs that target the excessive neutrophil accumulation could slow the progression of lung damage, thereby decreasing mortality during bacterial pneumonia.

Infections at mucosal sites are caused by a broad range of pathogens are primarily defended by the innate immune mechanisms. The key players of innate immunity are phagocytes such as neutrophils and macrophages. Recognition of the pathogen is the first step in a multistep paradigm leading to innate immunity in the lungs and this function is primarily performed by pattern recognition receptors, such as membrane bound toll-like receptors (TLRs) and cytosolic receptors NOD-like receptors (NLRs), Rig I like receptors (RLR), C-lectin receptors (CLR) and Scavenger receptors. TLRs were identified more than one decade ago, whereas cytosolic receptors were only recently been discovered [3]. Because of this, TLRs are the most extensively studied. In this article we will review our current understanding of the TLRs and NLRs in the context of bacterial recognition in the lungs.Neutrophils contribute to initial host defense in the lung during bacterial infections [4-6]. Neutrophils are stored in bone-marrow and are mobilized from the bone marrow into the circulation during infections.

*** This article was originally published in**
Balamayooran, T., Balamayooran, G and S. Jeyaseelan. **Review: Toll-like receptors and NOD-like receptors in pulmonary antibacterial immunity.** *Innate Immunity* June 2010 16: 201-210
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Neutrophil recruitment to the site of infection and inflammation is a multistep paradigm involving chemokine and cytokine production, upregulation of cell adhesion molecules, margination of neutrophils, rolling and diapedesis[7]. In the inflammatory foci activated neutrophils phagocytose microbes, degranulate, generate free radicals and degrade the microbes. Although neutrophils serve as the primary defense, their short life span (<6 h) renders it difficult to investigate molecular mechanisms upon interaction with bacterial pathogens. A recently described long term bone-marrow culture system will aid to advance our knowledge in neutrophil biology in the near future[8]. Although neutrophils are considered primary defenders of the innate immune system, a recent report showed that neutrophil-derived IL-18, along with dendritic cell-derived IL-12, stimulate natural killer cells to release IFNγ[9], showing their role in the induction of adaptive immunity. In addition to the intracellular killing mechanisms of neutrophils, recent studies showed that neutrophils form extracellular traps that can bind, confine and destroy extracellular bacteria (neutrophil extracellular traps; NET). NETs can be formed by activated neutrophils. In this context, it has been shown that LPS, phorbol myristate acetate (PMA) and chemokines, such as IL-8 [10]. These NETs are composed of chromatin meshwork containing several types of antimicrobial proteins from primary granules, including neutrophil elastase, cathepsin G and myepoperoxidase, secondary and tertiary granules, such as lacoferrins and gelatinase [10]. Although the formation of NETs is an exciting mechanism to destroy extracellular pathogens, forming NETs may be deleterious to the host because of extracellular noxious components released from neutrophils which can induce severe lung damage.

Toll like Receptors

TLRs play a pivotal role against a variety of exogenous and endogenous pathogens. TLRs are type 1 integral membrane glycoproteins which have an extracellular LRR domain and a signalling cytoplasmic domain homologus to interlukin 1 receptor and termed TIR domain[11]. There are 12 TLRs in mice and 10 TLRs in humans that have been reported so far[3]. TLRs function as homodimers with the exception of TLR2 which dimerises with TLR1

and TLR6 with different ligand specificity. TLRs activate similar but not identical molecules as in the IL-1R signaling cascade[12]. Stimulation of TLR ligands to their receptors recruit adaptor molecules to the cytoplasmic toll-interleukin 1 receptor (TIR) domain and trigger the downstream signalling cascade, activation of NF-κB and production of proinflammatory cytokines and chemokines. TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the cell surface and TLR3, TLR7 and TLR9 are located on the endosomal membrane. TLR1 TLR2 and TLR2 TLR6 heterodimers recognize lipoteichoic acid and lipoprotieins from Gram-positive bacteria and TLR4 recognizes lipopolysaccharide (LPS) of Gram-negative bacteria. TLR2 gene deficient mice showed high susceptibility to *Staphylococcus aureus* or *Streptococcus pneumonia* upon challenge[13]. However it protects only partially against *L. pneumophila[14-16].* TLR4 ligand LPS binds to TLR4 in association with LPS binding protein (LBP), CD14 and MD2[3, 17]. TLR4 plays an important role in the pulmonary immunity against *Klebsiella pneumoniae[18], Haemophillus influenzae[19]* and *Streptococcus pneumoniae[20]*. During *Acinetobacter baumanii* [21]and *Pseudomonas aeruginosa[22]* infections both TLR2 and TLR4 are shown to play an important role in the host defence. TLR5 is important for the recognition of flagellin, a constituent of flagella in motile bacteria. Mutations of TLR5 are associated with susceptibility to *Legionella pneumophila* [23]infections. TLRs 3, 7 and 8 recognize viral RNAs and their synthetic analogs. TLR9 is stimulated by bacterial unmethylated CpG DNA and protects the host against Gram-negative as well as Gram-positive bacterial infections[24-26]. Furthermore TLR11 is important for the recognition of profilin, and uropathogenic bacteria[3].

Engagement of microbial components to the TLRs triggers the downstream signalling pathways and induction of genes involved in the host defence. There are five distinct adaptor molecules (MyD88, TRIF, TIRAP, TRAM and SARM) that are associated with TLR signalling which are recruited to the cytoplasmic TIR domain of the TLRs. Among the adaptor molecules, MyD88 is essential for almost all TLRs and it is recruited to the TIR domain by another adaptor molecule, TIRAP, in the TLR2 and TLR4 signaling cascade. TLR3 and

TLR4 signalling involves the MyD88 independent pathway where the other adaptor, TRIF plays an essential role. TRAM is a key player in TRIF mediated MyD88 independent signalling. Recruitment of MyD88 facilitates the association of IL-1R associated kinases (IRAKs) IRAK4 and IRAK1 to the receptor complex, during this process IRAK4 and IRAK1 become activated and promote the interaction of TRAF6 to the complex. This complex interacts again with another preformed complex comprising of TAK1, TAB1 and TAB2[3]. This clustering activates IKK and subsequently activates NF-kB. Activation of TAK1 also activates mitogen associated protein kinase (MAPK) and Janus kinase (JNK). This activation results in the expression of growth factors, chemokines and cytokines and cell adhesion molecules. There are four different IRAKs (IRAK-1, IRAK-2, IRAK-M, and IRAK-4) that have been identified in mice and humans. Interestingly recent studies have shown that IRAK-M serves as a negative regulator of TLR signalling and IRAK-M gene deficient mice show increased inflammatory response[27].

Several studies have elucidated the roles of adaptor molecules involved in the TLR pathways, such as MyD88-dependent cascade (MyD88 and TIRAP) and MyD88-independent cascade (TRIF and TRAM) in bacterial infections. For example, MyD88 is essential for the host defence against *S. pneumoniae[28], S. aureus[29], E.coli [30], K. pneumoniae[6], H. influenzae[31], P. aeruginosa[29, 32]* and *L. pneumophila[15, 33]* whereas TIRAP, an upstream molecule in the MyD88 cascade, is important for pulmonary host defense against *E. coli* and *K. pneumoniae[6, 30].* In general, MyD88 is shown to be critical for the protection against bacterial infections than single or multiple TLRs (Table). Upon *E.coli[30]* and *P.aeruginosa[34]* challenge, TRIF plays an important role in the host defence. Regarding the relative importance of MyD88 and TRIF, MyD88 plays much more important role than TRIF against *K. pneumoniae* [35]. These findings suggest that pathogens utilize both MyD88-dependent and MyD88-independent cascades in the host via different bacterial components.

Surfactant proteins are a complex of lipoproteins that enhance pathogen clearance and primarily are expressed in the epithelial lining of the lung. The host-defence functions of surfactant are primarily mediated by SP-A and SP-D, which are members of the collectin family of proteins. These collectins opsonise bacterial pathogens in order to facilitate their phagocytic clearance. The surfactant proteins A and D potentially bind to several receptors and activate a number of signaling cascades, among which, TLR2 and TLR4 have shown to be important. Activation of TLRs by these ligands initiates a conserved series of responses that culminate the production of inflammatory cytokines [36].

NOD like receptors

NLRs comprise a large family of intracellular receptors that are characterised by the NOD family of proteins. They regulate both inflammation and apoptosis. There has been about 23 NLRs reported to date. The general structure of these proteins includes an amino-terminal caspase recruitment domain (CARD), pyrin, or baculovirus inhibitor repeat domains, a central NOD, and carboxyl-terminal leucine-rich repeats (LRRs) that detect specific PAMPs[37, 38]. The family contains NALP (NACHT-, LRR-, and pyrin domaincontaining proteins), NOD (nucleotide-binding oligomerization domain), CIITA (class II transactivator), IPAF (ICE-protease activating factor) and NAIP (neuronal apoptosis inhibitor protein). These receptors sense intracellular pathogens. The best characterised of the NODs are NOD1 and NOD2 where NOD1 has one CARD domain and NOD2 has two CARD domains in their amino terminus and they recognise different muropeptides (γ-D-glutamyl-mesodiaminopimelic acid and muramyl dipeptide) from the bacterial cell wall although how these peptides enter the cytosol is not clear[37, 38]. The NODs importance has been widely studied in their mutations associated with chronic inflammatory diseases such as Crohn's disease. NOD1 is ubiquitously expressed whereas NOD2 is primarily found in antigen presenting cells. Upon ligand binding to the NODs, the CARD containing adaptor molecule serine/threonine kinase RIP2 (also known as RICK, CARDIAK, CCK, and Ripk2) transduces signals for antimicrobial inflammatory responses independent of TLR signalling[39]. RIP2

gene deficient mice have shown increased susceptibility to intracellular *Listeria monocytogens*[40]. Similar observations were reported in NOD1 and NOD2 gene deficient mice against *Listeria monocytogens* infection[39]. NOD1 is critical for the host immunity during *Pseudomonas aeruginosa[41]* infection and NOD2 in *Mycobacterium tuberculosis[42].* NOD1 and NOD2 activation results in the NF-kB and MAPK activation and NOD mediated NF-kβ activation is essential for the protection of host during *Streptococcus pneumoniae* infection[43].

Besides NODs, there are other NLRs that play an important role in the antimicrobial host defense. The NALP subfamily has PYRIN instead of CARD in their N-terminus and has 14 members. NALP1, NALP2 and NALP3 form a multiprotein complex called "inflammasome" comprising ASC (apoptosis associated speck-like protein containing a CARD) and caspase. This complex activates the caspase1 and lead to the cleavage of prointerleukin (IL)-1β, proIL-18 and pro IL-33 to their biologically active form. NALP3 inflammasome is shown to be essential for host defence against *L. pneumophila* [44], *Francisella tulerensis* [45] and *Klebsiella pneumoniae [46]* whereas NALP1 is important for the host defence against *Bacillus anthracis* [47]. IPAF is another NLR that has a CARD domain and forms an inflammasome that recognises the bacterial flagellin of motile bacteria such as *Salmonella, Shigella, Legionella*, *Francisella* and *Pseudomonas[45, 48-53]*.

Transcription factors

Several transcription factors such as NF-κB, AP-1, STAT and IRFs are involved in the regulation of host defence mechanism. NF-κB and STAT proteins have been studied in detail. However NF-κB is considered as the central mediator of immune mechanisms. TLRs and NOD pathways converge to activate NF-κB dependent gene transcription to mediate immune responses. TLR2 and TLR4 were found to activate NF-κB in response to LPS stimulation, a response that is enhanced by the presence of CD14 and LBP[54, 55]. Furthermore, TLR5 regulates NF-κB activation during flagellin-induced sepsis [56]. Similarly TLR3 activated by poly I:C mediate NF-κB activation[57]. NF-κB family of structurally related

transcription factors occur as homodimers or heterodimers in the cytoplasm of almost all the mammalian cells[58]. The NF-κB commonly refers to p50-RelA heterodimer, which is one of the most avidly forming dimers present in cells. They control a large number of normal cellular processes, such as immune and inflammatory responses, developmental processes, cellular growth, and apoptosis. These NF-kB proteins are related through a highly conserved DNA-binding/dimerization domain called the Rel homology (RH) domain. NF-κB proteins' activity is primarily regulated by interaction with inhibitory IkB proteins. In cells NF-kB is present in a latent, inactive, IkB-bound complex form in the cytoplasm. The NF-κB-Iκ B interaction prevents NF-kB nuclear localisation as well as DNA binding ability. When a cell receives a signal, it can trigger multiple signalling cascades that can activate the serine-specific IkB kinase (IKK) and eventually ubiquitinate the IkB and promote its degradation. This facilitates activation of NF-kB, nuclear localisation and DNA binding. NF-kB activation turns on several proinflammatory genes, more specifically the neutrophil chemoattractants and result in the neutrophil accumulation in the site of infection[58]. Studies of NF-kB have shown that endogenous NF-κB is essential for the host defence against *E.coli* and *Streptococcus pneumoniae* pneumonia [59, 60]. It has been shown that NF-kB activity is affected by the cytokine receptor signalling such as TNF-α and IL-1 receptors during pneumococcal infection[61]. During *Pseudomonas aeruginosa[34]* and *Klebsiella pneumoniae[35]* infection TRIF, an adaptor molecule for TLR3 and TLR4, has been shown to reduce NF-κB activation and chemokine expression.

JAK/STAT pathway is an important mechanism for a wide array of cytokines and growth factors. Intracellular activation of JAK/STAT pathway occurs when ligand binding induces the multimerization of receptor subunits. Activation of JAKs subsequently phosphorylate and activate STATs. Similar to NF-κB, STATs are also latent transcription factors that reside in the cytoplasm until activated. Phosphorylated and dimerised STATs enter the nucleus and bind specific regulatory sequences to activate or repress transcription of target genes[62]. During *E.coli* pneumonia IL-6 is shown to activate STAT3 to promote

neutrophils recruitment into the lungs[63]. STAT4 is an essential component of the innate immune defence against *K. pneumoniae[64]* and *P. aeruginosa[65]* infections and bacterial clearance of *K. pneumoniae[64].*

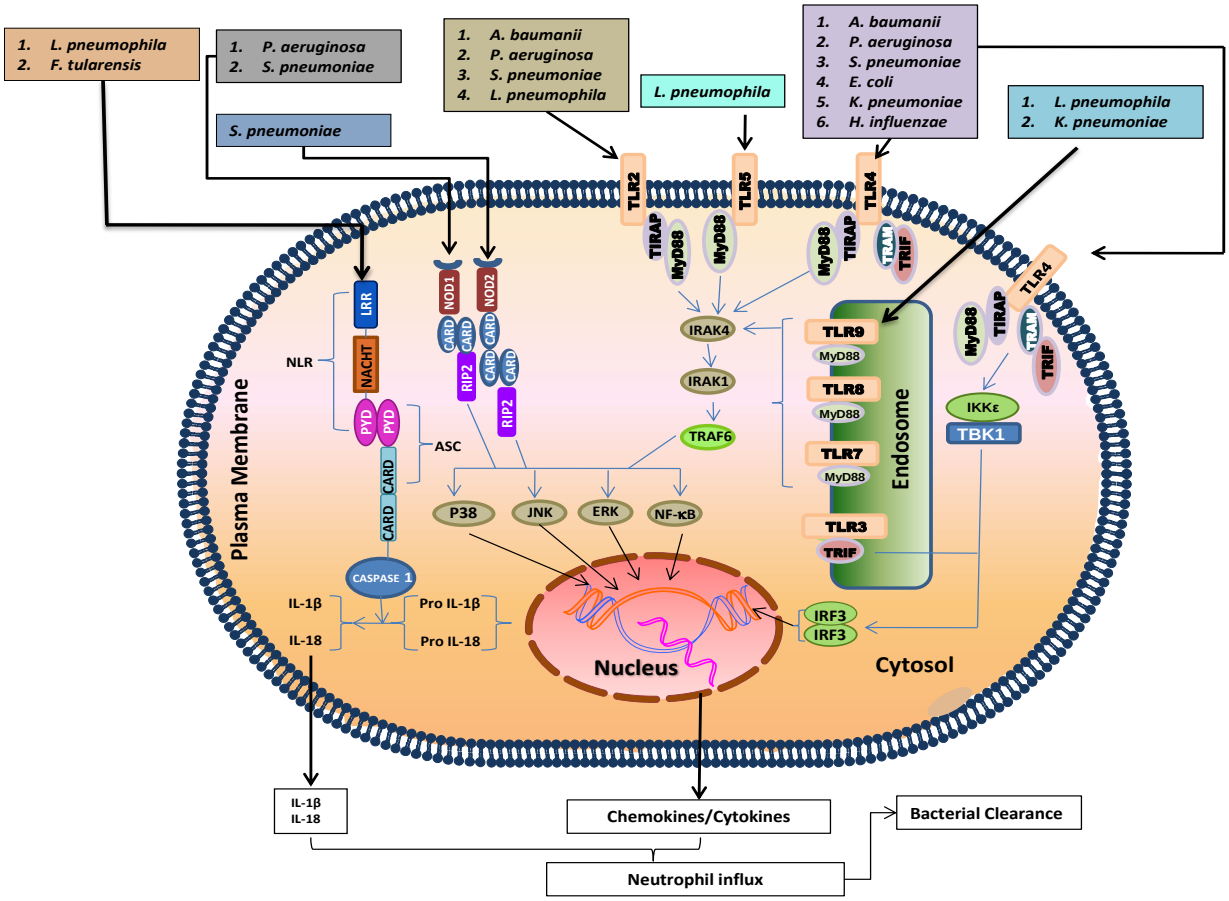

Figure 2.1: Respiratory pathogens are recognized by membrane bound and cytoplasmic pattern recognition receptors. Plasma membrane-bound TLRs (TLR2, TLR4 and TLR5) and endosome membrane-bound TLRs (TLR3, TLR7, TLR8 and TLR9) recognize bacterial pathogens in the lungs. TLR2, TLR4, TLR5, TLR6, TLR7 and TLR9 recruit MyD88 whereas TLR2 and TLR4 recruit both TIRAP and MyD88. TLR3 and TLR4 recruit TRIF to induce downstream signalling cascades. Binding of pathogens and/or PAMPs to TLRs leads to complex signaling cascades, which results in transcription of proinflammatory mediators and activation of MAP kinases. Cytosolic NLRs, such as NODs and NALPs, recognize bacterial pathogens in the lung, NLRs use ASC to induce signalling cascades, which results in transcription of proinflammatory mediators and activation of MAP kinases. These proinflammatory mediators, including chemokines, recruit neutrophils to the lung in order to clear the causative pathogen during bacterial infection.

Cytokines

Cytokines are a diverse group of soluble proteins or peptides that bring biological responses at very low concentrations. These cytokines have autocrine, paracrine or endocrine activities

to bring about immune functions. Specific binding of these cytokines to their cognate receptors result in signal transduction via secondary messengers, upregulation or downregulation of their receptors, cell proliferation and secretion. The major pro-inflammatory cytokines or the acute phase cytokines that are studied extensively are TNFα, IL-1β, IL-6, IL-8 and IFNγ. TNFα, IL-1β and IL-6 play an important role in vasodilatation, increasing the vascular permeability and upregulation of cellular adhesion molecules. IL-8 and IL-1β act as a potent chemokine and IFNγ promotes the intracellular phagocytic killing of microbes. During Gram-negative bacterial infection, TLR4 has been shown to regulate the expression of TNF-α and IL-1β [66, 67] whereas, during *L.pneumophila* infection, TLR5 and TLR9 mediate the expression of TNF-α [23, 24]. On the other hand, anti-inflammatory cytokines IL-10, TGF-β and IL-1Ra play a critical role to dampen the inflammatory response and resolve inflammation and aid in healing[12]. IL-12 is a proinflammatory cytokine that has been shown to have beneficial effects in the setting of host acquired immunity. Effective delivery of IL-12 to the murine lung during *Klebsiella pneumoniae* infection protects the mice[68]. During *L. pneumophila* infection, TLR9 seems to regulate IL-12 production in lungs[24]. The IL-12/IFNγ axis is important for the intracellular bacterial host defence and phagocytic destruction of the bacteria.

Recent studies have shown an emerging role of IL-23 in innate host defense during bacterial pneumonia. IL-23 is a heterodimer that has a p40 subunit identical to the IL-12 and a unique p19 subunit[69]. IL-23 is predominantly a cytokine that is produced by antigen presenting cells and it is shown to stimulate the production IL-17 (IL-17A and IL-17F) by Th17 and γδ T cells in a TLR dependent manner. These cytokines promote the production of many other cytokines and chemokines[70, 71]. IL-17 signalling is critical for the pulmonary host defence against and survival during *Klebsiella pneumoniae* infection[72, 73]. Studies have also shown that IL-23 plays a critical role in the pulmonary immunity against *Pseudomonas aeruginosa* infection*[74].* IL-23/IL-17 axis has been shown to regulate the expression of various other proinflammatory cytokines. In addition to IL-17A there are IL-

17B, IL-17C, IL-17D, IL-17E and IL-17F. IL-17 cytokine binds to type I cell surface receptor called IL-17R which has 3 different variants (IL-17RA, IL-17B and IL-17C). IL-17 signaling is critical for host defense against extracellular bacteria by regulating chemokine gradients for neutrophil emigration into infected tissue[75]. Recent reports demonstrate that IL-17 family of cytokines can be regulated by IL-23,IL-15 and IL-12[71].

Table 1: Role of Innate Immune Molecules in Acute Respiratory Bacterial Infection

TLR	Infection (References)	Phenotype[a]			
		Survival	Neutrophil Influx[b]	Bacterial Burden[c]	Bacterial Dissemination[d]
TLR2	*Acinetobacter baumannii [21]*	↓	↓	ND	ND
	Legionella pneumophila [14-16]	↓	↓	↑	↑
	Pseudomonas aeruginosa [22]	ND	NS	↑early	ND
	Streptococcus pneumoniae[13]	↓	↓	↑	↑
TLR4	*Acinetobacter baumannii [21]*	↓	↓	↑	↑
	Haemophillus influenzae [19]	↓	↓	↑	ND
	Klebsiella pneumoniae [18]	↓	ND	↑	ND
	Pseudomonas aeruginosa [22]	ND	↓late	NS	ND
	Streptococcus pneumoniae [20]	↓	↓	↑	ND
TLR5	*Legionella pneumophila [23]*	ND	↓early	NS	ND
TLR9	*Klebsiella pneumoniae [25]*	↓	NS	↑	↑
	Legionella pneumophila [24]	↓	NS	↑	ND
	Streptococcus pneumoniae [26]	↓	NS	↑	↑
TLR Adaptor					
MyD88	*Escherischia Coli [30]*	↓	↓	ND	ND
	Haemophilus influenzae [31]	ND	ND	↓	ND
	Klebsiella pneumoniae [35]	↓	↓	↓	↓
	Legionella pneumophila [15, 33]	↓	↓	↑	↑
	Pseudomonas aeruginosa [6, 29, 32]	↓	↓	↑	↑
	Staphylococcus aureus [29]	ND	↓	ND	ND
	Streptococcus pneumoniae [28]	↓	↓	↑	↑
TIRAP	*Klebsiella pneumoniae [6, 30]*	↓	↓	↑	↑
	Escherichia coli [6, 30]	ND	↓	↑	ND

Table 1 continued

TLR Adaptor	Infection (References)	Phenotype[a]			
		Survival	Neutrophil Influx[b]	Bacterial Burden[c]	Bacterial Dissemination[d]
TRIF	*Escherichia coli [30]*	↓	↓	↑	↑
	Pseudomonas aeruginosa [34]	ND	↓	↑	ND
	Klebsiella pneumoniae[35]	↓	↓	↑	↑
NLR					
NALP3	*Klebsiella pneumoniae[46]*	↓	↓	ND	ND
IPAF	*Pseudomonas aeruginosa[52]*	NS	ND	↑	↑
Transcription Factor					
NF-κB p50	*Escherichia coli [58, 59]*	↓	↓	NS	ND
NF- κB ReLa	*Streptococcus pneumoniae [76]*	ND	↓	↑	ND
STAT3	*E. coli [63]*	ND	↓	↑	ND
STAT4	*K. pneumoniae [64]*	↓	ND	↑	↑
	Pseudomonas aeruginosa [65]	ND	NS	↑	ND
Cytokine					
IL-23	*Pseudomonas aeruginosa [74]*	ND	↓	NS	NS
	Klebsiella pneumoniae [72]	↓	ND	ND	ND
IL-17	*Klebsiella pneumoniae [77]*	↓	↓	↑	ND
TNFα	*Streptococcus pneumoniae [61]*	↓	↓	↑	ND
IL-1	*Streptococcus pneumoniae [61]*	↓	↓	↑	ND
CXC chemokine receptor					
CXCR2	*Pseudomonas aeruginosa [78]*	↓	↓	ND	ND
	Legionella pneumophila [79]	↓	↓	NS	ND
	Norcadia asteroids [80]	↓	↓	↑	ND
CXC chemokine					
KC $	*Klebsiella pneumoniae [81]*	↓	↓	ND	ND
MIP2 #	*Klebsiella pneumoniae [82]*	↓	↓	↑	↑
	Norcadia asteroids [80]	NS	NS	NS	NS
Lungkine	*Klebsiella pneumoniae[83]*	↓	↓(24h) ** ↑(48h) **	↑	ND

[a]Phenotype was determined mainly by using gene-deficient or mutant mice after infection;
[b]Neutrophil influx was determined in BALF and/or Lung parenchyma;
[c]Bacterial burden was measured as CFUs in the lungs;
[d]Bacterial dissemination was measured as CFUs in blood or spleen;
**Signficant in airspaces but not in lung parenchyma
$ studied with transgenic mice; # Abs Only;ND: Not Determined; NS: No significant difference

Chemokines

Chemokines are cytokines that induce leukocyte infiltration into the site of infection/ inflammation. Chemokines promote the expression of cellular adhesion molecules and extravasation of leukocytes. There are four types of chemokines depending on the presence of cysteine towards the N-terminus (C, CC, CXC and CX3C chemokines) [76]. The members of ELR+CXC chemokines are neutrophil chemoattractants. In humans there are seven chemokines (IL-8; NAP-2; GRO α,β and γ; ENA-78 and GCP-2) that are reported [77]. Among these IL-8 is considered the most potent neutrophil chemoattractant. In mice, keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2, LPS-Induced CXC Chemokine (LIX/CXCL5), and lungkine are the four CXC chemokines associated with neutrophil recruitment to the lungs [78]. However, no rodent homolog of human IL-8 has been identified yet. Keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2, LPS-Induced CXC Chemokine (LIX; CXCL5), and lungkine are the four CXC chemokines described to date in mice[78]. KC and MIP-2 are myeloid cell derived chemokines, whereas lungkine and LIX are secreted by bronchial epithelial cells and type II alveolar epithelial cells respectively. There are two receptors CXCR1 and CXCR2 which bind to CXC chemokines and present in both humans and mice. Between these receptors, CXCR2 binds to all ELR+CXC chemokines and is essential for neutrophil infiltration during bacterial infection, it is involved in the host defence and neutrophil recruitment upon challenge of *P. aeruginosa* [79]*, Nocardia asteroides* [80] and *L. pneumophila* [81]. It has been shown that TLR2 downregulates CXCR2 and impairs neutrophil migration during polymicrobial sepsis [82]. Studies have also shown that KC, and MIP2 play an important role in the neutrophil accumulation during bacterial infection by using blocking peptides[83, 84]. However lungkine is not important for the neutrophil recruitment into the lung parenchyma during pneumonia [85]. Unlike KC and MIP-2, CXCL5, also known as LIX, is a relatively newly reported CXC chemokine/neutrophil chemoattractant. Upregulation of CXCL5 increases neutrophil recruitment during infections with *P.*

aeruginosa, *K. pneumoniae*, *L. pneumophila* and *Bordetella bronchiseptica*. Using TLR2 gene deficient mice, it has been shown that TLR2 regulates the expression of KC and MIP-2 during lipoteichoic acid (LTA) induced lung inflammation [86]. However, TLR4 is important for the regulation of KC and MIP-2 production during LPS induced inflammation, [67, 87]. Furthermore, in flagellin challenged mice, TLR5 is critical for the production of KC and MIp-2[88]. Although LPS-induced inflammation has also shown that LIX is an important molecule for neutrophil influx in the lungs [89], the role of LIX in neutrophil infiltration in the lungs during bacterial infections remains is unclear. Recently a CC chemokine, monocyte chemoattractant protein 1(MCP1) which is primarily a monocyte chemokine is shown to play a role in neutrophil chemotaxis [90].

Concluding remarks

Bacterial lung diseases are an essential public health concern. Neutrophils are one of the first cells to reach the site of bacterial infection in order to clear bacteria. Our understanding of the molecular mechanisms that regulate neutrophil recruitment during infection/inflammation has improved substantially over recent years. Emerging studies indicate complex roles for TLRs and NLRs in neutrophil accumulation during pneumonia. Although antibiotics are the rational treatment for pneumonias, antibiotic-resistant *S. pneumoniae*, *H. influenzae*, and *S. aureus* have been isolated from patients suffering from lower respiratory tract infections. The emergence of antibiotic-resistant pulmonary bacteria and the growing number of immunocompromised individuals have made the treatment of these infections difficult. As most of the TLR studies have been performed in murine models, the efficacy and safety of TLR therapies may not extrapolate to human responses. This is because of 1) differences between the human and murine immune system; 2) differences in the activation profile of human and mouse, such as TLR8, and also because 3) murine investigations are performed on in-bred strains that have minimal genetic variation. Though TLR9 agonists, such as CpG oligodeoxynucleotides have been shown to protect against

numerous infectious agents in murine models, no human clinical studies have been reported, to our knowledge, using TLR9 agonists in bacterial infections. In addition, TLR3, TLR7, TLR8 and TLR9 can be activated upon intracellular bacterial infection, resulting in the production of IFN-α, these receptor agonists can be targeted to control bacterial infections. The studies using TLR adaptor-deficient mice in response to bacterial infection reveal the potential for using cell permeable compounds to attenuate cytokine/chemokine production in order to reduce excessive neutrophil-mediated parenchymal damage. Unlike TLRs, NLRs have recently been identified and therefore, their therapeutic potential to reduce bacteria-mediated neutrophil influx in the lungs remains to be evaluated. The future challenge will be to apply our current understanding of TLRs and NLRs to design therapeutic methods to attenuate uncontrolled neutrophil migration to the lungs in ALI/ARDS patients.

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Chapter 3: Receptor-Interacting Protein 2 Controls Pulmonary Host Defense to *Escherichia coli* Infection via the Regulation of IL-17A**

Introduction

Lower respiratory tract infections cause more deaths than HIV, ischemic heart disease and diarrheal diseases (7, 40). Gram-negative bacterial pathogens are a common cause of nosocomial infections (48). Host defense against microbes is composed of two evolutionarily distinct arms, the innate and adaptive immune systems. Pattern recognition receptors (PRRs) are critical to induce effective innate and/or adaptive immune responses (2). PRRs, such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) can recognize pathogen associated molecular patterns (PAMPs) in microbes. This recognition triggers a cascade of events leading to the activation of transcription factors, production of chemokines/cytokines, up regulation of cell adhesion molecules, phagocytic cell infiltration and subsequent clearance of the microbes. Neutrophils are the primary innate immune cells to migrate towards the site of bacterial infection to augment host defense via complex signaling cascades (1, 14). However, excessive neutrophil recruitment and activation can lead to eventual mortality associated with acute lung injury (ALI) or acute respiratory distress syndrome (ARDS). In this regard, investigation of molecular and cellular mechanisms involved in neutrophil trafficking to the lungs is essential to designing improved therapies.

The adaptor molecule, receptor interacting protein 2 (RIP2), is a caspase recruitment domain (CARD) containing serine/threonine kinase (4, 28). A recent report show that RIP2 also has tyrosine kinase activity (56). Human RIP2 is a 61 kDa protein with 531 amino acids and is located on chromosome 8 (4). The murine homolog has 86% identity at the molecular level and 84% identity at the amino acid level and is located on chromosome 4 (4). RIP2 induces downstream signaling via the CARD domain (28). However, the role of RIP2 in contributes to innate immune mechanisms via both NOD1 and NOD2 signaling, but not through the TLR signaling (45).

****This article was originally published in**
Balamayooran, T., S. Batra, G. Balamayooran, S. Cai, K. S. Kobayashi, R. A. Flavell, and S. Jeyaseelan**. RIP2 Controls Pulmonary Host Defense to *E. coli* Infection via the Regulation of IL-17A.** *Infect. Immun*. November 2011 79:4588-4599
Official Journal of American Society of Microbiology
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Other studies have revealed that RIP2 functions downstream of TLRs to mediate NF-κB and MAPK activation (4, 18). In this context, RIP2 has been shown to be recruited to TLR2, TLR3 and TLR4 but not TLR9 in order to activate NF-κB, p38 MAPK, JNK and ERK (28). Additional studies have shown that lipo- polysaccharide (LPS) mediated TLR4 signaling involves RIP2,although its role in the signaling is not dependent on its kinase activity (32). Studies with pneumococcus have revealed that NOD2 recognition of *S. pneumoniae* involves RIP2 signaling (43).

RIP2 plays an essential role in mucosal immunity to intracellular pulmonary pathogens, such as *Listeria monocytogenes* (12)*, Legionella pneumophila* (3)*, Chlamydophila pneumoniae (53)* and *Mycobacterium tuberculosis (44)*. In another study using intraperitoneal infection with *Pseudomonas aeruginosa* and *E. coli* after LPS prestimulation showed higher inflammation in the peritoneum through NOD1/NOD2 signaling cascades and induced lethality during *Pseudomonas* infection via bacterial dissemination (46). Recent studies show that PGN obtained from gram-negative bacteria can induce NOD1 signaling in lung epithelium which protects mice after *S. pneumoniae* infection (33, 51). Despite these elegant studies, the role of RIP2 in acute extracellular gram-negative bacterial pneumonia has not been explored. Although recent reports suggest that IL-17A plays an essential role against gram-negative pathogens (60), the importance of RIP2 in IL-17A production during acute bacterial pneumonia has not been explored. Therefore, our hypothesis is that RIP2 is critical for the host defense during gram-negative pneumonia by regulating IL-17A production. Additional experimental data demonstrate that RIP2 controls neutrophil mobilization to the blood. Our findings provide new mechanistic insights on the importance of RIP2 in mediating lung inflammation and host defense against a gram-negative bacterial pathogen, *E. coli*.

Materials and Methods

Mice. RIP2$^{-/-}$ mice were generated as described earlier (28). All gene-deficient mice used in our experiments are on C57BL6 background after backcrossing with C57Bl/6 mice at least 10 generations. Because of this, C57BL6 mice were used as the wild-type (WT) controls. Eight to 10 wk-old female mice were used and animal studies were approved by the Louisiana State University Animal Care and Use Committee.

***Intratracheal instillation of bacteria*.** Bacteria were prepared for mouse inoculation as described in our previous reports (25). *E. coli* (ATCC 25922) was grown in trypticase soy broth (TSB) at 37°C overnight under constant agitation. Bacteria were harvested by centrifugation, washed two times in isotonic saline, and resuspended in saline at a concentration of 20 X10^6 CFU/ml. Mice were anesthetized by intraperitoneal (i.p). injection of a mixture containing 120 mg/kg ketamine and 16 mg/kg xylazine. The trachea was surgically exposed, and a total volume of 50 µl of saline containing10^6 CFU/mouse *E. coli* was administered. In control experiments, 50 µl of saline was instilled into the trachea. The initial mouse inoculums were confirmed by plating serial 10-fold dilutions on MacConkey and tryptic soy agar (TSA) plates. For enumerating bacterial colony forming units (CFUs) in the lungs, whole lungs were homogenized in 2 ml sterile saline for 30 s, and 20 µl of the resulting homogenates were plated by serial 10-fold dilutions on MacConkey and TSA plates. Bacterial colonies were counted after incubation at 37^{o}C for 18 to 24 h.

Cell counts. Bronchoalveolar lavage fluid (BALF) was collected to obtain airway cells for enumeration. The trachea was exposed, intubated and instilled with phosphate-buffered saline (PBS) containing heparin and dextrose in 0.8-ml aliquots 4 times. Approximately 3 ml of BALF was retrieved per mouse. Cytospin samples were subsequently prepared from BALF cells and stained with Diff-Quick (Fisher). Total leukocytes in BALF were determined using a hemocytometer whereas leukocyte subsets were examined by direct counting of stained slides based on cell nuclear morphology. Neutrophil recruitment to the lung parenchyma was assessed by myeloperoxidase (MPO) activity as described in our earlier reports (27)

***Lung pathology*.** The murine lungs were perfused from the right ventricle of heart with 10 ml isotonic saline at 24 h post-infection. Lungs were removed and fixed in 4% phosphate-buffered formalin. Fixed tissue samples were processed in paraffin blocks, and fine sections (5 µm in thick) were cut and stained with hematoxylin and eosin (H&E). Assessments of histopathology were performed in blinded fashion by a Veterinary Pathologist according to the following scoring scale: 0, No inflammatory cells (macrophages or neutrophils) present in section; 1, <5% of section is infiltrated by inflammatory cells; 2, 5–10% of section is infiltrated by inflammatory cells; and 3, >10% of section is infiltrated by inflammatory cells.

***Cytokines and chemokines*.** Cell-free BALF was used for the determination of TNF-α (eBioscience), IL-6 (eBioscience), MIP-2 (R&D systems) and LIX (R&D systems) proteins by enzyme-linked immunosorbent assay (ELISA). For the determination of IL-17A and IL-23 (eBioscience), lung homogenates were used. The minimum detection limit is 2 pg/ml of cytokine or chemokine protein.

***Western blotting*.** Tissue proteins were extracted by homogenising the lungs in a lysis buffer cocktail containing 0.1% Triton X-100 in PBS, complete protease and phosphatise inhibitor cocktail (Thermo Scientific, MA), and 1 mM dithiothreitol (DTT), followed by centrifugation at maximum speed using a microcentrifuge at 4^{o}C. The resulting supernatants were used for western blotting. Whole cell proteins were fractioned by 10-12% SDS-PAGE and transferred onto Immobilon-P (Millipore) polyvinylidene difluoride membranes. Membranes were blocked and probed with RIP2, phospho-RIP2, phospho-IKKα/β (Ser176/180), NF-κB, phospho-NF-κB, I$\kappa\beta\alpha$, ICAM-1, VCAM-1, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), phospho-MAPK/JNK (Thr183/Tyr185), STAT3, phospho-STAT3 (Ser727; Thr705), acetyl-STAT3 (Lys685) (1:1,000 dilution). Horseradish peroxidase (HRP)-conjugated appropriate secondary antibodies were used and the ECL enhanced chemiluminescence system was used to detect signals (Amersham, NJ). To demonstrate equal protein loading on gels, the blots were stripped and reprobed with antibody (Ab) specific for total p38 MAPK or GAPDH (glyceraldehyde-3-phosphat

dehydrogenase). The intensity of immunoreactive bands was determined using a Gel Digitizing Software (UN-SCAN-IT gel) from Silk Scientific, Inc.

***NF-κB DNA binding*.** Nuclear proteins were extracted from the lung tissue at 6 and 24 h post-*E. coli* administration as described in our publications (9, 10). A total of 7.5 µg nuclear extract was mixed with binding buffer, added to the precoated plate and incubated for 1 h at room temperature according to manufacturer's protocol (TransAM ELISA kit). Excess Ab was removed with wash buffer and plates were incubated with NF-κB/p65 antibody for 1 h. Plates were washed to remove excess Ab and HRP-conjugated anti-rabbit IgG was added. Plates were incubated for 1 h and subsequently read at 450 nm after adding the color developing reagent.

In-vitro experiments with bone marrow derived macrophages (BMDMs) and dendritic cells (BMDCs). Marrow cells were isolated from bone marrow of RIP2$^{-/-}$, and WT (control) mice and differentiated into macrophages for 7 days by adding macrophage colony –stimulating factor (M-CSF) and into dendritic cells (DCs) by adding IL-4 and M-CSF. A total of 1X10^{6}cells/well were used for each group at each time point for infection with *E. coli* at a multiplicity of infection (MOI) of 0.1 and incubated at 37˚C with slow agitation. At 10, 20, 30, 60 and 360 minutes cell pellets and supernatant were collected and cell pellets of BMDMs were processed for western blot analysis to determine the expression of RIP2 and activation of NF-κB and MAPKs. Supernatants from both BMDMs and BMDCs were used for the determination of cytokine/chemokines.

***Flow cytometry*.** A total of 50 µl of whole blood or perfused lung digest of WT and RIP2 $^{-/-}$ mice challenged with *E .coli* or saline (control) was aliquoted into tubes and Fc blocked. A total of 4 µl of mouse conjugated anti-mouse Gr-1/Ly6G, P-selectin/CD62P, LFA-1 (CD11a/CD18), CD11b and CXCR-2, (R&D Systems, MN) antibodies were added to tubes. Samples were vortexed and incubated for 30 min at room temperature in the dark. Cells were washed by adding 2 ml of 1X PBS and centrifuged at 200 X g for 8 min. Red blood cells (RBCs) were lysed using 2 ml of NH_4Cl lysing buffer to each sample tube, mixed well and incubated at room temperature for 10 min. Then, samples were centrifuged immediately at 200 x g for 8 min and the supernatant was removed. Cells

washed twice with PBS, fixed by adding 200 µl of cold 1% formaldehyde-PBS and stored at 1-6 ^{o}C for flurorescence-activated cell sorting (FACS) analysis.

***Determination of IL-17A producing cells using flow cytometry*:** The procedure of IL-17A producing cells has been reported earlier (47, 50)**.** Lung samples were obtained from both wild-type and RIP2$^{-/-}$ mice after 6 h post *E. coli* (10^6CFUs/animal) infection. They were minced, digested with collagenase and made into a single cell suspension and stimulated with PMA (50ng/ml), ionomycin (750ng) and Golgi stop (7µl/10 ml) for 5 hours. After stimulation, cells were surface stained with markers for IL-17A producing T cells (γδ, NKT and CD4 cells). Following incubation, cells were washed, fixed and permeabilized for intracellular staining. Permeabilized cells were subsequently incubated with IL-17A Ab for intracellular staining. Finally, cells were washed and resuspended for flow cytometry analysis.

***Exogenous IL-17A (rIL-17) Administration*:** RIP2 $^{-/-}$ mice were treated i.t with rIL-17A (1 µg/animal) 1 h after *E. coli* (10^6 CFUs/50µl/animal) and the control mice were treated with an equal volume of PBS. At 24 h post-infection, BALF was collected and processed for cellular enumeration and the determination of CFU.

Statistical analysis: Data were expressed as means $\pm$ SE. Data were analyzed by one-way or two-way ANOVA followed by Bonferroni's post hoc correction for multiple comparisons. Statistical calculations were performed using InStat software and GraphPad Prizm 4.0 (San Diego, CA). Differences were considered statistically significant when *P<0.05 between WT and knockout (KO) mice.

Results

RIP2 expression and activation in the lungs are induced upon E. coli infection

To investigate whether RIP2 expression is altered during *E. coli* pneumonia, WT (C57Bl/6) mice were infected i.t with 10^6 CFUs/animal *E. coli* and lungs were removed at 6 and 24 h post-infection

and processed for western blotting (Fig. 3.1A). Notably, we observed increase in expression and activation of RIP2 upon infection compared to saline challenged controls (Fig. 3.1A). Furthermore, we demonstrate enhanced RIP2 expression and activation in bone marrow-derived macrophages (BMDMs) following *E. coli* challenge (Fig.3.4A).

RIP2 limits bacterial burden in the lungs

To determine whether RIP2 is important for the bacterial clearance in the lungs, $RIP2^{-/-}$ mice and WT controls were instilled i.t. with *E. coli* (10^6 CFUs/mouse) and sacrificed at 6 and 24 h post-infection for bacterial culture. At 24 h after infection, bacterial burden in the lungs was higher in $RIP2^{-/-}$ mice compared to WT controls (Fig. 3.1B). However, there was no bacterial dissemination observed either in $RIP2^{-/-}$ or WT mice with this bacterial dose (data not shown).

RIP2 contributes to neutrophil trafficking to the lungs

Since there was attenuated bacterial clearance in $RIP2^{-/-}$ mice following *E. coli* infection, we determined whether RIP2 deficiency affects neutrophil transmigration to the lungs following infection. Mice were infected with *E. coli* and BALF was collected at designated time-points. At 6 and 24 h post-infection, there was a reduction in total leukocyte and neutrophil counts in the airspaces of $RIP2^{-/-}$ mice (Figs. 3.1C-D). Consistent with this, MPO activity in lung homogenates of $RIP2^{-/-}$ mice was reduced at 6 and 24 h post-infection (Fig. 3.1E). We next examined whether RIP2 deficiency affects pathologic consequences in the lungs during gram-negative bacterial pneumonia. $RIP2^{-/-}$ and WT mice were infected with *E. coli* i.t. at a dose of 10^6/mouse and the lungs were processed for histopthological studies at 24 h after infection. Unlike WT mice, $RIP2^{-/-}$ mice show reduced inflammation as evidenced by attenuated inflammatory cell accumulation and alveolar edema when compared to WT controls (Fig. 3.1F).

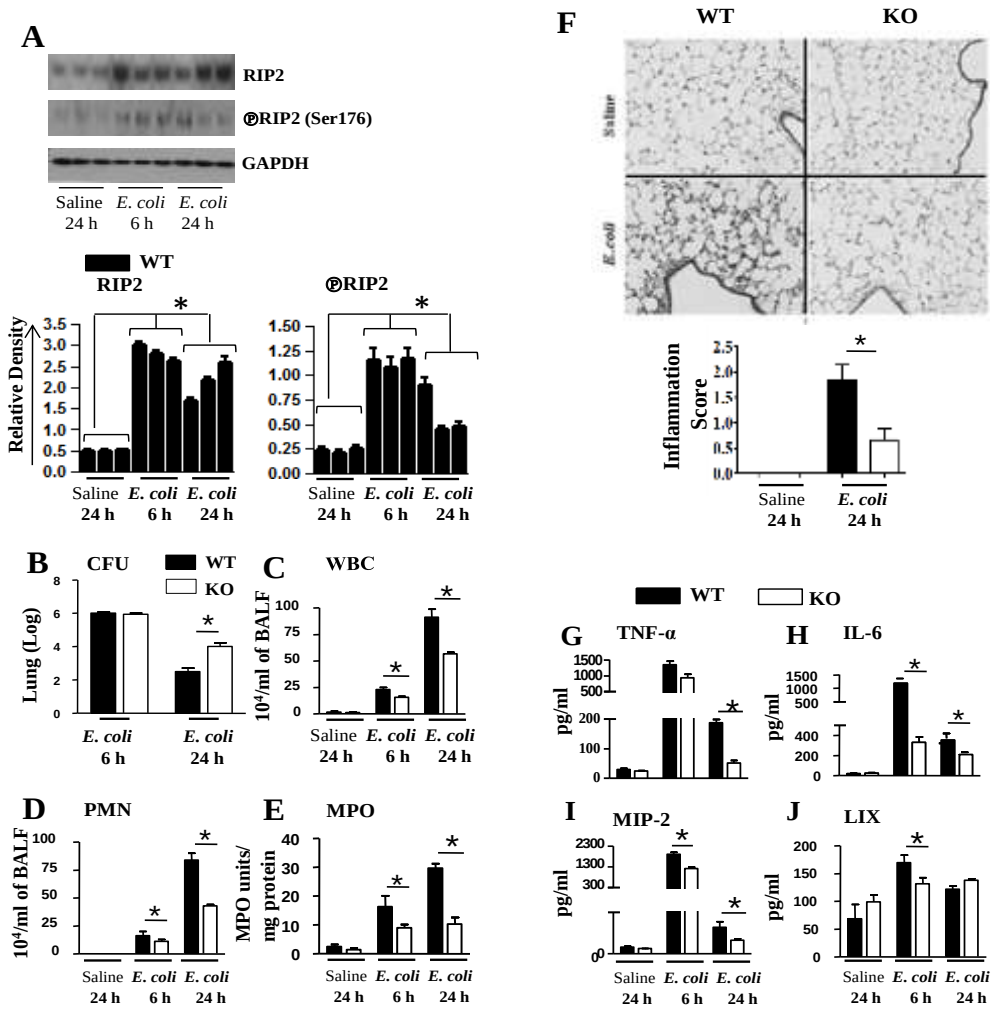

Figure 3.1. Importance of RIP2 in host defense during gram-negative pneumonia. A. RIP2 expression in C57Bl/6 (WT) mice at 6 and 24 h post-*E. coli* infection. WT mice were infected i.t with *E. coli* and lung samples were processed for western blotting using Abs against activated or regular form of RIP2. This is a representative blot from 2 separate experiments. *n*= 3 mice in each group in each experiment; *, *P*<0.05 between infected and saline challenge mice. **B.** Impaired bacterial clearance in the lungs in RIP2$^{-/-}$ mice. Lung homogenates at 6 and 24 h after infection were used to enumerate the bacterial colony forming units (n=5-6 mice/group/time-point). *indicates significant differences between WT and KO mice (p<0.05). **C-D**. Attenuated neutrophil recruitment to the lungs of RIP2$^{-/-}$ mice following *E. coli* infection. Mice were inoculated with *E. coli* (10^6 CFU/mouse), BALF was obtained at 6 and 24 h post-infection, and cell (total and differential) enumeration was performed to determine cellular recruitment to the lung (n=5-6 mice/group/time-point; p<0.05). **E.** Reduced MPO in the lungs of RIP2$^{-/-}$ mice after *E. coli* infection. Lung MPO was determined in lung homogenates (n=4-5 mice/group/time-point; p<0.05). **F**. Attenuated lung histology in RIP2$^{-/-}$ mice following *E. coli* inoculation. Mice were inoculated with *E. coli* (10^6 CFU/mouse), lungs were obtained at 24 h post-infection, stained with H&E and inflammation scoring in histological sections was performed by a veterinary pathologist. These are representative sections of 6 mice in each condition with identical results (Magnification ×200). **G-J.** Impaired cytokine and chemokine responses in the airspaces following *E. coli* infection. BALF was collected from the lungs after i.t. instillation of *E. coli* (10^6 CFU/mouse) at designated time points. TNF-α (G), IL-6 (H), MIP-2 (I) and LIX (J) levels in BALF were quantified by sandwich ELISA. Asterisks indicate significant difference between WT and KO mice (p<0.05; n = 5-7 mice/group/time-point).

***RIP2$^{-/-}$ mice show decreased expression of proinflammatory cytokines and chemokines in the lung*.** Neutrophil recruitment to the infected site is mediated by cytokines and chemokines. Therefore, the next step was to determine whether RIP2 has a dominant role in the regulation of cytokine and chemokine expression. Sandwich ELISA was used to determine the levels of cytokines (TNF-α and IL-6) and chemokines (MIP-2 and LIX) in BALF. Figure 1 shows reduced levels of TNF-α and IL-6 in RIP2$^{-/-}$ mice compared to their controls post-infection (G-J). The potent neutrophil chemokine, MIP-2, level is reduced in BALF of RIP2$^{-/-}$ mice at 24 h post-infection although the LIX levels were reduced only at 6 h (Figs.3.1G-J). These findings show that RIP2 regulates the expression of proinflammatory cytokines and chemokines.

IL-17A is a cytokine mainly produced by Th17 cells and has been implicated to be critical in the production of cytokines and neutrophil chemokines, such as IL-1β, granulocyte colony-stimulating factor (G-CSF) and MIP-2 (60). Since cytokine/chemokine production can be mediated through IL-17A, we determined IL-17A levels in lung homogenates of RIP2$^{-/-}$ mice upon *E. coli* infection. IL-17A levels were substantially reduced in the lungs of RIP2$^{-/-}$ mice (Fig. 3.2A). Since IL-17A production can be induced by IL-23 dependent or independent signaling cascades (22, 24), IL-23 levels were determined in the lungs after bacterial infection. RIP2$^{-/-}$ show attenuated levels of IL-23 in the lungs compared to WT lungs following infection (Fig. 3.2B). These results indicate that RIP2 regulates the IL-23/IL-17A axis.

RIP2 deficiency reduces STAT-3 activation in the lung

STAT-3 activation and IL-6 production are two critical events for the production of IL-17A (59). Recent studies have shown that IL-6 regulates the activation of STAT-3 in the lungs after *E. coli* infection (49). In this context, we found reduced IL-6 expression and phosphorylated STAT-3 levels in the lungs of RIP2$^{-/-}$ mice following *E. coli* infection (Fig. 3.2C-D). Our results correlate with earlier studies which demonstrate that serine and tyrosine phosphorylation as well as acetylation of STAT3 is critical for the development of Th17 cells (19, 34). Our results also indicate that RIP2 is important

for the activation of STAT3 by tyrosine phosphorylation and acetylation but not serine phosphorylation of STAT3 (Fig. 3.2C-D).

RIP2 deficiency impairs activation of NF-κB and MAPKs and expression of cellular adhesion molecules in the lung

Expression of proinflammatory mediators is regulated by a number of transcription factors in which NF-κB is well characterized in the context of infection and inflammation. RIP2 activates numerous transcription factors, including NF-κB that contributes to immune and inflammatory responses (36). Therefore, we examined the activation of NF-κB in lung homogenates of RIP2$^{-/-}$ and WT mice by NF-κB DNA binding assay and western blotting. We found that NF-κB activation was reduced in the lungs of RIP2$^{-/-}$ mice at 6 and 24 h following bacterial infection (Figs. 3.3A-C).

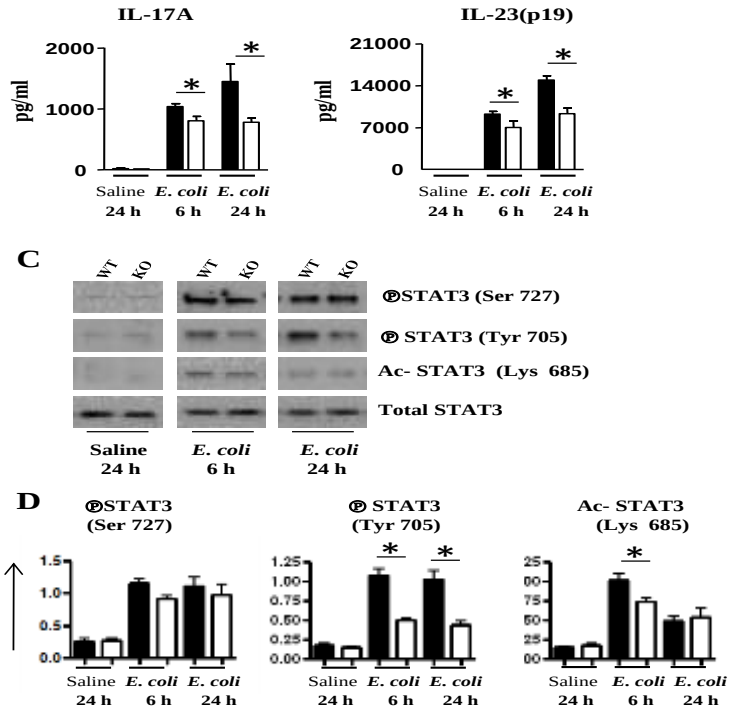

Figure 3.2. Production of IL-17A and IL-23 and activation of STAT3 in the lungs following *E. coli* infection. A-B. Reduced IL-17A and IL-23 levels in lung paranchyma of RIP2$^{-/-}$ mice following infection. Whole lung homogenates of WT and RIP2$^{-/-}$ mice at 6 and 24 h post *E. coli* infection were used to determine the IL-17A and IL-23 levels by ELISA (n=5-7 mice/group/time-point). **C.** Decreased STAT-3 activation in the lungs of RIP2$^{-/-}$ mice following *E. coli* infection. STAT-3 activation was determined by western blotting of lung homogenates obtained from the lungs of RIP2$^{-/-}$ and WT mice after *E. coli* infection. (n=4-6/group/time-point). **D.** Densitometric analysis of expression of phospho-STAT-3 (ser and thr) and acetyl-STAT-3 using blots from 3 separate experiments. The data obtained were normalized against total STAT-3 and expressed as mean ± SE. *denotes the differences between WT and KO mice ($p<0.05$).

Activation of MAPKs is involved in the expression of cytokines/chemokines. Activation of p38, ERK and JNK in RIP2$^{-/-}$ and WT mice upon *E. coli* challenge was determined by western blot analysis. There was reduced activation of p38 and JNK observed at 6 h in the *E. coli* infected lungs of RIP2$^{-/-}$ compared to WT mice (Figs. 3.3A-C). These observations exhibit an important role for RIP2 in the activation of p38 and JNK but not ERK.

Cytokines and chemokines produced in response to infection promote up regulation of cellular adhesion molecules on resident cells, such as endothelium and myeloid cells, such as leukocytes (14). To investigate whether RIP2 deficiency decreases the expression of the cellular adhesion molecules, *E. coli* infected lungs were processed for western blotting. ICAM-1 and VCAM-1 expression was attenuated in the lungs of RIP2$^{-/-}$ mice compared to WT mice after infection (Figs. 3A-C).

RIP2 deficient BMDMs show reduced activation of NF-κB and MAPKs and IL-23 and IL-6 levels following E. coli infection

In order to further validate our *in-vivo* findings, we utilized bone marrow-derived macrophages (BMDM) to determine the activation of transcription factors, including NF-κB, MAPKs and the expression of cytokines (IL-6 and IL-23). BMDMs obtained from RIP2$^{-/-}$ mice demonstrate decreased activation of NF-κB and MAPKs (Fig.3.4C-D), production of IL-6 and IL-23 (Fig.3.4E) and expression of ICAM-1 when BMDMs are infected with 0.1 MOI of *E. coli*.

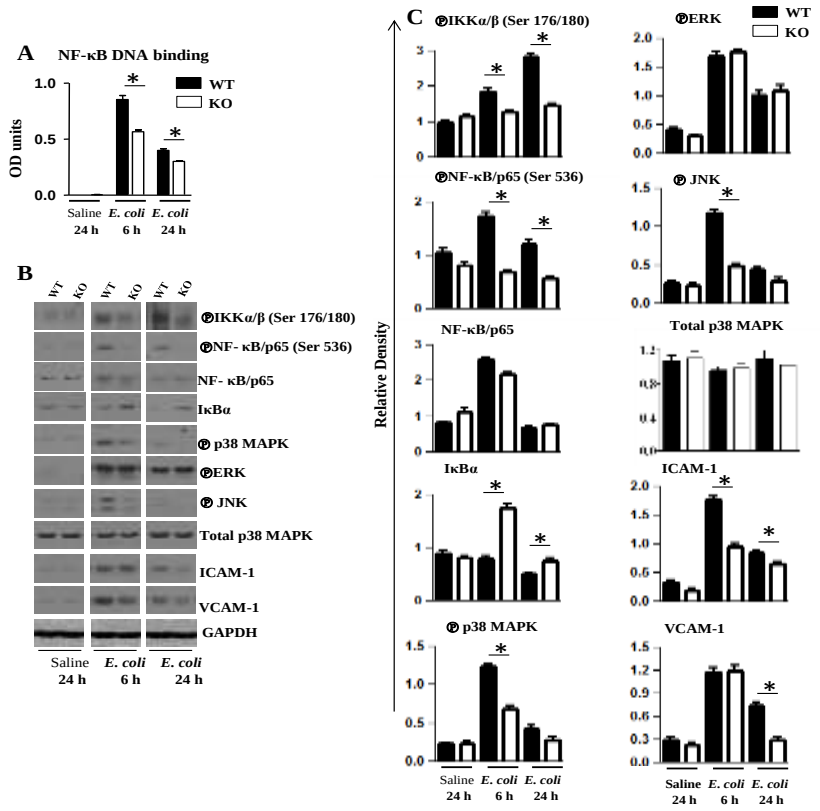

Figure 3.3. **Activation of NF-κB and MAPKs and upregulation of ICAM-1 and VCAM-1 in the lungs during *E. coli* infection**. A-B. Attenuated NF-κB activation in the lungs of RIP2$^{-/-}$ mice following infection. Whole lung homogenates of RIP2$^{-/-}$ and WT mice at 6 and 24 h after *E coli* infection were used for NF-κB binding assay and the proteins were run on a SDS-PAGE gel and membranes were blotted with appropriate Abs to determine activation of NF-κB and MAPKs as well as expression of ICAM-1 and VCAM-1 (B). This blot is representative of 3 independent experiments. **C.** Densitometric analysis of NF-κB and MAPK activation as well as ICAM-1 and VCAM-1 expression. Densitometry was performed from 3 separate blots/experiments. The results obtained were normalized against GAPDH and expressed as mean ± SE. *denotes the differences between WT and KO mice (*p<0.05).

RIP2$^{-/-}$ mice show reduced IL-6 and IL-23 production by dendritic cells post-E. coli infection

Since IL-23 has been shown to be primarily produced by dendritic cells as well (30, 58), we determined whether RIP2 has any role in IL-23 production during *E. coli* infection in dendritic cells. Using BMDCs, we observed increased IL-6 and IL-23 production by BMDCs following infection and these cytokines levels were significantly reduced in RIP2$^{-/-}$ DCs, demonstrating a role for RIP2 in IL-6 and IL-23 production during *E. coli* infection (Fig. 3.4F).

RIP2 deficiency decreases IL-17A producing cells in the lungs in response to E. coli infection

Although we observed reduced IL-17A levels in the lungs of RIP2$^{-/-}$ mice following *E. coli* infection, the T cell subsets which produce IL-17A in the lung during infection were not determined. In order to

find out the T cell types are involved in RIP2 mediated IL-17A production, we performed flow cytometry using intracellular and surface staining. Interestingly, we found that natural killer cells (NK) and gamma delta (γδ) cells were the predominant sources of IL-17A production in the lungs at 6 h following *E. coli* infection (Fig. 3.5A-B). Furthermore, the IL-17A producing cell numbers were significantly lower in RIP2$^{-/-}$ mice following infection (Fig. 3.5A-B).

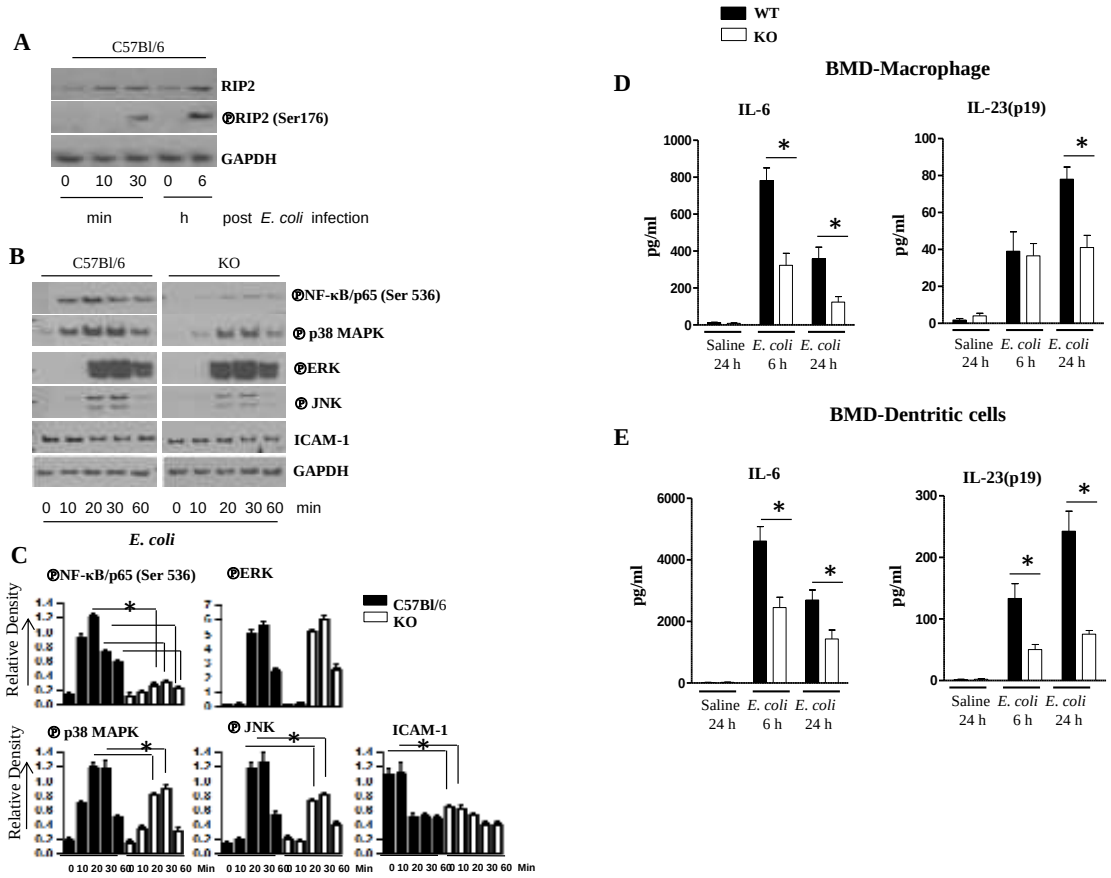

Figure 3.4. RIP2 expression and phosphorylation, NF-κB and MAPK activation, ICAM-1 expression and IL-6 and IL-23 production in macrophages and dendritic cells. A-B. Decreased RIP2 expression and phosphorylation in bone marrow-derived macrophages (BMDMs) upon *E. coli* infection. **C.** Reduced activation of NF-κB and MAPKs in BMDMs obtained from RIP2 $^{-/-}$ mice following infection with *E. coli*. Representative Western blot from 3 separate experiments were shown. **D.** Densitometric analysis of NF-κB and MAPK activation in BMDMs of WT and RIP2 $^{-/-}$ mice. Relative densities normalized against GAPDH are representative of 3 independent experiments. **E-F.** Attenuated IL-6 and IL-23 levels in culture supernatants obtained from BMDMs (E) and BMDCs (F) of and RIP2$^{-/-}$ mice following *E. coli* infection. Experiments were performed in triplicate wells. For experiments A-F, 5-6 mice/group was used; *denotes the differences between WT and RIP2 $^{-/-}$ mice ($p<0.05$).

RIP2 is essential for neutrophil mobilization during E. coli infection

A large pool of mature neutrophils is stored in the bone marrow. These neutrophils can rapidly be mobilized to the blood during infection and/or inflammation, resulting in substantial increase in circulating neutrophil numbers (60). Since we observed reduced neutrophil recruitment in the lungs of RIP2$^{-/-}$ mice during *E. coli* infection, this could also be due to attenuated release of neutrophils to the blood from the marrow. To investigate this, we examined the numbers of neutrophils in the blood using flow cytometry. Although neutrophil numbers in the blood remain the same in RIP2$^{-/-}$ and WT mice following saline challenge, the mobilization/release of neutrophils to the blood was impaired in RIP2$^{-/-}$ mice after *E. coli* infection (Figs 3.6A-B).

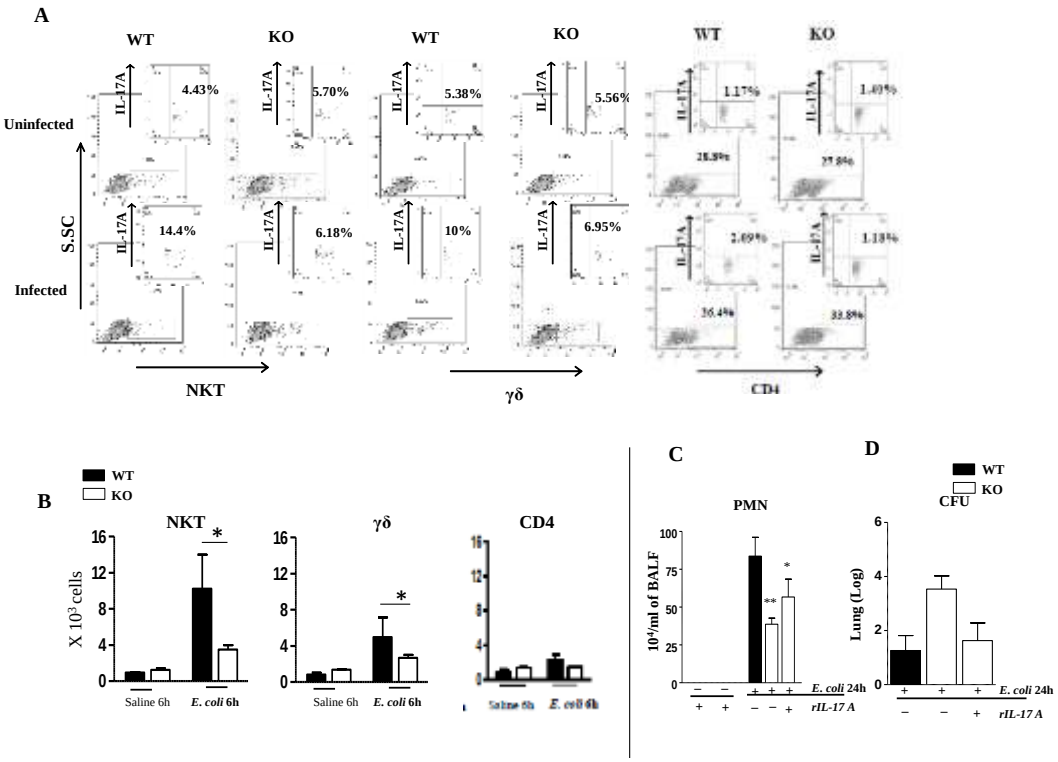

Figure 3.5. RIP2 regulates neutrophil accumulation and bacterial clearance in the lungs during *E. coli* infection. **A-B.** Reduced IL-17A producing T cells (NKT γδ cells and CD4) in the lungs of RIP2$^{-/-}$ mice upon *E. coli* infection. Flow cytometric analysis was performed in cells obtained from whole lung homogenates as described in *Materials and Methods*. **C-D**. Enhanced neutrophil recruitment in airspaces **(C)** and bacterial clearance **(D)** following exogenous administration of rIL-17A in *E. coli*- infected RIP2 $^{-/-}$ mice. Mice were infected with 10^6 CFUs/mouse i.t. and administered rIL-17A 1 h later, and BALF was collected at 24 h post-infection. For experiments A-E, a total of 7-9 mice/group was used. *, $p<0.05$ compared to CXCL5$^{-/-}$ mice.

RIP2 deficiency does not affect the expression of P selectin/CD62P, LFA-1, CD11b and CXCR2 expression on lung and blood neutrophils during E. coli pneumonia

Neutrophil migration is a multistep process which is associated with the expression of cellular adhesion molecules expressed on resident and myeloid cells, such as neutrophils. To determine whether attenuated neutrophil accumulation in the lungs of RIP2$^{-/-}$ mice was due to decreased expression of cell adhesion molecules on neutrophils, we determined CD62P, LFA-1 (CD11a/CD18), CD11b, and CXCR2 on neutrophils obtained from blood and lungs following *E. coli* infection. We did not observe substantial neutrophils in the lungs after digestion (data not shown). We did not see differences in the expression of these cellular adhesion molecules on neutrophils between RIP2$^{-/-}$ and WT mice (Figs.3.6C-F). These observations exclude the possibility that expression of CD62P, CD11b, LFA-1 and CXCR2 on neutrophils as a mechanism for attenuated neutrophil recruitment to the lungs during *E. coli* infection (Figs. 3.6C-F).

Exogenous IL-17A administration restores neutrophil numbers and improves bacterial clearance in RIP2 $^{-/-}$ mice following E. coli infection.

Since we found reduced IL-17A levels associated with lower neutrophil numbers and impaired bacterial clearance in lungs of RIP2 $^{-/-}$ mice following *E. coli* infection, we determined whether administration of exogenous recombinant IL-17A rescues the neutrophil numbers and bacterial clearance in RIP2$^{-/-}$ mice. We treated RIP2$^{-/-}$ i.t. with rIL-17A 1 h post-*E. coli* infection and determined neutrophil numbers and lung CFUs. We observed increased neutrophil numbers in the BALF of RIP2$^{-/-}$ mice following rIL-17A treatment, compared to RIP2$^{-/-}$ mice upon *E. coli* infection (Fig.3.5C). Moreover rIL-17A treatment restored bacterial clearance by RIP2$^{-/-}$ mice following infection (Fig. 3.5D).

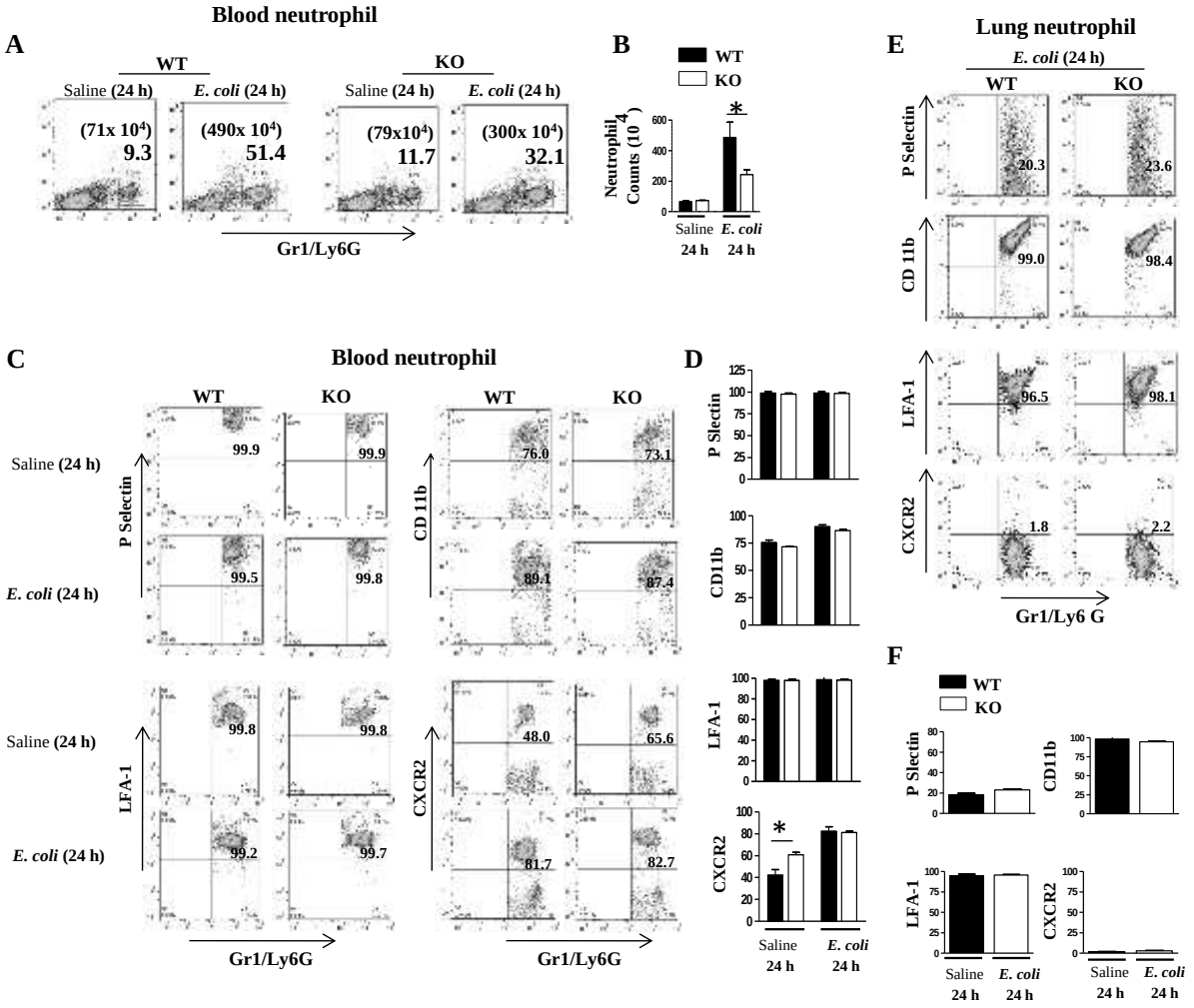

Figure 3.6. RIP2$^{-/-}$ show reduced neutrophil counts in the blood following *E. coli* infection. A-B. Flow cytometric analysis was performed in blood obtained from WT and RIP2 $^{-/-}$ mice at 24 h after i.t *E. coli* (1X10^6 CFU/mouse) infection. Blood cells were stained with antibodies against Gr-1/Ly6G. A total of three mice/group/time-point were used in each of 3 independent experiments ($p<0.05$). *denotes significance between WT and KO mice. **C-F.** Expression of P selectin/CD62L, LFA-1, CD11b and CXCR2 by flow cytometric analysis of blood and lung neutrophils obtained from WT and KO mice at 24 h after i.t *E. coli* (1X10^6 CFU/mouse) infection. Neutrophil population was gated and analyzed for the expression of each adhesion molecule. Data shown here is a representation of 3 individual experiments where n=3 mice/group/time-point and $p<0.05$.

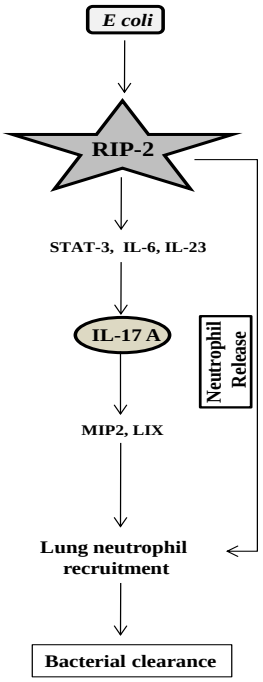

Figure 3.7. Schematic showing the role of RIP2 in neutrophil recruitment and neutrophil release during *E. coli* infection. *E. coli* infection induces IL-17A production through RIP2 mediated STAT-3 activation and IL-6 and IL-23 production. IL-17A in turn regulates the production of CXC chemokines (MIP-2 and LIX) and neutrophil release resulting in neutrophil recruitment and subsequent bacterial clearance in the lungs. RIP2 also plays an important role in neutrophil release from the marrow during pulmonary infection.

Discussion

The immune system has complex mechanisms by which it is capable to sense and respond to microbes. Pattern recognition receptors, such as TLRs and NLRs contribute to multifaceted innate immune response (2, 16). However, the signaling cascades induced by interaction of microbes with these receptors are complex. Although numerous studies from our laboratory (10, 26, 27) and others (8, 41) have explored the TLR-mediated signaling events in detail, limited studies have delineated the role of NLRs in the context of pulmonary bacterial infections. In particular, the role of NLRs in gram-negative extracellular pathogens has not been investigated. Although IL-17A is critical for the host defense against gram-negative bacteria (60), whether RIP2 can regulate IL-17A in the lung during acute bacterial pneumonia is not clear. In this regard, we determined the importance of RIP2 in an experimental model of acute bacterial pneumonia using *E. coli*. Our observations identify roles of RIP2 signaling in neutrophil accumulation in the lungs initiating innate immunity against Gram-negative pneumonia. First, in the absence of RIP2 signaling, lung infection

with *E. coli* showed decreased neutrophil accumulation in the lungs. Second, RIP2 also causes innate host defense against this pathogen via IL-23 and IL-17A production.

The RIP family members have been identified as a group of kinases that can induce NF-κB activation (36). RIP2's tissue distribution is broad and it is highly expressed at the mRNA level in numerous tissues, such as placenta, kidney, pancreas and brain (36). RIP2 mRNA was up regulated upon activation of murine macrophages by LPS (12, 28). Our data show that *E. coli* infection regulates the expression and activation of RIP2 in the lungs and in macrophages. These data are in agreement with previous reports showing that TLR ligands, such as LPS, lipoteichoic acid (LTA) and peptidoglycan (PGN) stimulation in mice increases the expression of RIP2 (12, 28) although RIP2 activation was not determined by earlier studies. Subsequent studies (28) have demonstrated that RIP2 deficiency impairs both TLR and NLR mediated signalling although, Park *et al*. have shown that RIP2 is involved in NOD1 and NOD2 signaling but not TLR signaling (45).

It has been well documented that bone marrow and resident cells in the lung produce neutrophil chemoattractants (15). In this regard, hematopoietic cells produce neutrophil chemoattractants, including KC and MIP-2, whereas the resident cells produce the neutrophil chemoattractants, LIX and lungkine. We reported that KC produced by both myeloid and resident cells are important for neutrophil-dependent inflammation during *Klebsiella pneumoniae* pneumonia (9). However, the expression of pattern recognition receptors and their adaptors on these cell types is not well documented. We demonstrated in our earlier studies that MD-2 in both cell types is important for neutrophil-mediated inflammation in the lungs. Additional investigations have shown that MyD88 derived from hematopoietic cells is more important for LPS-induced bronchial constriction and expression of TNF-α and IL-12p40 (42), whereas both hematopoietic and resident cell–derived MyD88 are important for LPS-induced neutrophil influx (49, 54). Shimada *et al*. (53) have reported that hematopoietic cell-derived RIP2 is important for the clearance of *C. pneumophila* in the lungs although the role of these cells in neutrophil sequestration to the lungs was not examined (53). Recent studies have shown that lung epithelium also plays a central role in protecting the host against bacterial challenge (31). In particular, it has been shown that Gram-

negative bacterial PGN stimulates NOD1 signaling in lung epithelium and cause resistance to *S. pneumoniae* infection (33, 51). The role of these cell type-derived RIP2 in neutrophil recruitment to the lungs during acute bacterial pneumonia caused by extracellular Gram-negative pathogens need to be determined by future investigations.

The initial crucial mechanism that supports mucosal host defense in the lung against pathogens is successful recruitment of neutrophils from the bloodstream (57). Neutrophil accumulation within capillaries and migration into lung parenchyma and subsequently to the alveolar spaces during lung infection is a multistep process that involves shape/size changes of neutrophils, retention in capillaries, adhesion to endothelium, and transmigration into the alveolus (6, 57). In this regard, using neutrophil depletion, we previously reported that neutrophils are critical to control bacterial clearance in the lungs following *E. coli* infection (5) . In our study, however, we found reduced neutrophil recruitment, cytokine/chemokine production and VCAM-1 and ICAM-1 expression(14). Neutrophils bind to ICAM-1, E-selectin, and VCAM-1 expressed on endothelium. In particular, ICAM-1 and VCAM-1 are not constitutively expressed on endothelium but these cell adhesion molecules can be up regulated by LPS and other inflammatory mediators, such as TNF-α (14, 57). The reduction of TNF-α in the lungs at both early (6 h) and late (24 h) time-points in response to *E. coli* infection suggest that TNF-α may have caused this upregulation in addition to LPS. Although neutrophil recruitment to the site of infection depends on the expression of cellular adhesion molecules, such as P-selectin, LFA-1, CD11b and CXCR2 on neutrophils, we did not see changes of these molecules on neutrophils between $RIP2^{-/-}$ and WT mice after infection. These findings indicate that cellular adhesion molecule expression on resident or other myeloid cells to play an important role for neutrophil recruitment to the lungs during *E. coli* infection.

IL-17A has been shown to be critical for the neutrophil mediated host defense during Gram-negative bacterial infection (22, 60). The primary T cells which produce IL-17A during bacterial infection have been recognized as $\gamma\delta$, NK and CD4+ cells (17, 47). IL-17A cell differentiation and recruitment to tissues are critical events to regulate the inflammation via Th17 cells (60). Although

there are reports on the role of RIP2 in Th1/Th2 differentiation, the role of RIP2 in Th17 differentiation is not well understood (12, 21, 28). Although IL-17A has a plethora of proinflammatory functions, the key function of IL-17A is to stimulate the production of proinflammatory cytokines and chemokines, such as IL-1β, G-CSF and MIP-2 (60). It is also clear that G-CSF produced by IL-17A is important for neutrophil release from the marrow (60) and *E. coli* infection induces IL-17A mediated neutrophil recruitment (52). Our findings clearly show reduced IL-17A production along with lower numbers of IL-17A producing cells (NK and γδ) in the lungs of RIP2 $^{-/-}$ mice following *E. coli* infection. Additional experiments showed that exogenous administration of RIP2 $^{-/-}$ mice with rIL-17A partially rescued neutrophil mediated host defense. Several possibilities could be attributed to these findings: 1) more IL-17A may be needed for complete rescue; and 2) RIP2 may have an IL-17A independent role in neutrophil mediated host defense. Further studies are warranted to examine these possibilities. Our findings also demonstrate that RIP2 indeed regulates IL-17A production and neutrophil influx in the lungs via MIP-2 and/or LIX production.

Th17 cell development involves the activation of transcription factor STAT-3 which can be regulated through IL-6 (13, 35) and IL-23 (29, 39). Stat3 is a latent cytoplasmic transcription factor which migrates to the nucleus and exerts it's transcriptional activity upon stimulation (29, 39). STAT3 can be activated via phopshorylation of serine 727 and tyrosine 705 residues as well acetylation of lys 685 residue (11, 55). Our data support STAT tyrosine phosphorylation and acetylation in the lungs following *E. coli* infection. IL-17A can be produced in an IL-23 dependent and independent manner (22, 24). Consistent with this, our results show that RIP2$^{-/-}$ mice exhibit reduced IL-6 along with attenuated levels of IL-23, IL-17A and STAT-3 activation in the lungs following *E. coli* infection. We found that BMDMs and BMDCs produce substantial IL-23 in response to *E. coli* infection although BMDCs produce more IL-23 than macrophages. These results are in agreement with the report demonstrating that both BMDMs and BMDCs can produce IL-23 in response to *Streptococcus pneumoniae* infection (58). The results validate previous observations that IL-17A production is regulated through the IL-6-IL-23 axis in other pathological settings (49). Since we found reduced neutrophil numbers in the blood of RIP2$^{-/-}$ mice and IL-17A levels in the

lungs following *E. coli* infection, these observations suggest that RIP2-dependent IL-17A signaling contributes to neutrophil release to the blood

Furthermore, one key question arising from our studies is that how NLR mediated signaling is activated upon *E. coli* infection. The majority of NOD proteins have specific domains composed of leucine rich repeats (LRRs) for ligand binding (6). Several diverse bacterial NOD1 and NOD2 ligands can activate the RIP2 signaling cascades, including peptidoglycan, a component of Gram-negative bacterial cell wall and muramyldipeptide (MDP), a structure found in all bacteria (6, 20, 23, 38). Although the mechanisms involved in the recognition of extracellular pathogen by an intracellular receptor (NOD2) is not clear, at least three different mechanism could be attributed to the recognition of pathogens and/or their components, such as 1) bacterial MDP can be released to the cytosol and stimulate the intracellular receptors; 2) *E. coli* and/or their product can be leaked into the cytosol from the phagosomes; and 3) a cross-talk between TLRs and NLRs after initial bacterial recognition by TLRs. Future studies are required to identify the exact mechanisms of host recognition of extracellular pathogens by NLRs.

In conclusion, our data reveal the complex nature of the innate immune system to recognize extracellular pathogens. Based on our findings, we propose that *E. coli* activation of RIP2 leads to IL-17A production via IL-6, IL-23 and STAT-3 activation. In turn, IL-17A stimulates the production of CXC chemokines (MIP-2 and LIX) as well as neutrophil release to cause neutrophil mediated host defense against this bacterial pathogen (Figure 5). Although we did not investigate the role of RIP2 in the induction of adaptive immune responses, recent studies have shown such a role for RIP2. For example, RIP2 has a central role in T cell mediated acquired responses against bacterial pathogens (4, 12, 28). Furthermore, RIP2 deficient T cells have severely reduced NF-κB activation, IL-2 production, proliferation and differentiation (12, 28).

Observations from the current study have both clinical and therapeutic potential in terms of novel therapeutic targets to modulate host defense during bacterial infections. In this regard, targeting a single adaptor molecule, such as RIP2 to augment the host defense could be a viable

strategy. Since RIP2 is implicated in several other diseases, including graft rejection, Crohn's disease, collagen-induced arthritis and type I diabetes (4, 37), RIP2 can be used as a potential therapeutic target to augment the host defense during bacterial infections of the lungs.

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Chapter 4: NOD2 Signaling Contributes to Host Defense in the Lungs against *Escherichia coli* Infection***

Introduction

Gram-negative bacterial pneumonia is a leading cause of mortality in the United States and worldwide (10, 47). Neutrophil recruitment to tissues is essential for effective innate defense against bacteria (1, 16). Neutrophil trafficking to the lungs is a sequentially regulated network of signals beginning with the recognition of microbes (1, 16). The pattern recognition receptors, such as toll-like receptors (TLRs) and NOD-like receptors (NLRs) detect pathogen associated molecular patterns (PAMPS) present on bacteria and initiate cellular signaling cascades in the host leading to bacterial clearance (1). NOD2 (caspase-1 recruitment domain [CARD] 15) is among the NLRs that detect muramyl dipeptide (MDP), a conserved glycoprotein of bacterial cell wall (8, 40). NOD2 has a C-terminal leucine rich repeat (LRR) that interacts with microbial components, central ATP-dependant oligomerization domain and two CARDs at the N-terminus to transduce intracellular signals (8, 40). NOD2 is a cytosolic receptor expressed mainly in leukocytes, dendritic cells and epithelial cells (14, 30) although the relationship between NOD2 mediated signaling and host defense against pulmonary extracellular bacterial infection has not been elucidated.

When ligands interact with NOD2, it gets oligomerized and binds to RIP2 kinase (aka RICK or CARDIAK) by CARD-CARD interaction which activates a signaling cascade that leads to NF-κB activation and cytokine/chemokine transcription (8, 40). Interestingly, an earlier report shows that NOD2 activation promotes membrane recruitment of RIP2 and the NOD2/RIP2 complex signals from the plasma membrane (36). Subsequent studies demonstrate that NOD2 activation enhances TLR 2, 3 and 4 mediated signaling (44, 52, 63).

In a murine model of uveitis, it has been shown that NOD2 regulates the production of IFN-γ (59).

*****This article was originally published in**
Balamayooran Theivanthiran, Sanjay Batra, Gayathriy Balamayooran, Shanshan Cai, Koichi Kobayashi, Richard A. Flavell, and Samithamby Jeyaseelan. **NOD2 Signaling Contributes to Host Defense in the Lungs against Escherichia coli Infection**
Infect. Immun. July 2012 80:2558-2569
Official Journal of American Society of Microbiology
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Tak-1, a member of MAPK family, involved in TNF-α, IL-1β and TLR signaling, has been shown to be a central mediator of NOD2 signaling in epidermal cells (32). Furthermore, NOD2 has been shown to be responsible for IL-6 production and activation of STAT-3 (15, 27). In addition, the cytokine IL-32 enhanced NOD2 mediated cytokine response upon MDP challenge (51).

Prior studies demonstrated that NOD2 is an essential mediator of host defense against a variety of intracellular pathogens such as *Listeria monocytogenes* (56), *Mycobacterium tuberculosis (23, 54),*and *Legionella pneumophila* (9). In addition, it has been demonstrated that murine macrophages require NOD2 to respond against *Staphylococcus aureus* infection (31). $NOD2^{-/-}$ mice showed enhanced susceptibility to *Staphylococcus aureus* infection due to increased bacterial burden and defective neutrophil phagocytosis. In an aerosolized infection with *Legionella pneumophila*, $NOD2^{-/-}$ mice display reduced neutrophil recruitment, cytokine production and increased bacterial burden when compared to controls (22). Recent work revealed that NOD2/RIP2 signaling is essential for survival and clearance of *Chlamydophila pneumoniae* from the lungs (62). Nevertheless, much less is known about NOD2 in acute pneumonia caused by extracellular Gram-negative pathogen, such as *E .coli*.

In this study, we explored the role of NOD2 in neutrophil mediated host defense against pulmonary *E. coli* infection. We found enhanced bacterial burden and reduced neutrophil influx, cytokine response, NF-κB and MAPK activation in the lungs of $NOD2^{-/-}$ mice compared to wild-type mice upon *E. coli* infection. Furthermore, we found that $NOD2^{-/-}$ neutrophils show defective phagocytosis and bacterial killing along with reduced elastase, H_2O_2, MPO and nitric oxide (NO) production as well as expression and activation of NADPH oxidase subunits. As compared with $NOD2^{-/-}$ mice, $NOD2/RIP2^{-/-}$ mice exhibit similar bacterial burden and attenuated neutrophil accumulation in the lungs following *E. coli* infection. As compared with $NOD2^{-/-}$ macrophages, macrophages obtained from $NOD2/RIP2^{-}$

$^{/-}$ mice indicate similarly attenuated activation as evidenced by reduced NF-κB and MAPKs following *E. coli* infection.

Materials and Methods

Mice: NOD2$^{-/-}$ and RIP2$^{-/-}$ mice used in the experiment were generated on a C57BL6 background (33, 34); therefore C57BL/6 mice were used as wild-type controls. NOD2/RIP2 mice were generated by interbreeding mice containing these targeted mutations. Eight to 10 wk-old female mice were used in all experiments. Animal studies were approved by the Louisiana State University Animal Care and Use Committee. The mice were ranging from 19 to 25 g in weight at the time of experiments.

***Intratracheal instillations*:** Mice were anesthetized by intraperitoneal (i.p.) injection of a solution containing 250 mg/kg ketamine/xylazine mixture. The trachea was surgically exposed, and a total volume of 50 µl of muramyl peptide (MDP; 100 ng/mouse), LPS (100 ng/mouse) or 10^6 CFUs/mouse *E. coli* (American Type Culture Collection 25922) was administered. Saline alone (50 µl) was instilled into the trachea as a control.

***Preparation of Bacteria*:** *E. coli* was prepared for mouse inoculation, as described in previous studies (2, 5, 12). Bacteria were grown in trypticase soy broth (TSB) at 37°C overnight under constant agitation. Bacteria were harvested by centrifugation, washed twice in sterile isotonic saline, and resuspended in sterile 0.9% saline at a concentration of 20 X 10^6 CFUs/ml. The initial mouse inoculums were confirmed by plating serial 10-fold dilutions on MacConkey and tryptic soy agar (TSA) plates. For enumerating bacterial CFUs in the lungs, whole lungs were homogenized in 2 ml sterile saline for 30 s, and 20 µl of the resulting homogenates were plated by serial 10-fold dilutions on MacConkey and TSA plates. Bacterial colonies were counted after incubation at 37^{o}C for 24 h.

Neutrophil Recruitment: Bronchoalveolar lavage fluid (BALF) was collected to obtain BAL cells for enumeration of total leukocytes and neutrophils. The trachea was exposed and intubated using a 1.7-mm-outerdiameter polyethylene catheter. BAL collection was performed by instilling PBS containing heparin and dextrose in 0.8-ml aliquots.

Approximately 3 ml of lavage fluid was retrieved from each mouse. Cytospin samples were subsequently prepared from BAL cells and stained with Diff-Quick (Fisher). Differential cell counts were determined by direct counting of stained slides. Total leukocytes in BALF were determined using a hemocytometer (2, 5, 11, 12).

***MPO assay*.** An MPO assay was performed as described previously (2, 5, 11, 12). Whole lungs were weighed, frozen at −70°C, and then homogenized in 1 ml of HTAB buffer for 30 s (50 mg of tissue/ml of HTAB). The samples were vortexed, 1 ml of homogenate was transferred into each microcentrifuge tube and centrifuged at 20,000 × *g* for 4 min. Seven microliters of supernatant was transferred into a flat-bottom 96-well plate, and 200 µl of a *O*-dianisidine hydrochloride solution was added immediately before the activity was determined. The MPO activity as measured as optical density at 450 nm was expressed in units per milligram of lung tissue.

***Cytokine and Chemokine expression*:** Cell-free BALF was used for the determination of TNF-α (eBioscience), IL-6 (eBioscience), KC/CXCL1 (R&D systems), MIP-2/CXCL2 (R&D systems) and LIX/CXCL5 (R&D systems) by sandwich ELISA. The minimum detection limit is 2 pg/ml of cytokine or chemokine protein (2, 5, 11, 12, 29).

***NF-κB DNA binding assay*:** This technique has been described in our earlier publications (2, 5, 11, 12, 28, 29). Briefly, nuclear proteins were extracted from the lung tissue collected at 6 and 24 h post-*E. coli* or saline challenge. A total of 7.5 µg nuclear extract was mixed with binding buffer, added to the precoated plate (with the DNA binding motif of NF-κB) and incubated for 1 h at room temperature according to manufacturer's protocol (TransAM ELISA kit). Wells were then washed, and plates were incubated with NF-κB/p65 antibody for 1 h. Plates were then washed three times with wash buffer and HRP-conjugated anti-rabbit IgG was added to each well and incubated for 1 h. Plates were read at 450 nm after adding the developing reagent.

***Lung pathology*:** The lungs were perfused from the right ventricle of heart with 10 ml isotonic saline after 24 h post-infection from NOD2$^{-/-}$ and WT mice. Lungs were then removed and fixed in 4% phosphate-buffered formalin. Fixed tissue samples were processed in paraffin blocks, and fine sections (5 µm in thickness) were cut with a microtome and stained with hematoxylin and eosin (H&E). Assessments of histopathology were performed by a Veterinary Pathologist in blinded fashion according to the following scoring scale: 0, No inflammatory cells (macrophages or neutrophils) present in section; 1, <5% of section is infiltrated by inflammatory cells; 2, 5–10% of section is infiltrated by inflammatory cells; and 3, >10% of section is infiltrated by inflammatory cells as described in our earlier publications (2, 11).

***Whole cell protein extraction and Immunoblotting*:** Whole cell proteins were extracted by homogenizing the lungs in a lysis buffer cocktail containing 0.1% Triton X-100 in PBS, complete protease inhibitor cocktail (Thermo Scientific, Waltham, MA 02454), complete phosphatase inhibitor cocktail (Thermo Scientific**,** Waltham, MA 02454), and 1 mM DTT. Protein extracts from neutrophils were prepared by lysing cells with Urea/CHAPS/Tris buffer containing protease and phosphatise inhibitor cocktails. Cell lysates were centrifuged at maximum speed in a microcentrifuge at 4^{o}C. The resulting supernatants were used for Immunoblotting. Whole cell proteins were fractioned by 10-12% SDS-PAGE and electrophoretically transferred on to Immobilon-P (Millipore) polyvinylidene difluoride membranes. Membranes were blocked and probed with VCAM-1, ICAM-1, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), phospho-MAPK/JNK (Thr183/Tyr185), NF-κB, phospho-NF-κB (Ser536), IκBα, phospho-IκBa (Ser32) phospho-p47^{phox}, p47^{phox}, p67^{phox}, and NOD-2 (1:1,000 dilution). The primary Ab was detected by autoradiographic film with appropriate HRP-conjugated secondary antibody and the ECL chemiluminescent system (Amersham Pharmacia Biotech, Piscataway, NJ). To demonstrate equal protein loading on gels, the blots were stripped and reprobed with Ab specific for total p38, laminin or GAPDH (2, 5, 11, 12).

***Neutrophil purification and E. coli killing assay*.** To evaluate the ability of neutrophils to kill *E. coli,* a killing assay was performed. Murine neutrophils were purified by negative magnetic selection. Wild-type and NOD2$^{-/-}$ mice were sacrificed and their femurs and tibias were flushed to obtain bone marrow cells. Cell suspension was passed through a 0.77 µM filter and was resuspended in RoboSep buffer (PBS without Ca^{2+}/Mg^{2+}, 2% FBS, 1 mM EDTA). Neutrophils were purified by using a custom mixture containing Abs to CD5, CD4, CD45R/B220, TER119, F4/80, CD11c, c-KIT (Cat No# 19709). Briefly, bone marrow cells were incubated in RoboSep buffer containing 5% normal rat serum along with the custom Ab cocktail, biotin selection cocktail and magnetic colloid according to manufacturer's instructions. Samples were then placed in an EasySep magnet. After three minutes incubation samples highly enriched with neutrophils were separated by pouring into a new tube. Fractions were FACS analysed to determine the purity of the cells (>92% purity was confirmed). Purified neutrophils were used for neutrophil mediated bacterial killing. These neutrophils also used to detect Elastase, H_2O_2, MPO, and Nitric oxide (NO) release along with the expression of NADPH oxidase subunits and NOD2.

For bacterial killing, 1×10^6 neutrophils were pooled with 10% *v/v* FBS, and 1×10^6 opsonized bacteria were added to 10×75 mm polypropylene tubes (BD Biosciences). The capped tubes were incubated in a shaking water bath at 37°C with continuous agitation for 0, 30, 60, or 120 min. Samples were removed at each time point and placed in an ice bath. A portion of sample was spun at 100Xg for 10 min to collect the viable bacteria in media the remaining sample was washed in PBS three times to remove the extracellular and adherent bacteria. The neutrophil pellet was resuspended in 1 ml of PBS and by four freeze thawing cycles the cells were ruptured and the lysate was used for subsequent culture and enumeration of bacteria. Colony counting of viable bacteria was determined by plating 20 µl aliquots of five diluted samples on McConkey agar and TSA plates. The number of colonies was enumerated after incubation at 37°C overnight (20).

Neutrophil elastase (NE) activity. Culture media obtained from neutrophil mediated killing assay was collected at different time intervals was stored at -80 °C until use. N-methoxysuccinyl–Ala-Ala-Pro-Val p-nitroaniline (Sigma Chemical) was used as substrate for NE as this compound is not hydrolized by cathepsin G. Hydrolysis of substrate by NE was determined spectrophotometrically at 405 nm. In brief, 1 mM substrate was incubated with 0.2 ml sample in 0.1 M tris (hydroxyl-methyl) aminomethane. HCl buffer (pH 8.0) containing 0.5 M NaCl at 37˚C for 24 h. Optical density was determined and activity of NE was determined, where one enzyme unit is defined as quantity of enzyme that liberated 1 µmol of p-nitroaniline in 24 h (65).

***Determination of hydrogen peroxide levels*.** Hydrogen peroxide levels were measured to evaluate reactive oxygen species production by using Fluorescent H_2O_2/Peroxidase Detection Kit (Cell Technology, Inc, Mountain View, CA). The Fluoro H_2O_2 detection kit utilizes a non-fluorescent detection reagent to detect H_2O_2. H_2O_2 oxidizes the detection reagent in a 1:1 stoichiometry to produce a fluorescent product resorufin which is catalyzed by peroxidase in a homogeneous no wash assay system. To evaluate the generation of ROS in control and infected tissues, fifty micro litres of lung homogenates were incubated with 50µl reaction cocktail (10mM detection reagent, 10U/ml horseradish peroxidase (HRP), 100 U SOD) for five minutes at room temperature in dark. After incubation the fluorescence was measured at excitation 540 nm and emission 595 nm in a fluorescent plate reader (49).

MPO activity in neutrophils. MPO release by the neutrophils was measured as previously described (2, 5, 11, 12, 26). Briefly culture media obtained at different time intervals was stored at –80 °C until assay. Reaction mixture consisted of 100µl of culture media, 50µl of 1.0% hexadecyltrimethylammoniumbromide (HTAB) (Sigma Chemical, St. Louis, MO) in 100 mmol/l phosphate buffer (pH 6.0) and 50 µl of 50 mmol/l potassium phosphate buffer (pH 6.0) containing o-dianisidine hydrochloride (0.167 mg/ml; Sigma Chemical, St. Louis, MO, USA) and 0.0005% hydrogen peroxide. Absorbance change at 460 nm was monitored with spectrophotometer (Hitachi, Tokyo, Japan) at every 5 mins interval at room temperature.

***NO release by neutrophils*.** The NO assay was performed as described in previous reports (7, 38, 39). Neutrophils isolated from bone marrow were infected with 1 multiplicity of infection (MOI) *E. coli* for different time intervals. Media were collected at designated time points for the detection of NO2 and NO3 anions using a colorimetric assay kit (Cayman Chemical Company). A standard curve was plotted by diluting standards with incubation media (7, 38, 39).

***Bone marrow-derived macrophage culture*.** Bone marrow cells from $NOD2^{-/-}$, and WT (control) mice were differentiated into macrophages by adding M-CSF for 7 days. A total of 1×10^6 cells/well were used for each group at each time point for infection with 0.1 MOI of *E. coli* and incubated at 37˚C with slow agitation. At 10, 20, 30, and 60 minutes cell pellets were purified and processed for western blot analysis to determine the activation of NF-κB and MAPKs (5, 11).

***Fractionation of cytoplasmic and membrane proteins*.** A FractionPREP™ Cell fractionation kit (Biovision, San Francisco) was used for preparation of the cytoplasmic and membrane fractions following the vendor's recommendations. Briefly, $2X10^6$ *E. coli* infected bone marrow neurophils were suspended in the cytosol extract buffer, vortexed, and incubated for 10 mins on ice. Following centrifugation, the cytosolic fraction was separated and membrane fractions were suspended in Urea/CHAPS/Tris buffer and protein contents were quantified. Equal proteins were resolved by SDS-PAGE and membranes were probed with antibodies for $p47^{phox}$, GAPDH or pan-Cadherin (control).

***Phagocytic Index*.** Neutrophils were purified from bone marrow by negative selection. A total of 1×10^6 neutrophils were infected with 1×10^6 opsonized *E. coli.* At 30 min post-incubation, the cells were washed in PBS three times and centrifuged at 200 x *g* (to remove extracellular bacteria), deposited onto microscope slides in a cytocentrifuge at 200 x *g* (Thermo Shandon), and fixed with methanol, and stained by Diff-Quick (Fisher Scientific, Pittsburgh, PA) method. The number of neutrophils that ingested bacteria and the number of ingested bacteria per cell were counted under a light microscope using the oil emersion lens.

***Statistical analysis*:** All data are expressed as means ± standard errors of the means (SEM). The intensity of western blot bands was determined using gel digitizing software (UN-SCAN-IT gel) from Silk Scientific, Inc., UT. Data were analyzed by ANOVA, followed by Bonferroni's *post hoc* analysis for multiple comparisons. Differences in data values were defined significant at a *P* value of less than 0.05 using Graph pad Prism 4.

Results

***NOD2 is necessary for neutrophil accumulation in the lungs in response to MDP but not LPS challenge*.** As a first step to explore the function of NOD2 in the lungs, we used $NOD2^{-/-}$ mice and challenged them with intratracheal muramyl dipeptide (MDP), a NOD2 specific agonist, LPS, a canonical TLR4 agonist or a combination of MDP and LPS. Mice were sacrificed at 24 h post-infection and BALF was collected for total leukocyte and neutrophil counts. At 24 h post-infection, neutrophils were increased in BALF following MDP and LPS challenge although LPS caused more neutrophil numbers in BALF as compared with MDP (Figs. 4.1A-B). Our data show that LPS-induced neutrophil influx in the pulmonary airways is not dependent on NOD2 whereas MDP-induced neutrophil migration to the airways is dependent on NOD2 (Figs. 4.1A-B). When MDP was co-administered with LPS intratracheally, we observed a synergistic neutrophil influx in the lungs which is dependent on NOD2 (Figs. 4.1A-B). These results reveal NOD2 signaling cascade is independent of TLR4 signaling cascade in the lungs.

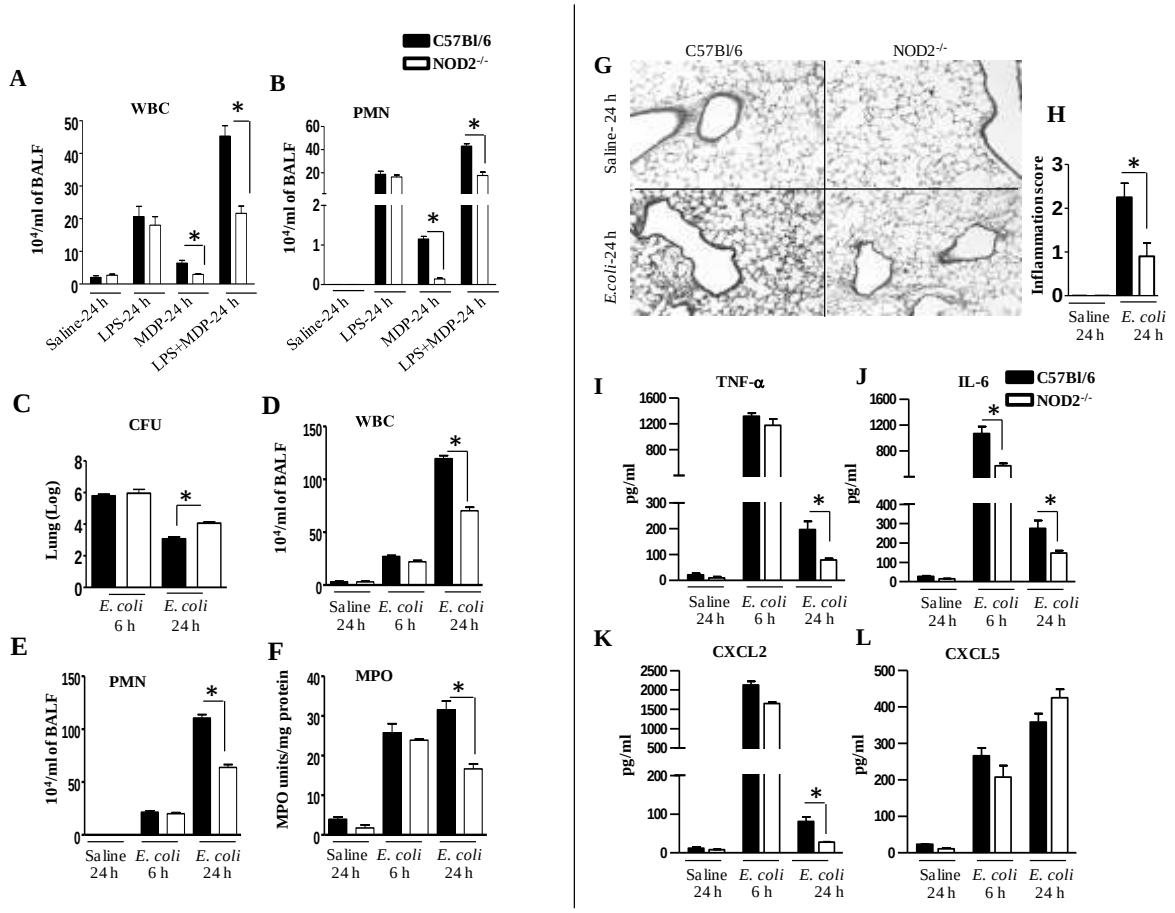

Figure 4.1. Importance of NOD2 in host defense against pulmonary Gram-negative infections. A-B. Total leukocytes (A) and neutrophils in BALF (B) obtained from NOD2$^{-/-}$ mice following LPS (100 ng), MDP (100 ng) or LPS+MDP (100 ng each). Mice were challenged and BALF was collected at 24 h post-challenge. (n=5-6 mice/group). *indicates significant difference between NOD2$^{-/-}$ and WT mice; p<0.05. **C.** Bacterial burden in the lungs of NOD2$^{-/-}$ mice compared to WT controls following *E. coli* infection. Mice were infected with intratracheal administration of *E. coli* (10^6 CFUs/mouse). Lungs were collected and homogenized at designated times and the number of viable bacteria were enumerated (n=4-5 mice/group/time-point). *indicates significant difference between KO and WT mice; p<0.05. **D-E.** Cellular infiltration in the lungs of NOD2$^{-/-}$ mice following *E. coli* infection. Mice were inoculated with *E. coli* (10^6 CFU/mouse), BALF was obtained at 6 and 24 h post-infection, and cell enumeration was performed to determine neutrophil and macrophage recruitment to the lungs (n=4-6 mice/group; p<0.05). **F.** Neutrophil accumulation in lung parenchyma following *E. coli* infection. Lungs were homogenized and MPO activity was measured as described in *Materials and Methods*. (n=3 mice/group; p<0.05). **G-H**. Lung histology in NOD2$^{-/-}$ mice following *E. coli* infection. Mice were inoculated with *E. coli* (10^6 CFUs/mouse), lungs were obtained at 24 h post-infection, and histological examination was performed by a veterinary pathologist in a blinded manner. This picture is a representative of 3 separate experiments with identical results (G). The degree of inflammation in lung sections from 3 separate lung sections are schon (H). **I-L.** Cytokine and chemokine levels in the lungs following *E. coli* infection. Mice were infected by intratracheal instillation of *E.* coli (10^6 CFUs/mouse), and BALF was collected from the lungs at designated time points. Concentrations (pg/ml) of TNF-α (I), IL-6 (J), CXCL2 (K) and CXCL5 (L) in BALF were quantified by sandwich ELISA. Asterisks indicate significant difference between NOD2$^{-/-}$ and WT mice (p<0.05; n = 4-6 mice in each group at each time-point).

***NOD2$^{-/-}$ mice show increased bacterial burden and decreased neutrophil influx in the lungs*.** In initial experiments, we determined if NOD2 is important for bacterial clearance from the lungs. In this regard, NOD2$^{-/-}$ mice and their wild-type controls were intratracheally instilled with *E. coli* (10^6 CFUs/animal). Mice were sacrificed at both 6 and 24 h post-infection and the lungs were removed and processed for bacterial culture. At 24 h post-infection, the bacterial burden in the lungs was increased in NOD2$^{-/-}$ mice compared to wild-type controls (Fig. 4.1C). However no bacterial dissemination was observed in mice with this bacterial dose (data not shown). To examine if NOD2 deficiency affects neutrophil recruitment to the lungs during Gram-negative bacterial infection, NOD2$^{-/-}$ mice were intratracheally infected with *E. coli* and BALF was collected at 6 and 24 h post-infection. At 24 h post-infection, there was a reduction in total leukocyte and neutrophil counts in NOD2$^{-/-}$ mice (Figs: 4.1D-E). This result was further confirmed by reduced myeloperoxidase (MPO) activity in lung homogenates of NOD2$^{-/-}$ mice (Fig. 4.1F). Since there was a reduction in neutrophil recruitment in NOD2$^{-/-}$ mice following *E. coli* challenge, we determined whether NOD2 deficiency affects the severity of lung inflammation during *E. coli* infection. NOD2$^{-/-}$ mice showed reduced inflammation in lung sections as evidenced by less neutrophil recruitment and alveolar edema as compared to their wild-type controls following *E. coli* infection (Figs. 4.1G-H).

***NOD2$^{-/-}$ mice display impaired cytokine and chemokine production*.** In the above experiments, we demonstrated that NOD2 participates in modulating bacterial clearance and neutrophil accumulation in pulmonary airspaces. As neutrophil recruitment to the infected focus is a multistep sequence beginning with cytokine and chemokine expression (1, 3, 17), we sought to determine the levels of the cytokines (TNF-α and IL-6) and neutrophil chemoattractants (CXCL1, CXCL2 and CXCL5) in BALF. As shown in Figs 1I-L, NOD2$^{-/-}$ mice exhibit reduced levels of cytokines in BALF as compared to their littermate controls following *E. coli* infection. The potent neutrophil chemoattractant, CXCL2, level was also reduced in the BALF of NOD2$^{-/-}$ mice following bacterial infection (Fig. 4.1K).

***NOD2$^{-/-}$ mice exhibit reduced activation of NF-κB, MAPKs and expression of cellular adhesion molecules*.** Expression of proinflammatory mediators is primarily regulated by transcription factors (6, 58). Of these, NF-κB has been identified as a critical transcription factor important for inflammation (6, 58). Given the participation of NOD2 in cytokine and chemokine expression in the lungs, we next asked if NOD2 is required for NF-kB activation during *E. coli* infection. NOD2$^{-/-}$ mice showed reduced NF-κB DNA binding activity and NF-κB activation in the lungs following *E. coli* infection (Figs. 4.2A-C). In addition to NF-κB activation, MAPK activation is required to induce cytokines and chemokines (17, 58). As shown in Figs. 4.2D-E, reduced activation of JNK and p38 was observed in the lungs of *E. coli* infected NOD2$^{-/-}$ mice compared to wild-type controls. These observations indicate that NOD2 contributes to *E. coli*- induced NF-κB and MAPK activation.

Cytokines and chemokines produced in the lungs in response to infection are shown to promote expression of cellular adhesion molecules on vascular endothelium and leukocytes (3, 17). To determine if NOD2 deficiency affects cellular adhesion molecule expression, lung homogenates were processed for western blot analysis. Not only ICAM-1 but also VCAM-1 expression was decreased in the lungs of NOD2$^{-/-}$ mice as compared to wild-type controls following *E. coli* infection (Figs. 4.2F-G).

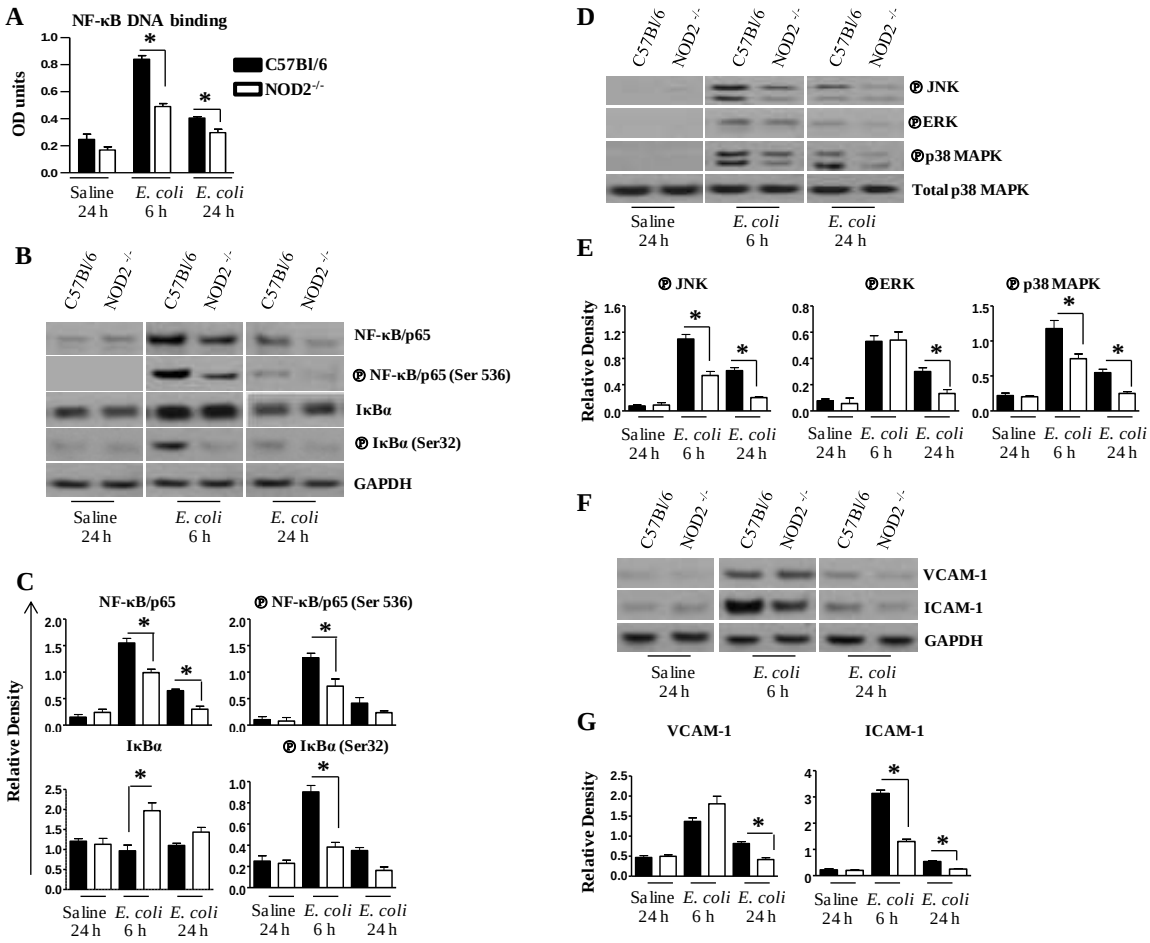

Figure 4.2. Activation of NF-κB and MAPKs and upregulation of ICAM-1 and VCAM-1 in the lungs of NOD2-/- mice following *E. coli* infection. **A-C.** Activation of NF-κB in the lung following infection with *E. coli*. Lung homogenates and nuclear lysates from NOD2$^{-/-}$ mice and their controls were prepared at 6 and 24 h after infection with *E coli*. NF-κB binding assay was performed in nuclear lysates from the lungs (A) and activation of NF-κB was determined using western blots of lung homogenates. The blot is a representative of 3 independent experiments with identical results (B). Relative densities normalized against GAPDH are representatives of 3 independent experiments/blots (C). **D-E.** Activation of MAPKs in the lungs following *E. coli* infection. Total proteins in the lungs were isolated from NOD2$^{-/-}$ or control mice at 6 and 24 h after infection with *E coli*, resolved on an SDS-PAGE and the membrane was blotted with the Abs against activated/phosphorylated form of MAPKs as described in *Materials and Methods*. This is a representative blot of 3 separate experiments with identical results (D). Densitometric analysis of MAPK activation was performed from 3 separate blots (E). *denotes the difference between NOD2$^{-/-}$ mice and their WT controls ($p<0.05$). **F-G.** Expression of ICAM-1 and VCAM-1 in the lungs in response to *E. coli* challenge. Infected lungs were homogenized, total proteins were isolated, resolved on SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membrane was blotted with Abs against ICAM-1, VCAM-1 and GAPDH. This is a representative blot of 3 independent experiments with identical results (F). Densitometric analysis was performed in 3 blots to demonstrate the relative expression of ICAM-1 and VCAM-1 in the lungs against GAPDH following *E. coli* infection (G).

***Neutrophils from NOD2$^{-/-}$ mice display impaired bacterial killing*.** We assessed if NOD2 deficiency affects phagocytosis and/or bacterial killing ability of neutrophils. To explore this, bone marrow neutrophils isolated from NOD2$^{-/-}$ or wild type (WT) mice and were stimulated with *E. coli* at an MOI of 1.0. We found higher extracellular CFUs in the supernatant of NOD2$^{-/-}$ neutrophils compared to supernatants from wild-type (control) neutrophils at 60 minutes post-infection (Fig. 4.3A). As compared to WT controls, significantly lower intracellular CFUs were observed at 30, 60 and 120 minutes post-infection in NOD2$^{-/-}$ mice (Fig. 4.3B). NOD2$^{-/-}$ neutrophils also show reduced phagocytosis of *E. coli* at 30 min post infection when compared to the WT neutrophils (Fig. 4.3C). We have also measured the levels of antimicrobial compounds, including myeloperoxidase, H_2O_2 nitrate, and elastase in isolated neutrophils (Figs. 4.3D-G). Our data suggest that NOD2 controls bacterial killing of *E. coli* via the generation myeloperoxidase, H_2O_2 and elastase and Nitrate.

***NOD2 regulates NADPH oxidase expression and activation*.**

Since we observed impaired bacterial killing and production of antimicrobial compounds, we wished to determine whether NOD2 can regulate the expression of NADPH oxidase subunits. NADPH oxidase is an enzyme complex that is important for the generation of reactive oxygen species and thereby critical for the killing of bacteria (25). In this regard, we determined the expression levels of p47^{phox} and p67^{phox} both in the lungs and neutrophils. Our data show that decreased expression of p47^{phox} and p67^{phox} in the lungs of NOD2$^{-/-}$ mice when compared to wild-type controls (Figs. 4.4A-B). In a similar manner, we observed reduced expression of p47^{phox} and p67^{phox} in purified NOD2$^{-/-}$ neutrophils (Figs. 4.4E-F). Furthermore, we observed reduced phosphorylation of p47^{phox} both in the lungs (*in-vivo*) and in isolated neutrophils (*in-vitro*) (Figs 4.4C, D, G, H). We observed that neutrophils challenged with *E. coli* show enhanced membrane translocation of p47^{phox} (Figs. 4.4G-H). Our data therefore demonstrate that NOD2 is essential for NADPH oxidase activation as determined by phosphoryation and membrane translocation of p47^{phox} in neutrophils

following *E. coli* infection. We also observed increased NOD2 expression in neutrophils upon *E. coli* infection (Figs. 4.4E-F).

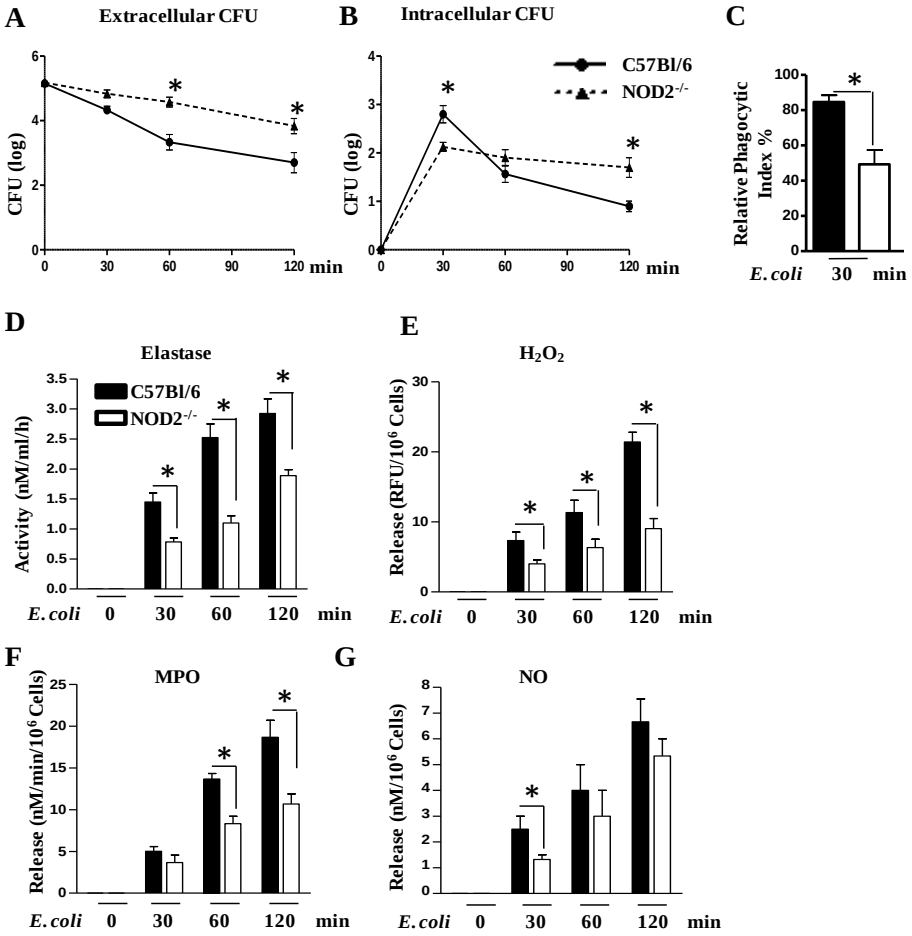

Figure 4.3. Bacterial killing by neutrophils and the generation of antimicrobial mediators in neutrophils following *E. coli* infection. A-B. Bacterial killing by neutrophils obtained from WT and NOD2$^{-/-}$ mice following *E. coli* infection. Neutrophils were isolated from the bone marrow using negative selection as described in *Materials and Methods*. These neutrophils were infected with *E. coli* (MOI of 1) and extracellular CFUs (A), intracellular CFUs (B) were determined at 30, 60 and 120 min post-infection. **C.** Relative phagocytic index of neutrophils from NOD2$^{-/-}$ and WT neutrophils following 30 min *E. coli* infection. Bone marrow neutrophils were infected with 1 MOI of *E. coli* and relative phagocytic index was determined as described in *Materials and Methods.* The data obtained from three independent experiments. **D-G.** Release of elastase, H_2O_2 MPO and nitric oxide were measured in the culture media of infected neutrophils at 30, 60 and 120 min post-infection. A total of 4-5 mice were used (*p<0.05)

***NOD2/RIP2$^{-/-}$ mice display enhanced bacterial burden and attenuated lung inflammation following E. coli infection*.** Although we found that RIP2 augments

neutrophil-mediated bacterial clearance from the lungs following *E. coli* infection (5), the role of RIP2 in NOD2-mediated signaling during *E. coli* infection has not determined. To explore this, we generated NOD2/RIP2$^{-/-}$ mice. When compared to NOD2$^{-/-}$ mice, NOD2/RIP2$^{-/-}$ mice display similar bacterial burden and reduction in neutrophil trafficking and cytokine/chemokine expression following intrapulmonary *E. coli* infection (Figs. 4.5A-E). These observations were accompanied by a similar degree of reduction in activation of NF-κB and MAPKs between NOD2/RIP2$^{-/-}$ and NOD2$^{-/-}$ mice (Figs. 4.5F-I). These observations suggest that RIP2 is a primary mediator of NOD2 signaling in NF-κB and MAPK activation.

***Bone marrow-derived macrophage function is compromised in NOD2/RIP2$^{-/-}$ mice following E. coli infection*.** The above experiments demonstrated that RIP2 participates in NOD2-mediated signaling in the lungs upon *E. coli* infection. Macrophages are the first cells interact with pathogens in the lungs (41). Therefore, we determined if NOD2 and RIP-2 have similar effects on macrophage activation by challenging them with *E. coli* (1 MOI) for different lengths of time. As shown in Figs. 4.6A-B, macrophages obtained from NOD2/RIP2$^{-/-}$ mice show reduced activation of NF-κB and MAPK at 60 mins after infection as compared to NOD2$^{-/-}$ mice. Expression of TNF-α, IL-6 and CXCL2 was reduced in macrophages obtained from NOD2-/- and NOD2/RIP2$^{-/-}$ mice (Figs 4.6C-E)

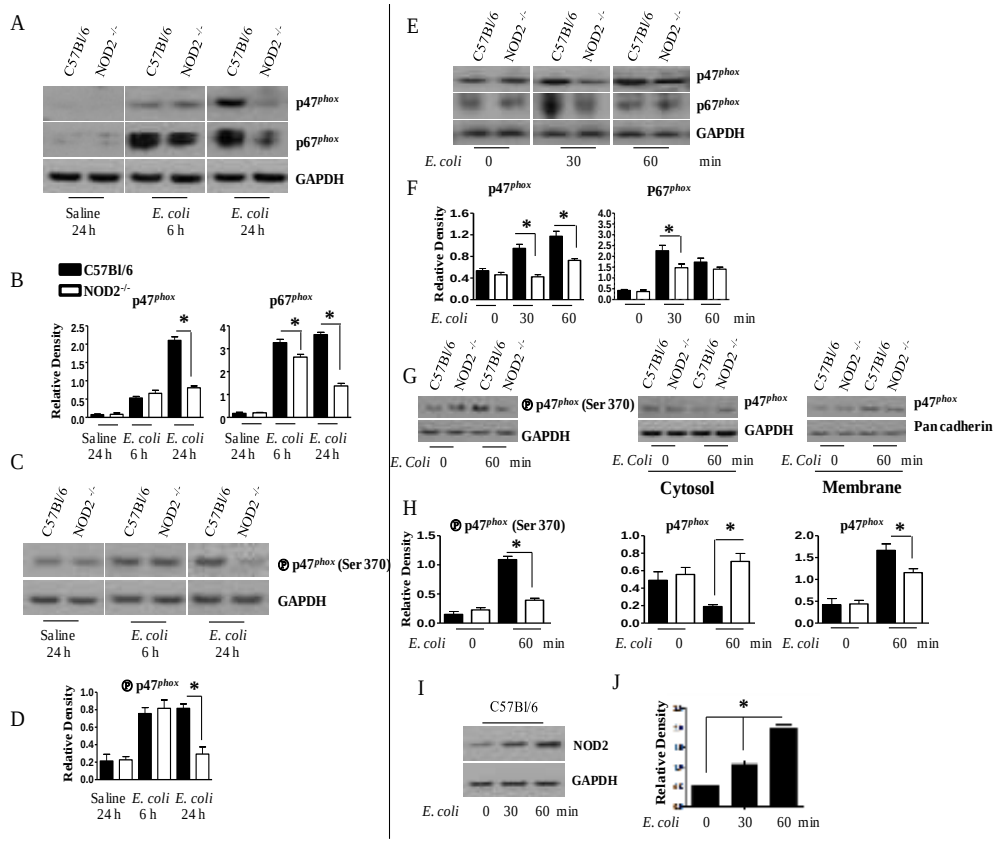

Figure 4.4. Expression and activation of NADPH oxidase components in the lungs and neutrophils in response to *E. coli* infection. A-B. Expression of p67^{phox} and p47^{phox} in the lungs of NOD2$^{-/-}$ mice after *E . coli* infection. Resolved lung homogenates were transferred onto a nitrocellulose membrane and the membrane was blotted with the appropriate Abs as described in *Materials and Methods*. The data are a representative blot of 3 separate experiments with identical results. Densitometric quantitative analysis was performed in 3 blots to demonstrate the expression of p47^{phox} and p67^{phox} in the lung following *E. coli* infection. Data shown here is a representation of 3 separate blots. **C.** Activation of NADPH oxidase in the lungs following *E.coli* infection. Lung homogenates were processed for western blot analysis using phospho**-**p47^{phox} (Ser-370) antibodies as described in *Materials and Methods*. The blot is a representative of 3 separate experiments with identical results. **D.** Densitometry of activated p47^{phox} following *E. coli* infection. **E** Expression of p47^{phox} and p67^{phox} in WT and NOD2$^{-/-}$ neutrophils upon *E. coli* infection. Bone marrow neutrophils were stimulated with *E. coli* for 30 and 60 mins and cell lysates were prepared for western blotting using Ab against p67^{phox} and p47^{phox}.This is a representative blot of 3 separate experiments. **F.** Relative p47^{phox} and p67^{phox} expression in neutrophils using 3 independent blots (**p*<0.05). **G-H.** Phosphorylation of p47^{phox} in neutrophils upon *E. coli* infection. Data is a representation of three separate blots. Bone marrow neutrophils were challenged with *E. coli* (MOI of 1). Cytosol and membrane fractions were separated, resolved on an SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were probed with antibodies against p47^{phox}, pan-Cadherin or GAPDH. This is a representative blot of 3 experiments with identical results Densitometric analysis shows protein expression normalized against GAPDH and pan-Cadherin respectively from 3 independent blots. **I.** Expression of NOD2 in neutrophils purified from bone marrow of WT by immunoblotting upon *E. coli* infection. Purified neutrophils were stimulated with *E. coli* for 30 and 60 mins and cell lysates were used for western blotting using Ab against NOD2.This is a representative blot of 3 separate blots from independent experiments. **J.** Relative NOD2 expression in stimulated neutrophils using 3 independent blots to determine quantitatively.

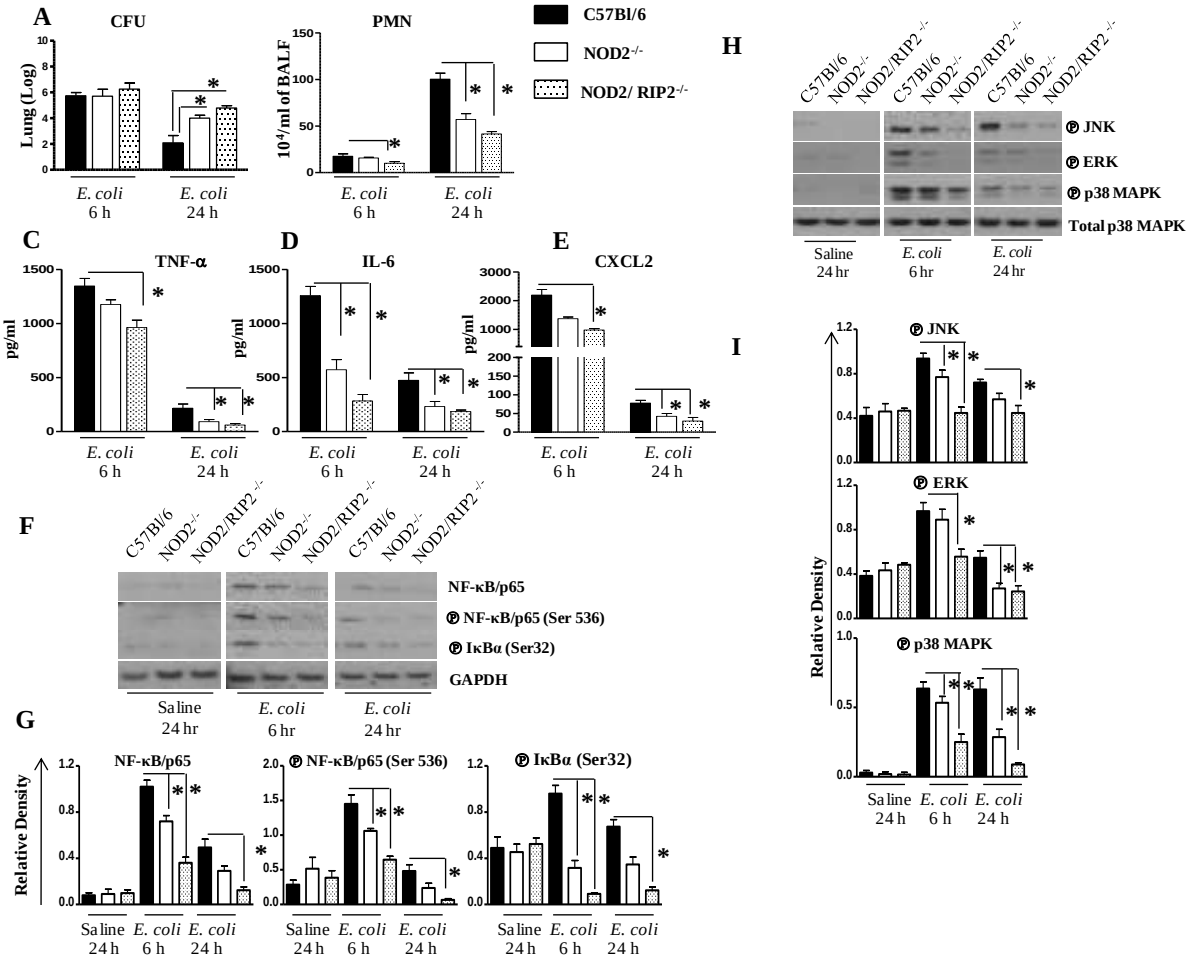

Figure 4.5. Importance of NOD2/RIP2 axis in host defense against pulmonary *E. coli* infection. A. Impaired bacterial clearance in the lungs in NOD2/RIP2$^{-/-}$ mice. Lung homogenates at 6 and 24 h after infection were used to enumerate the bacterial CFU (n = 6 or 8 mice/group/time point). Asterisks indicate significant differences between WT (C57BL/6) and RIP2$^{-/-}$ (KO) mice ($P < 0.05$). **B.** Cellular recruitment into the lungs of NOD2/RIP2$^{-/-}$ mice at 6 and 24 h after i.t *E. coli* (10^6 CFUs/mouse) infection.n=4-6/group. **C-E.** Cytokine and chemokine responses in the airspaces following *E. coli* infection. BALF was collected from the lungs after i.t. instillation of *E. coli* (10^6 CFU/mouse) at designated time points. TNF-α (C), IL-6 (D) and CXCL2 (E) levels in BALF were quantified by sandwich ELISA. Asterisks indicate significant difference between WT and KO mice (n=3-5 and *p<0.05).**F-G.** Activation of NF-κB in lung homogenates following *E. coli* infection. Data shown here is a representation of 3 separate blots with identical results (F). Densitometric analysis of western blots normalized against GAPDH. Data are from 3 individual experiments/blots (*p<0.05) (G). H-I. Activation of MAPKs in lung homogenates after stimulation with *E. coli*. This is a representative blot of 3 separate blots with identical results (F). Densitometric analysis of western blots normalized against GAPDH. Data are from 3 individual experiments/blots (*p<0.05)

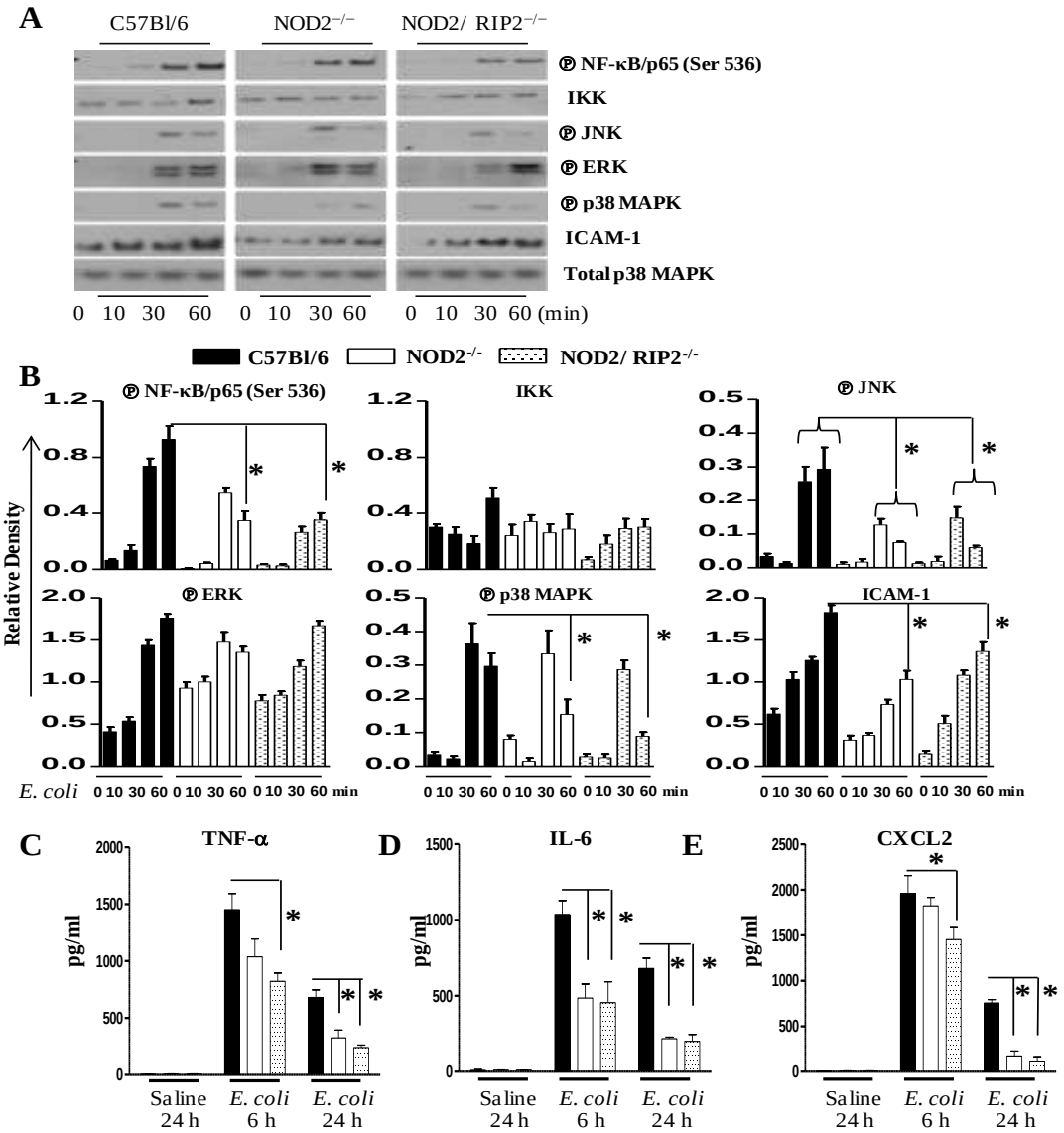

Figure 4.6. **Activation of NF-κB and MAPKs and upregulation of ICAM-1 in BMDMs obtained from NOD2$^{-/-}$ and NOD2/RIP2$^{-/-}$ mice following *E. coli* infection**. **A.** Activation of NF-κB, MAPKs and cellular adhesion molecules in the bone marrow-derived macrophages from WT, NOD2$^{-/-}$ and NOD2/RIP2$^{-/-}$ mice upon *E. coli* infection. Data shown here is a representation of 3 separate blots from 3 individual experiments. **B.** Densitometric analysis of NF-κB and MAPK activation in BMDMs of WT, NOD2$^{-/-}$ and NOD2/RIP2$^{-/-}$ mice. Relative densities normalized against total p38 MAPK are representative of 3 independent experiments. **C.** Levels of TNF-α, IL-6 and CXCL2 in BMDMs of WT, NOD2$^{-/-}$ and NOD2/RIP2 $^{-/-}$ mice following *E. coli* infection. Experiments performed in triplicate wells in each experiments (n=3 mice/group).

Discussion

NOD1 and NOD2 are structurally related cytoplasmic proteins (40, 57). Both NOD1 and NOD2 have CARDs in the N-terminus, a central nucleotide oligomerisation domain, and a C-

terminal LRR domain to recognize PAMPs (40, 57). The primary function of NOD1 and NOD2 is to serve as pattern recognition receptors for bacteria and/or their products. NOD1 and NOD2 detect microbial cell wall components diaminopimelate (DAP) and peptidoglycan/muramyl dipeptide (MDP), respectively (13, 24). In the current report, we describe a new role for NOD2 in modulating neutrophil-dependent host defense in the lungs in response to extracellular Gram-negative (*E. coli*) infection. Consistent with the important role that NOD2 plays in augmenting host defense in the lungs, we found that NOD2-RIP2 axis is important to mediate pulmonary defense against *E. coli* infection.

PGN is a known inducer of host resistance against bacteria and serves as an immune-adjuvant to immunoglobulin production (13). The biological properties of PGN showed that PGN components, such as DMP and MDP are the potent immunostimulatory components (13). Subsequent studies revealed that MDP can interact with NOD2 to induce downstream signalling (24). In previous studies, NOD2 signaling has been associated with either higher or lower tissue inflammation (19, 21, 64) as this reflects different bacterial strains used in experimental infection models. For example, NOD2 is important for different organisms, including intracellular pathogens that resides in the cytosol (21), and intracellular organisms that evades phagosomal-lysomal fusion (45). Both NOD1 and NOD2 modulate pulmonary immune response to *Legionella pneumophila* whereas NOD2 contributes to host resistance to mycobacterial infection via both innate and adaptive immune mechanisms (19). The different host responses to these bacterial pathogens may be attributed to the quantity, location, and structure of peptidoglycan (PGN). In the current investigation, impaired bacterial clearance and reductions in inflammatory cell recruitment were observed in NOD2$^{-/-}$ mice compared to their wild-type counterparts. Whether different structures of MDP from *E. coli* alter their detection by NOD2 is the subject of future investigation. We have chosen *E. coli* (ATCC 25992) strain in this investigation because this strain has been shown to cause neutrophil recruitment without capillary leakage even with a higher dose 10^6 CFUs/animal (53).

One important question arising from our studies related to the mechanisms by which NOD2-mediated signaling is activated upon *E. coli* infection. Although the mechanisms involved in the recognition of extracellular pathogen by an intracellular receptor (NOD2) are not entirely clear but at least three possibilities could be outlined: (i) bacterial MDP can be released to the cytosol and stimulate the intracellular receptors; (ii) *E. coli* cells and/or their products can be leaked into the cytosol from the endosomes or phagolysosomes (37, 60); and (iii) cross-talk between TLRs and NLRs could occur after initial bacterial recognition by TLRs.

Recruitment of neutrophils to the lungs appears to play a pivotal role in mediating efficient innate immunity against extracellular pathogens, including *E. coli* (4). To examine if differences in CFUs were due to differences in neutrophil influx in the lungs, we examined neutrophil accumulation in the pulmonary airspaces by performing BALF and in lung parenchyma by measuring MPO activity. Although NOD2 contributes to innate antibacterial immunity against *E. coli*, $NOD2^{-/-}$ mice show 50% reduced neutrophil recruitment to intrapulmonary *E. coli* infection. This observation emphasizes the importance of other PRRs recognizing pathogen-associated molecular patterns (PAMPs) of *E. coli*, including plasma membrane- or endosome-bound TLRs. In this regard, we reported the importance of TLRs in *E. coli* infection by the reduced bacterial clearance from the lungs along with attenuated accumulation of neutrophils in mice deficient in TIRAP or MyD88 (28). There are several reports indicating the cross-talk between TLR and NOD molecules (27, 44, 52, 63), and recent work revealed that TLR2 and NOD2 were redundant in the detection of Gram-positive bacteria (*S. aureus*) and cytokine production by macrophages (27, 44, 52, 63).

Our data reveal reduced neutrophil influx into the airspaces of $NOD2^{-/-}$ mice when treated i.t with LPS and MDP together. Our results are in agreement with the earlier reports (50) that LPS and MDP together increased substantial neutrophil influx when compared to the treatment of each ligand alone, suggesting that TLR and NOD2 signaling can serve as an amplifier to each other. Thus, analysis of NOD2 function in the absence or presence of

other PRRs may be required to unravel the importance of NOD2 in antibacterial host defense.

Recent studies have highlighted the complex nature RIP2 signaling as this molecule plays a central role in both TLR and NOD signaling pathways (5). Both NOD1 and NOD2 contain caspase-1 recruitment (CARD) domains that can activate downstream signaling through RIP2 (33). We demonstrate earlier that RIP2$^{-/-}$ mice show substantially decreased bacterial clearance from the lungs and neutrophil migration following *E. coli* infection (5). These findings brought the questions regarding the upstream molecules which cause RIP2-dependent signaling cascades that augment innate immune responses. The findings that NOD2$^{-/-}$ and NOD2/RIP2$^{-/-}$ mice show similar degree of impairment in neutrophil-mediated host defense suggest that RIP2 is primarily an adaptor for NOD2 cascades. Although not statistically significant, NOD2/RIP2$^{-/-}$ mice display more reduction in neutrophil recruitment to the lungs than NOD2$^{-/-}$ mice, suggesting a minor role of RIP2 in signaling cascades other than the NOD2-mediated ones. As compared with NOD2$^{-/-}$ mice, NOD1$^{-/-}$ mice showed no significant reduction in neutrophil numbers in BALF following *E. coli* infection (data not shown). These results suggest that unlike NOD2 plays a major role in host defense against *E. coli* infection.

Neutrophil activation is a critical first step to clear bacteria from infected tissues (46, 48). In our previous reports we have shown that neutrophil depletion renders the mice highly susceptible to *Klebsiella pneumoniae* (2, 11). The intriguing observation from the current study is that NOD2$^{-/-}$ neutrophils show inherent defect in intracellular bacterial killing. In this regard, a previous report demonstrates that adherent-invasive *E. coli* clearance is not different between NOD2$^{-/-}$ monocytes and wild-type monocytes (55). On the other hand, in a cutaneous *Staphylococcus aureus* infection model, it has been shown that NOD2$^{-/-}$ neutrophils were defective in killing the bacteria *in vitro* (27). Strain differences of *E. coli* and different cell type used in the other studies may have contributed to the differences in outcomes. The findings from the current studies that NOD2$^{-/-}$ neutrophils demonstrate

reduced phagocytosis following *E. coli* infection conform a previous report in which NOD2 deficient neutrophils have defective phagocytosis after *Staphylococcus aureus* infection (18).

Intracellular bacterial killing by neutrophils is primarily mediated via the generation of reactive oxygen (RO) and reactive nitrogen species (ROS) (35, 42). The importance of the NADPH oxidase to the host defense is shown by frequent infections resulting from the impaired killing of microbes in patients with chronic granulomatous disease (CGD) (61). The chronic granulomatous disease results in an impaired NADPH oxidase is due to mutations in the subunits of the NADPH oxidase (43). Findings from the current study suggests that NOD2-dependent expression and activation of NADPH oxidase complex along with the production of elastase, H_2O_2, MPO and nitrate are important mechanisms of intracellular *E. coli* killing by neutrophils. However, the extent to which MPO is released and whether it retains extracellular activity that contributes to bacterial killing have not been fully established.

In conclusion, this investigation revealed that NOD2 may act as an important regulator during *E. coli* infection in the lungs. Activation of NOD2 resulted in NF-κB-dependent cytokine/chemokines expression and neutrophil recruitment as well as activation in the lungs in a RIP2-dependent fashion. Delineating such novel mechanisms would facilitate to device a better immunotherapy to overcome the complications of bacterial pneumonia.

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Chapter-5: Protective Role of RIP-2 in *Klebsiella* induced Pneumonia

Introduction

Klebsiella pneumoniae (Kp) is one of the most important causative agents of pneumonia. It causes local inflammation in the lung and disseminates to vital organs leading to death [1, 2]. Kp is the second most common cause of Gram-negative sepsis [3, 4] and causes significant burden on healthcare due to high morbidity and mortality, especially in children and immune compromised individuals [4]. Treating Kp has been become difficult because of the emergence of the antibiotic resistant Kp strain or superbug[5]. Therefore, understanding the host immune mechanism is a prerequisite for developing improved therapies and prevention measures.

During acute bacterial pneumonia, neutrophils play a critical role in clearing the bacteria from the lung and systemic organs [6-8]. Early neutrophil recruitment and neutrophil-mediated bacterial killing have been shown to be critical in eliminating bacteria [9-11]. PRRs, such as TLRs [12]and NLRs[13, 14] are important host sensors that initiate innate defense during bacterial infections. Receptor interacting protein 2 (RIP-2) is a serine/threonine kinase that functions as an adaptor for TLR and NOD (NOD1 and NOD2) signaling pathways[15]. RIP2 has been shown to play an essential role in mucosal immunity to intrapulmonary pathogens, such as *Listeria monocytogenes[16] , Legionella pneumophila[17] , Chlamydophila pneumoniae [18]* and *Mycobacterium tuberculosis [19]*. In another study LPS pre-stimulation showed reduced inflammation in the peritoneum through NOD1/NOD2 signaling and induced lethality during *Pseudomonas* infection due to bacterial dissemination[20]. In our recent study, we have shown that RIP-2 regulates IL-17A and neutrophil recruitment in the lung during *E. coli* pneumonia[21]. We also reported that an upstream molecule of RIP-2, namely; NOD2, regulates neutrophil dependent host defense during *E. coli* pneumonia [22]. However, the murine model of *E. coli* pneumonia has some limitations because *E. coli* gets cleared from the lung and does not disseminate into bloodstream. In addition, as compared with other Gram-negative pulmonary pathogens, *E. coli* is not a significant primary pathogen because *it* does not multiply in the lungs,

disseminate to distal organs upon intrapulmonary administration, and can be cleared from the lungs rapidly. Therefore, we have chosen the primary pathogen Kp which multiplies in the lungs and disseminate to the bloodstream following intrapulmonary inoculation that leads to evaluate both local and systemic responses following pulmonary inoculation.

In the present investigation, we investigated whether RIP-2 signaling is essential for the immune response during pneumonic *Klebsiella* infection. We also determined the role of NOD2/RIP-2 axis in IL-17A regulation during Kp pneumonia. In this regard, we used RIP-2, NOD2 and NOD2-RIP-2 double knockout mice in the studies. The results show that RIP-2 mice are more susceptible to Kp lung infection with attenuated neutrophil recruitment to the lung along with increased bacterial dissemination. We also observed that RIP2 modifies the immune responses by regulating the activation of NF-κB and the infammasome. We also show that RIP-2 mediated IL-17A regulation occurs through STAT3 activation and IL-6.

Materials and Methods

***Mice*:** Eight to ten-week-old female RIP-$2^{-/-}$ [23] mice alongwith the age- and gender-matched WT (C57Bl/6) counterparts (19-25g by weight) were used as controls. All animal experiments were approved by the Louisiana State University Animal Care and Use Committee.

Murine model of infection: Bacteria were prepared for mouse inoculation, as described in previous studies [24, 25]. Kp serotype 2 (American Type Culture Collection (ATCC) 43816) was grown in trypticase soy broth (TSB) at 37°C overnight under constant agitation. Bacteria were harvested, washed, and resuspended in sterile 0.9% saline at a concentration of 20 $X10^6$ CFU/ml. Mouse strains were anesthetized with intraperitoneal (i.p) administration of ketamine/xylazine (250 mg/kg), followed by intratracheal (i.t.) inoculation of 50 µl of bacteria (10^3 CFU/mouse), while control mice were inoculated with 50 µl of saline. The initial mouse

inocula were confirmed by plating serial 10-fold dilutions on MacConkey and tryptic soy agar (TSA) plates. For enumerating bacterial CFU in the lung and spleen, whole lungs and spleens were homogenized in 2 ml sterile saline and 20 µl of the resulting homogenates were plated by serial 10-fold dilutions on MacConkey and TSA plates. In a similar manner, spleens were homogenized for for bacterial culture. Bacterial colonies were counted after overnight incubation at 37^{o}C.

Bronchoalveolar lavage fluid (BALF) collection*:* BALF was collected and total and differential cell counts and cytokine/chemokine levels were determined. Approximately 3 ml of lavage fluid was retrieved per mouse. Total leukocytes in BALF were determined using a hemocytometer. Cytospin samples were subsequently prepared from BALF cells and stained with Diff-Quick (Fisher). Differential cell counts were determined by microscopic observation of of stained samples. For analysis of cytokines/chemokines levels, the remainder (2 ml) of the undiluted cell-free BALF was passed via a 0.22-µm filter and used immediately or stored at -80^{o}C[26-29].

Lung pathology: The lungs were perfused from the right ventricle of heart with 10 ml isotonic saline at 24 h post- infections as described earlier [24]. Lungs were then removed and fixed in 4% phosphate-buffered formalin. Fixed tissue samples were processed and stained with hematoxylin and eosin (H&E).

Measurement of cytokines and chemokines: We used BALF and lungs that were obtained from animals after *Kp* infection or saline instillation by specific ELISA for IL-17A, TNF-α, IL-6, (eBiosciences, PA) and for KC,GCSF and MIP-2 (R&D systems, MN). The minimum detection limit for cytokines is 8 pg/ml [28, 29].

Immunoblotting: The lungs were collected at the designated time points and used for immunoblotting, as described in our previous publications [24, 26]. The primary antibodies (Abs) to phospho-NF-κB/p65(ser 536), NF-κB/p65, phospho-IKKα/β (ser 176/180), IKKβ, phospho-

IκBα (ser 32), IκBα, VCAM-1, ICAM-1, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185) were added at a 1:1,000 dilution. The primary Abs to total p38, pan Cadherin and GAPDH (Santa Cruz Biotechnology, CA) were added at 1:5,000 dilution. Immunostaining was performed using the appropriate secondary Ab at a dilution of 1:2,000 and developed with ECL plus western blot detection system (Amersham Pharmacia Biotech, Piscataway, NJ). To demonstrate equal protein loading on gels, the blots were stripped and reprobed with Ab specific for total p38, pan Cadherin or GAPDH.

***Blood neutrophil count**:* Total leukocyte count was determined in 100 μl of blood. Differential leukocyte count was determined using cytospin slides made from each sample after staining with Diff-Quik reagents. Total leukocyte count was calculated based on neutrophil percentage and total blood volume (weight X 0.06%= total blood volume). Total leukocyte count X neutrophil percentage X total blood volume = absolute blood neutrophil count.

Flow cytometry: A total of 50 μl of whole blood or BALF from WT or RIP-$2^{-/-}$ mice treated with saline or Kp was aliquoted into flow cytometry tubes and Fc blocked. 10 μl of mouse-conjugated anti-mouse Gr-1, and CXCR-2 (R&D, Minneapolis,MN) antibodies were added to appropriate tubes. Samples were vortexed and incubated for 30 min at room temperature in the dark. Cells were washed in 2 ml PBS and centrifuged at 1000 rpm (200 x g)/8 min. Red Blood cells were lysed by adding 2 ml NH_4Cl lysing buffer to each sample tube, mixing well and incubating at room temperature for 10 min. The samples were then centrifuged immediately at 1000 rpm (200 x g) for 8 min and the supernatant was removed. Cells were washed twice with PBS. Cells were fixed by adding 200 μl of cold 1% formaldehyde-PBS and stored at 2-6 ^{o}C for FACS analysis [28, 30].

Bone marrow derived macrophages (BMDMs: Marrow cells were isolated from the bone marrow of RIP-2$^{-/-}$, and WT (control) mice and differentiated into macrophages for 7 days by adding M-CSF. A total of 1X10^{6}cells/well were used for each group at each time point for infection with 0.1 MOI of Kp and incubated at 37˚C with slow agitation. At 2h and 4h, cell pellets and supernatants were collected and the cell pellets of BMDMs were processed for western blot analysis to determine the expression of RIP-2 and activation of NF-κB and MAPKs. Supernatants from BMDMs were used for the determination of cytokine and chemokine levels.

Determination of IL-17A producing cells using flow cytometry: The procedures involving of IL-17A producing cells have been reported earlier [31, 32]**.** Lung samples obtained from both WT type and RIP-2$^{-/-}$ mice after 48 h post Kp (10^{3}CFUs/animal) infection were minced and digested with collagenase. Resulting single cell suspension was stimulated with PMA (50ng/ml), ionomycin (750ng) and golgi stop (7µl/10 ml) for 5 hours. Stimulated cells were surface stained with markers for IL-17A producing T (γδ and CD4) cells. Following incubation, cells were washed, fixed and permeabilized for intracellular staining. Permeabilized cells were subsequently incubated with an IL-17A Ab for intracellular staining. Finally, cells were washed and resuspended for flow cytometry analysis.

Exogenous IL-17 (rIL-17)/ IL-6 (rIL-6) administration: RIP-2 $^{-/-}$ mice were treated i.t with rIL-17A /rIL-6 (1 µg/animal) 1 h after Kp (10^{3} CFUs/50µl/animal) and the control mice were treated with an equal volume of PBS. At 24 and 48h post-infection, BALF was collected and processed for cellular enumeration and the determination of CFUs.

Statistics: Data are expressed as mean $\pm$ SE. The intensity of immunoreactive bands was determined using Gel Digitizing Software (UN-SCAN-IT gel^{TM}) from Silk Scientific, Inc, Utah, USA. Data were analyzed by one- way ANOVA followed by Bonferroni's post- hoc analysis for multiple comparisons using InStat software and GraphPad Prism 4.0. $p<0.05$ were considered significant. Survival curves were compared by Wilcoxon signed- Rank test.

Results

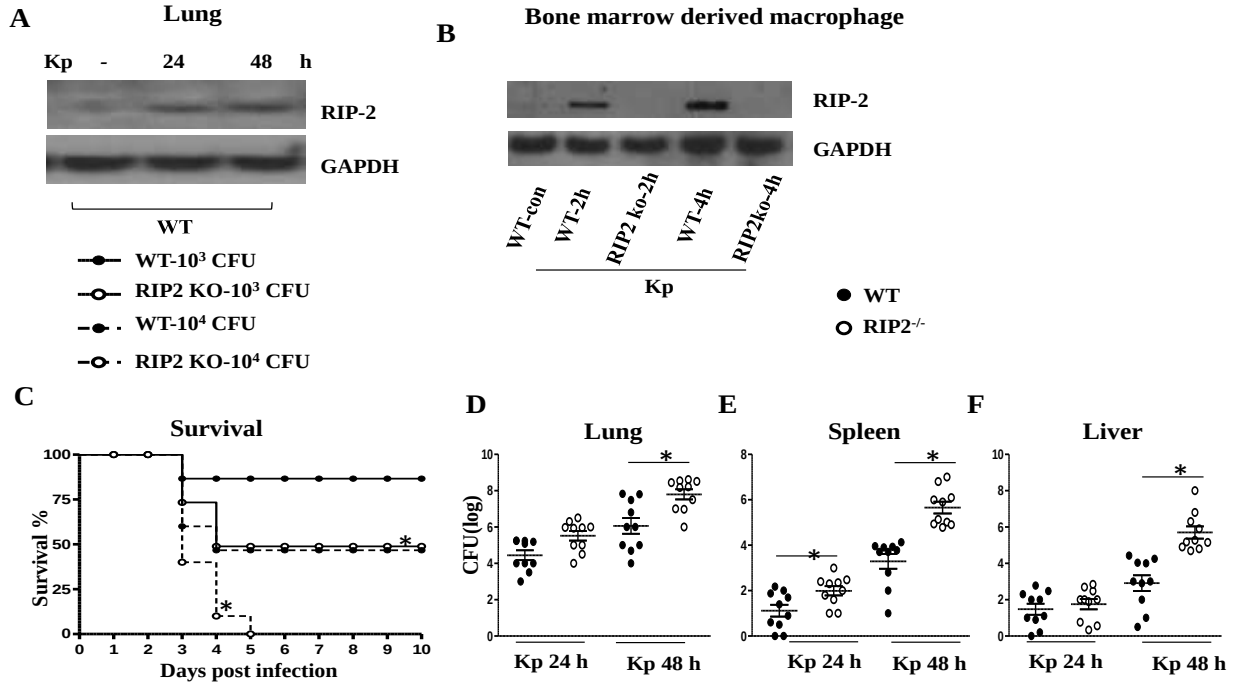

Figure 5.1: RIP-2 is important for host defense against pulmonary Kp infection: **A**. RIP2 protein expression in C57Bl/6 (WT) mice at 24 and 48 h post-Kp infection. WT mice were infected i.t with Kp and lung samples were processed for western blotting using an Ab against RIP-2. This is a representative blot from 3 separate experiments. (*n*= 3 mice/group); *, *P*<0.05 between infected and saline challenged (control) mice. **B**. RIP2 expression in bone marrow derived macrophages (BMDMs) from C57Bl/6 (WT) and RIP2 $^{-/-}$ mice at 2 and 4 h post-Kp infection. BMDMs from RIP2$^{-/-}$ and WT mice were infected with Kp and cell pellets were processed for western blotting using Abs against activated or regular form of RIP2. This is a representative blot from 3 independent experiments. *n*= 3 mice/ group (out of place-combine this with 5.7) *, *P*<0.05 between infected and saline-challenged mice. **C**. Enhanced mortality in RIP-2$^{-/-}$ mice following i.t. Kp infection. RIP-2$^{-/-}$ and WT (C57Bl/6) mice were i.t. inoculated with 10^3 CFU/mouse of Kp, and survival was monitored for 10 days. *n*=14 mice in each group. *, *P*<0.05 determined by Wilcoxon signal-rank Test. **D-F**. Impaired bacterial clearance in the lung, spleen and liver RIP-2$^{-/-}$ mice after Kp infection (10^3/mouse). Bacterial CFUs in mice were determined in tissues of RIP2$^{-/-}$ and WT mice after Kp infection. (n=4-6 mice at each time point. *, Significant differences between RIP-2$^{-/-}$ and WT mice (P<0.05). Median values represented by a horizontal line.

RIP-2-deficient mice show higher mortality and attenuated bacterial clearance.

To investigate whether RIP2 expression is altered during Kp infection , WT (C57Bl/6) were infected i.t with 10^3 Kp CFUs, lungs were harvested at 24 and 48 h post-infection and processed

for western blotting (Fig. 5.1A). Notably, we observed increase in RIP2 expression upon infection compared to saline- challenged controls.

We next assessed whether RIP-2 is protective in mice against i.t. Kp infection of the lungs. We infected RIP-2 mice and WT controls with 10^3 Kp CFUs and monitored them for up to 14 days. We observed RIP-2 mice were more susceptible to Kp infection compared to the WT controls. KO mice showed 60% mortality (10^3 cfu/ animal) whereas 100% of KO mice succumbed to a higher inoculum (10^4 cfu). To determine whether this impaired survival is due to defect in bacterial clearance from the lung and/or bacterial dissemination, we examined the bacterial burden in the lung and extra pulmonary organs, such as spleen and liver. RIP-$2^{-/-}$ mice displayed bacterial loads in the lungs and enhanced bacterial dissemination distal organs (Figs. 5.1B-E).

Impact of RIP-2 deletion in neutrophil numbers and cytokine/ chemokine levels in lungs during Kp infection.

Neutrophils plays a critical role in the clearing bacteria from tissues. Since RIP-$2^{-/-}$ mice demonstrated a higher bacterial burden in the lung, we determined the neutrophil influx into the lung after 24 h and 48h post-Kp infection. We found reduced neutrophil recruitment in the lungs at the designated time-points after infection in the knockout mice compared to WT mice (Figs. 5.2F-H). Histopathology of the lungs of RIP-$2^{-/-}$ mice showed reduction in cellular influx and alveolar edema in the lung sections (Fig. 5.2I-J). Chemokine production drives neutrophil influx into the lungs. Sandwich ELISA was used to determine the levels of neutrophil chemokines (MIP-2, KC and LIX) in BALF. The level of MIP-2, a potent neutrophil chemokine is reduced in BALF of RIP-$2^{-/-}$ mice at 24 and 48h post-infection (Fig. 5.2 E). Figure 1 G-J illustrates reduced level of IL-6 and IL-1β in RIP$2^{-/-}$ mice compared to their littermate controls after infection.

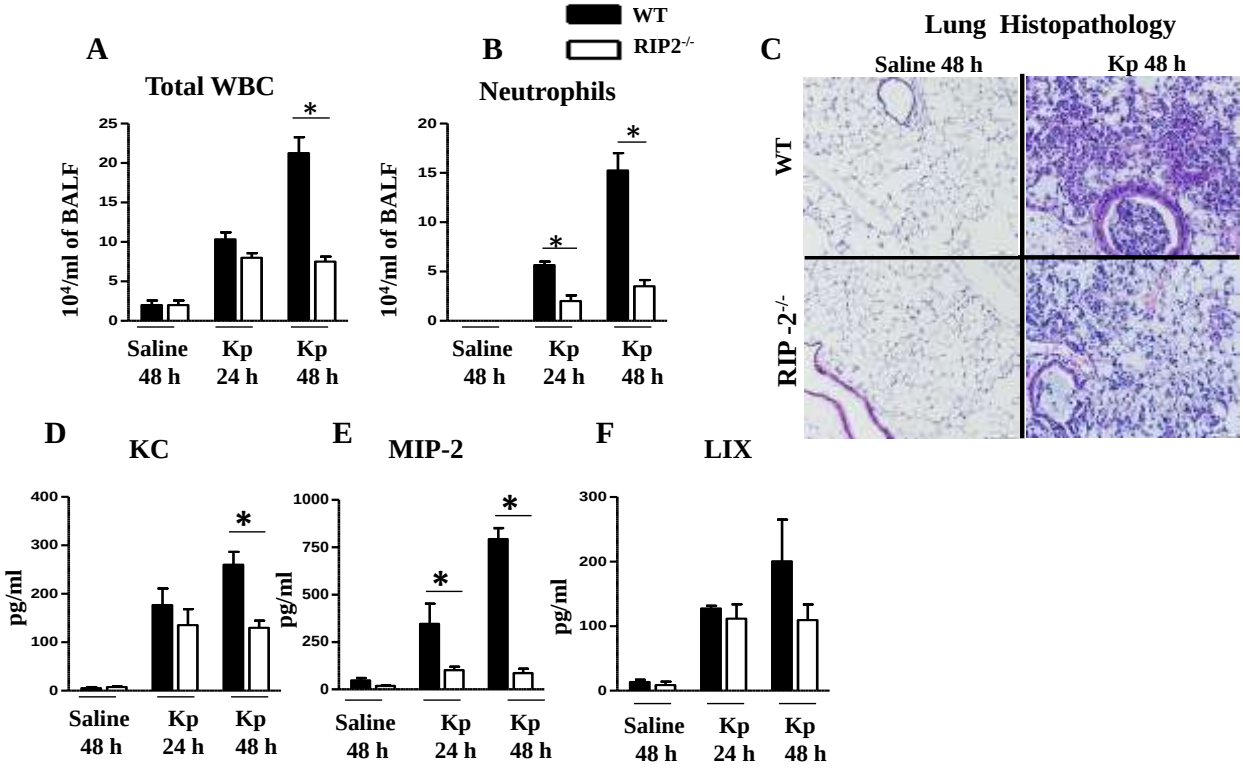

Figure 5.2: Impaired neutrophil recruitment and cytokine responses in the lungs of RIP-2$^{-/-}$ mice after infection with Kp.

A-B.Total leukocyte and neutrophil numbers in the lungs of RIP2$^{-/-}$ mice after Kp inoculation. Both RIP-2$^{-/-}$ and WT animals underwent BALF or lung tissue collection at 24 or 48 h after challenge with Kp(10^3 CFU/mouse). (n=6-8 mice/group). (* indicates $p<0.05$ compared with RIP-2$^{-/-}$ mice). **C**. Lung histology in RIP-2$^{-/-}$ mice following Kp inoculation. H&E stained lung sections from mice challenged with Kp or saline for 48 h are shown. Leukocyte numbers and alveolar edema as well as bacterial numbers were calculated in lung sections as described in Materials and Methods. Representative images of 4 mice in each condition with comparable results (magnification x200) are shown. **D-F**. Chemokine (MIP-2, KC and LIX) concentrations in BALF were measured by sandwich ELISA after Kp infection. A total of4-6 animals were used at each time point. Significant differences between RIP-2$^{-/-}$ and WT mice are indicated by asterisks ($p<0.05$).

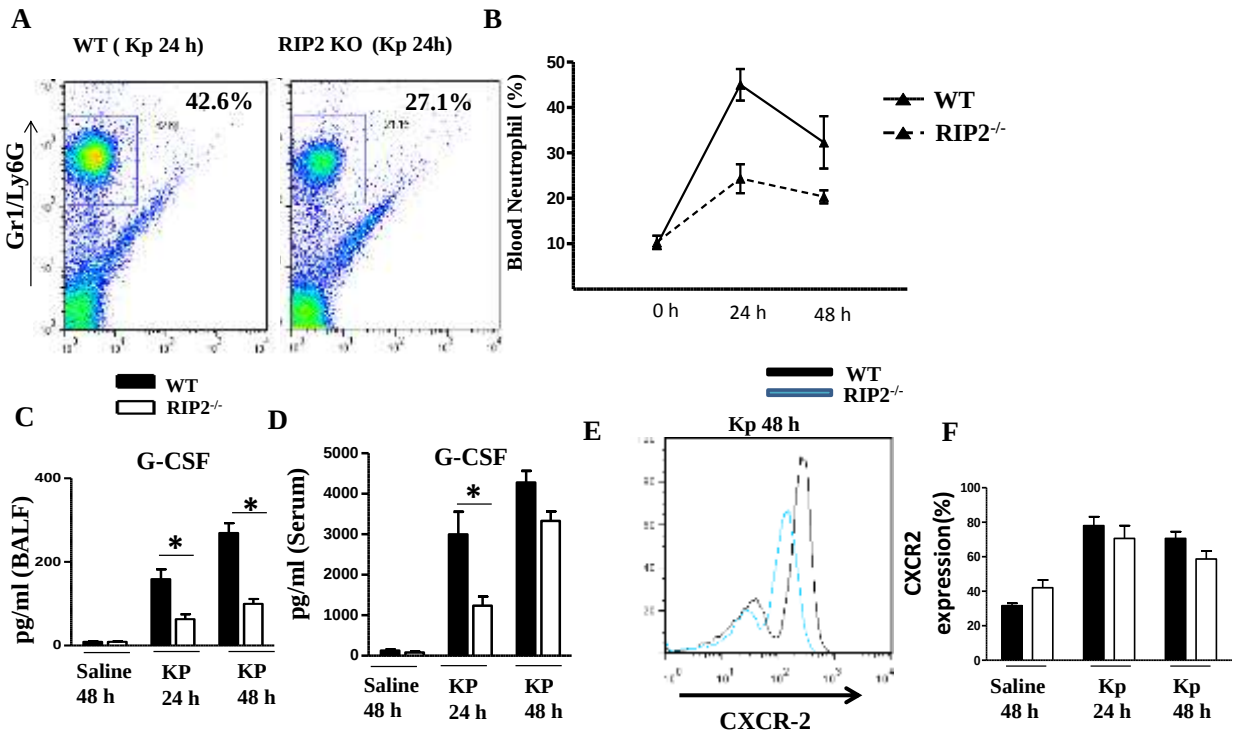

Figure 5.3: Decreased blood neutrophil numbers and G-CSF concentrations in BALF after Kp infection of the lung. A-B Blood neutrophil percentage and absolute blood neutrophil numbers were determined following i.t. Kp infection. Neutrophils were gated and the % of Gr-1/Ly6G positive cells was determined**. C.** G-CSF protein concentration in BALF was determined by using a sandwich ELISA. Data shown here are a representive of 3 individual experiments where n=4-6 and *p<0.05.**D** Flow cytometric analysis of blood from WT mice at 24 and 48 h after i.t. Kp (10^3 CFU/mouse) infection using tagged antibodies against CXCR2. This is representative of 3 independent experiments with comparable results.

Reduced blood neutrophil numbers and CXCR2 expression on neutrophils of RIP2 KO mice

Intrapulmonary Kp infection results in bacterial dissemination to the bloodstream. During Kp infection, blood neutrophil numbers are increased in WT mice, which is an important protective mechanism[33]. CXCR2 is a chemokine receptor that attracts neutrophils to the site of infection from the blood [10]. Although, we observed RIP-$2^{-/-}$ mice have low blood neutrophil numbers after Kp infection (5.3A-B), RIP-2 deficiency did not affect CXCR2 expression on neutrophils (5.3D,E). Since we found attenuated neutrophil numbers in blood, we also determined the

GCSF levels in the lung. RIP-$2^{-/-}$ mice showed reduced GCSF level compared to WT controls in the blood(5.3C). These findings show that RIP-2 regulates blood neutrophil numbers during Kp via the modulation of G-CSF levels following infection.

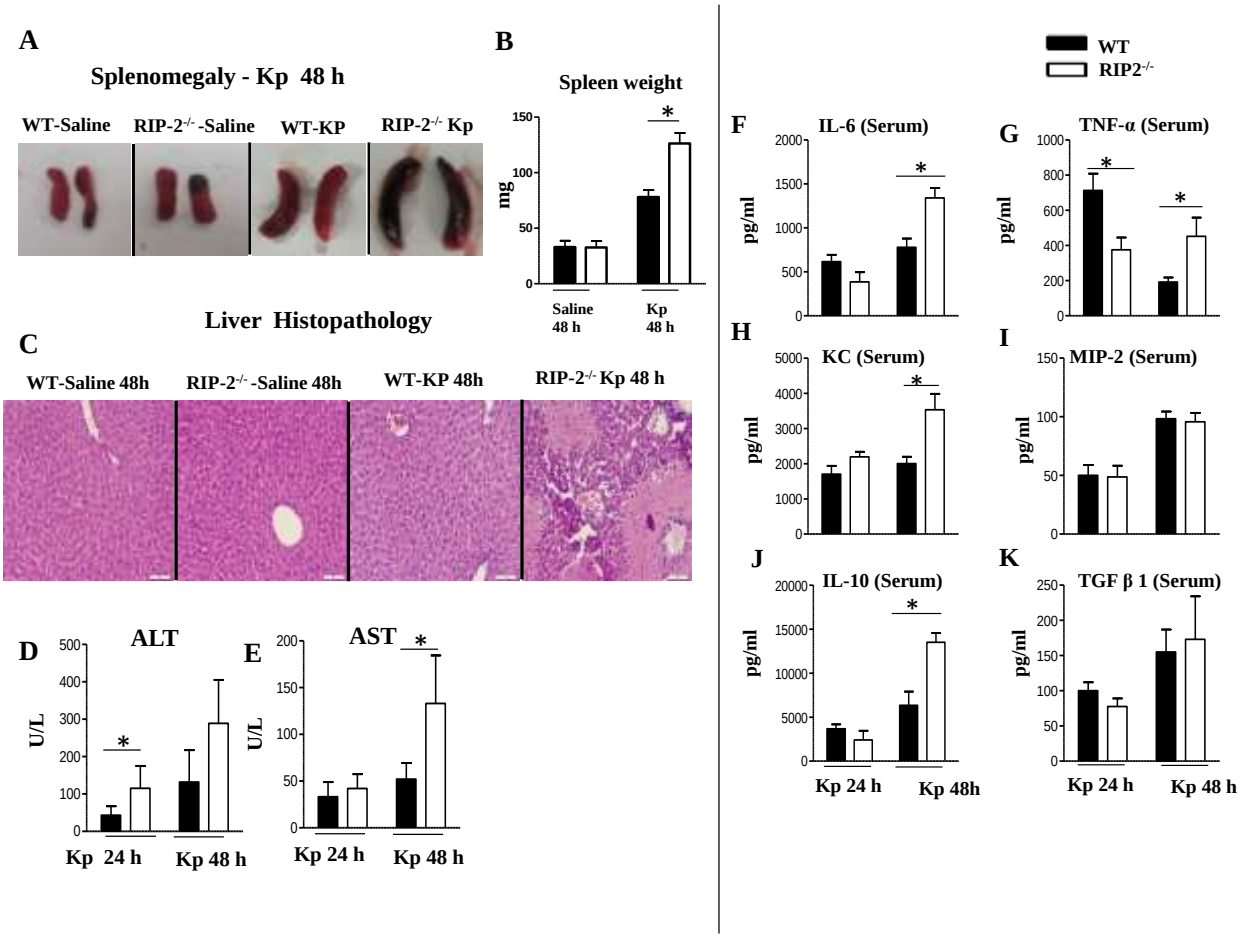

Figure 5.4: Increased pathological changes accompanied by altered inflammatory mediators in RIP-$2^{-/-}$ mice after infection with Kp. A. Splenomegaly was observed in RIP$2^{-/-}$ mice 48h after Kp infection. **B.** Histopathological section of liver showing coagulative necrosis in RIP$2^{-/-}$ mice 48h post Kp infection. WT and RIP-$2^{-/-}$ mice were infected with Kp (10^3 CFU/mouse) for 24 and 48 h. (n=6-8 mice/group). (* indicates $p<0.05$ compared with RIP-$2^{-/-}$ mice). **C-D** Liver enzymes ALT and AST levels after Kp infection. ALT and AST levels were determined in serum of RIP-$2^{-/-}$ mice and WT mice after i.t Kp infection. Data is expressed as mean $\pm$ SEM. n=6-8 mice/group, *$p<0.05$. **E-K.** Cytokine (IL-6, TNF–α, IL-1β, IL-10 and TGF-β) and chemokine (MIP-2 and KC) concentrations in serum were measured by sandwich ELISA after infection with Kp. (n=4-6 animals per time point). Significant differences between RIP-$2^{-/-}$ and WT mice are indicated (*, $p<0.05$).

RIP2$^{-/-}$ mice show pathological changes in distal organs

Since RIP-2 $^{-/-}$ mice showed extensive dissemination, we measured the systemic responses during Kp lung infection. We found that RIP-2 deficient mice showed increased levels of cytokines TNF-α and IL-6, as well as the chemokine KC in the serum after 48 h of infection(Fig 5.4E-J). We also observed splenomegaly in RIP-2 deficient mice compared to WT mice. In liver histopathology, RIP-2 $^{-/-}$ mice showed multifocal coagulative necrosis, vasculitis, occasional thrombi and intralesional bacteria compared to WT controls (5.4 A-D). These findings clearly demonstrate that RIP-2 is involved in the systemic immune responses during Kp infection.

RIP-2 regulates IL-17A production in the lungs following Kp infection

IL-17A is shown to be an important cytokine that plays a critical role in containing Kp by regulating lung and blood neutrophil numbers[8]. We also reported that RIP-2 regulates IL-17A during *E. coli* pneumonia [21].Therefore, we determined IL-17A levels and the numbers of IL-17A producing cells in the lung. We found RIP-2 deficiency reduced IL-17A level and numbers of major IL-17A producing cells (CD4 and γδ cells) during Kp infection(Fig 5.5 A-D and I). We also determined whether lack of RIP-2 in IL-17A producing cells or a RIP-2 deficiency in cells that are responsible for cytokine production(IL-1β,IL-6) drive the induction of IL-17 producing cells during an acute bacterial infection. Intriguingly, we used in-vitro co-culture system to study this possibility, we used naïve CD4 cells and CD11C positive cells in different combinations and treated them with heat killed Kp. We found that the defect in RIP-2 of CD11C cells showed reduced IL-1β and IL-6 production (Fig 5.5 E-G) that is associated with reduced IL-17A levels compared to the WT control.

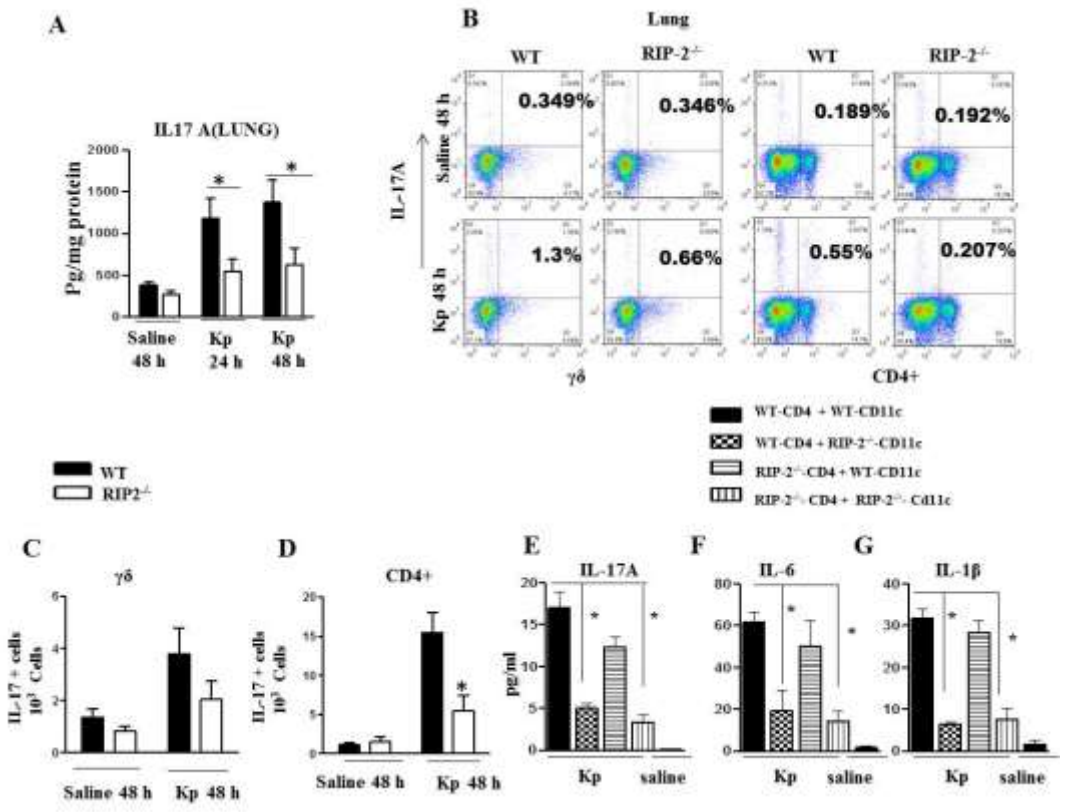

Figure 5.5: RIP-2 regulates IL-17A production in the lungs following Kp infection A. Reduced IL-17A levels in the BALF of RIP-$2^{-/-}$ mice at 24 and 48 h post-Kp infection. (n=5-7 mice/group/time-point). **B-E.** Numbers of IL-17A producing cells in the lungs following Kp infection. Lung tissues were obtained from WT and RIP-$2^{-/-}$ mice at 48 h post-Kp infection and digested, flow cytometric analysis was performed in cells obtained from the lung digest. Reduced IL-17A producing T cells (γδand CD4+ cells) in the lungs of RIP-$2^{-/-}$ mice upon Kp infection. **F-H**. Cytokine (IL-17A, IL-6 and IL-1β) levels in the supernatants from $CD4^{+}$/$CD11C^{+}$ co-culture. $CD4^{+}$cells and $CD11C^{+}$ cells were purified from spleens of RIP-$2^{-/-}$ and WT mice and they were co-cultured in different combinations with Kp as described in the materials and methods. (n=6-8 mice/group, *p<0.05).

Exogenous IL-17A restores the host defense in RIP-2 deficient mice.

We determined whether IL-17A is sufficient to rescue RIP-2 deficieincy during Kp infection(Fig 5.6A). Thus RIP-2 deficient mice administered with exogenous IL-17A after Kp infection. Exogenous IL-17A administration in RIP-$2^{-/-}$ mice partially rescued neutrophil numbers in lung and blood (Fig 5.6 B, G and H) and chemokines KC, MIP2 and GCSF in the lung (Fig 5.6 C-D

and I) as compared with the RIP2 KO mice treated with vehicle control (BSA). Furthermore, we observed that exogenous IL-17A partially but significantly rescued bacterial dissemination and

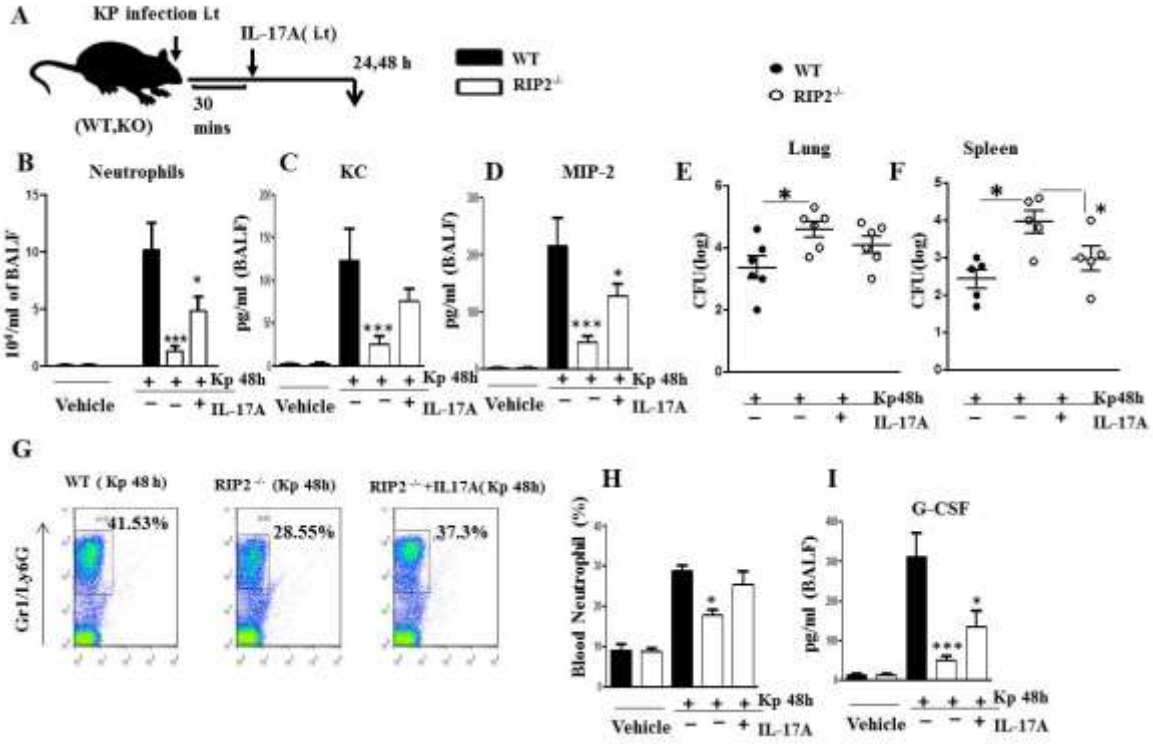

Figure 5.6: Exogenous IL-17A rescues host defense in RIP-2$^{-/-}$ mice following Kp infection **A**. Schematic diagram describing the treatment plan. Mice were infected with 10^6 CFUs/mouse i.t. and administered rIL-17A or vehicle (BSA 1 h later, and BALF was collected at 24 h post-infection. For experiments A-E, a total of 7-9 mice/group was used. *, $p<0.05$. **B**. Enhanced neutrophil recruitment in airspaces **(C-D)** chemokine levels (KC and MIP2) and bacterial clearance **(E)** following exogenous administration of rIL-17A in Kp- infected RIP-2 $^{-/-}$ mice. **F-G**. Blood neutrophil percentage and absolute blood neutrophil numbers were determined following i.t. Kp infection and rIL-17A (1 μg/mouse) (A-B)**. H.** G-CSF protein concentrations in BALF and in serum were determined by using a sandwich ELISA. Data shown here are a representation of 3 individual experiments where n=4-6 and *$p<0.05$.

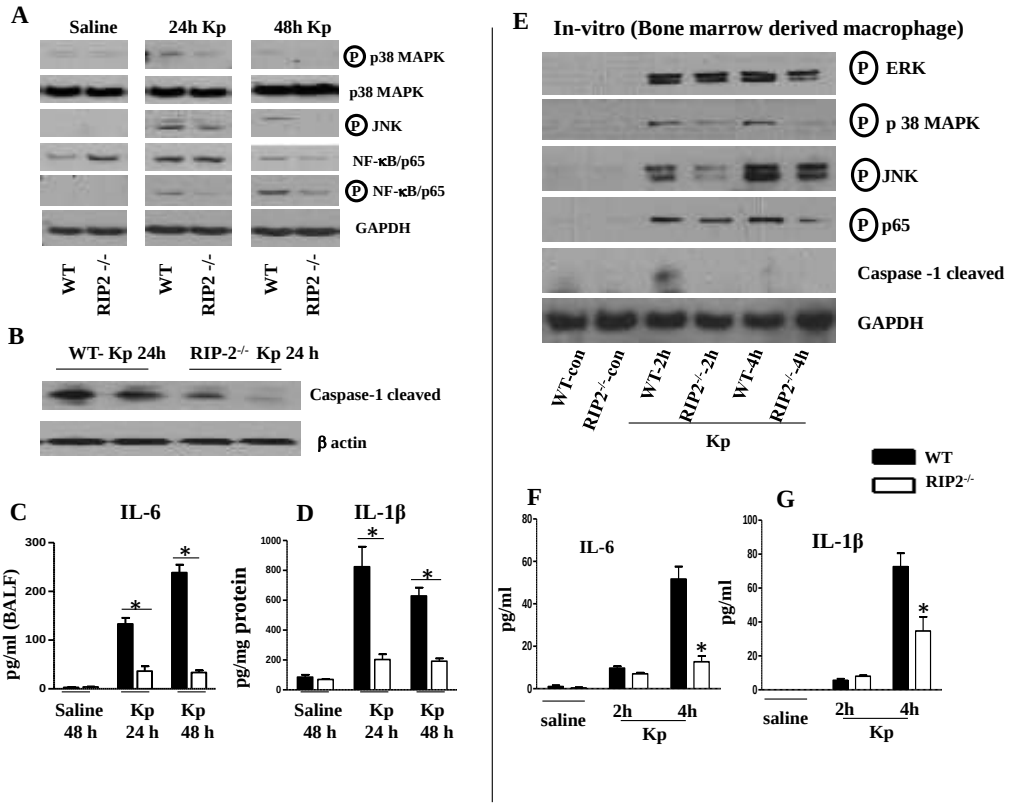

Figure 5.7 RIP2 regulates the activation NF-κB, MAPKs and inflammasome cascades following Kp infection of the lungs. **A-B**. Activation of NF-κB, MAPKs and the inflammasome (caspase 1 cleavage and IL-1β production) in the lung following infection with Kp. Lung homogenates from RIP-2$^{-/-}$ mice and their controls were prepared at 24 and 48 h after infection with Kp. NF-κB expression and phosphorylation of NF-κB and phosphorylation of JNK and p38 MAPKs were determined using western blots of lung homogenates. The blots are representative of 3 independent experiments with similar results. **C-D**. Cytokine (IL-6, and IL-1β) concentrations in BALF were measured by a sandwich ELISA after infection with Kp. (n=4-6 animals were used at each time point). Significant differences between RIP-2$^{-/-}$ and WT mice are indicated by asterisks ($p<0.05$). **E.** Reduced activation of NF-κB, MAPKs and the inflammasome cascades in BMDMs obtained from RIP-2$^{-/-}$ mice following infection with Kp. Representative western blot from 3 separate experiments were shown. **F-G.** Attenuated IL-6 and IL-1β levels in culture supernatants obtained from BMDMs of RIP2$^{-/-}$ mice following Kp infection. Experiments were performed in triplicate wells. *denotes the differences between WT and RIP-2 $^{-/-}$ mice ($p<0.05$).

Essential role of RIP-2 in the production of IL-6 and IL- 1β during Kp lung infection

We found that IL-6 and IL-1β is highly regulated by RIP-2 in the in-vitro co culture system. IL-6 and IL-1β are known to induce IL-17A production [34]. We sought to determine the level of IL-6 and IL-1β in the lung as well as in bone marrow derived macrophage during Kp infection. IL-6 is produced by the activation of NF-κB and MAPKs. IL-1β is produced in pro form and activated by caspase-1 [13]. Therefore, we determined the role of RIP-2 in NF-κB and MAPKs activation. We also found IL-6 and IL-1β were significantly reduced in the lung of RIP-2-deficient mice compare to WT mice during Kp infection (Fig 5.7 C,D). Furthermore, RIP-2 mice show reduced activation of NF-κB MAPKs and caspase-1 activation (Fig 5.7 A,B). In addition, RIP-2-deficient macrophages demonstrate reduced NF-κB, MAPKs and caspase-1 activation accompanied by low levels of IL-1β and IL-6 when compared to WT controls (Fig 5.7 E-G).

Exogenous IL-6 increases IL-17A levels and neutrophil numbers in RIP-2 deficient mice during Kp infection*.*

Previous studies have shown that IL-6 regulates IL-17A production through STAT3 tyrosine kinase activation[35]. STAT3 tyrosine phosphorylation is shown to be important for the induction of IL-17A producing cells from naïve T cells[34, 36]. Therefore, we sought to determine whether recombinant IL-6 can restore IL-17A levels, STAT3 activation and neutrophil numbers in RIP-2 deficient mice. We treated RIP-2 deficient mice with rIL-6 1h after Kp infection and found that exogenous IL-6 alone was able to rescue IL-17A levels and neutrophil numbers in the lung (Fig 5.8A-C). We found that STAT3 tyrosine phosphorylation is also rescued in the RIP-2 KO mice after rIL-6 treatment (Fig 5.8D).

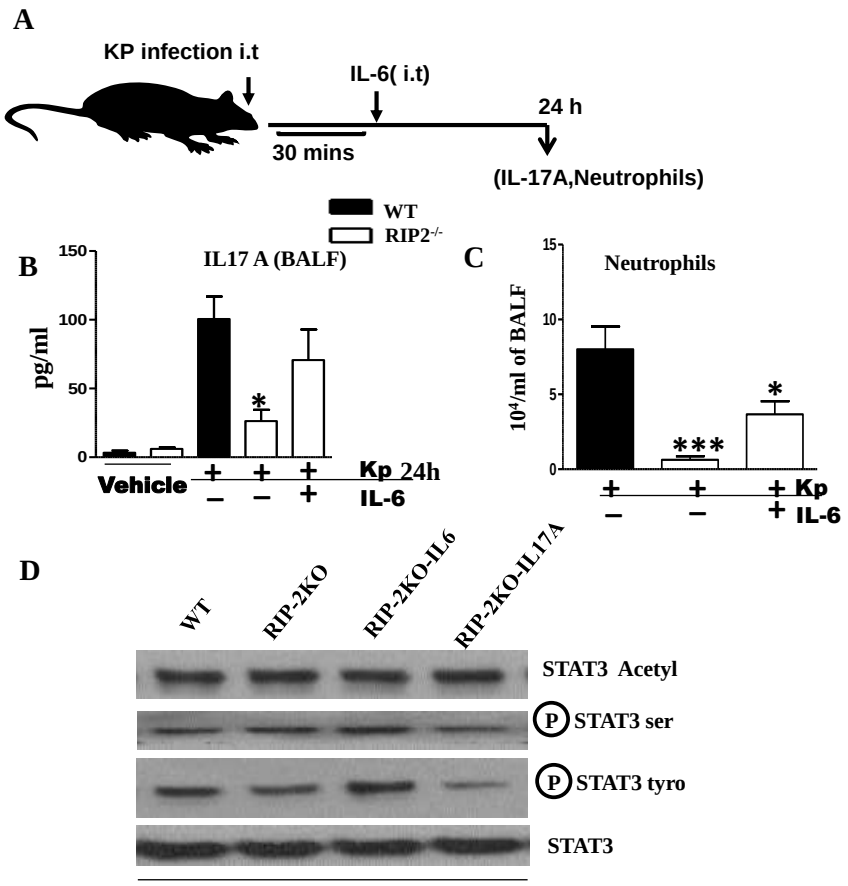

Figure 5.8: Impaired IL-17A levels, STAT3 activation and neutrophil influx in the lungs of RIP2$^{-/-}$ mice are restored by rIL-6. (A) IL-17A levels WT and RIP-2$^{-/-}$ mice infected with 10^3CFUs of Kp and administered with rIL-6 (1 μg/mouse) or vehicle (BSA) 1 h later, sampled at 24 and 48h post infection. **B.** IL-17A levels in BALF determined by sandwich ELISA. Data are presented as mean ± SE (n=5-6 mice/group). (* indicates $p<0.05$ compared with BSA (vehicle) administered mice. **C**. Neutrophil infiltration in air spaces at 48 h after i.t. treatment with rIL-6 (1 μg/mouse) or BSA control. **D.** Activation of STAT3 in lungs post Kp infection and IL-6 treatment. Data shown here is a representation of 3 individual experiments where n=4-6 and *$p<0.05$.

Impact of NOD2 in host defense during Kp lung infection.

We sought to determine the role of NOD2, the effector upstream of RIP-2, during Kp lung infection. We found that NOD2 knockout mice show higher mortality and less neutrophil recruitment in the lung after Kp infection. Our data also demonstrate that NOD2 mediated neutrophil recruitment mainly depends on IL-17A and that exogenous IL-17A treatment restores

the number of neutrophil in the lungs of NOD-2$^{-/-}$ mice to WT-like levels. However, we did not see significant differences between the KO and WT mice with regard to IL-1β levels.

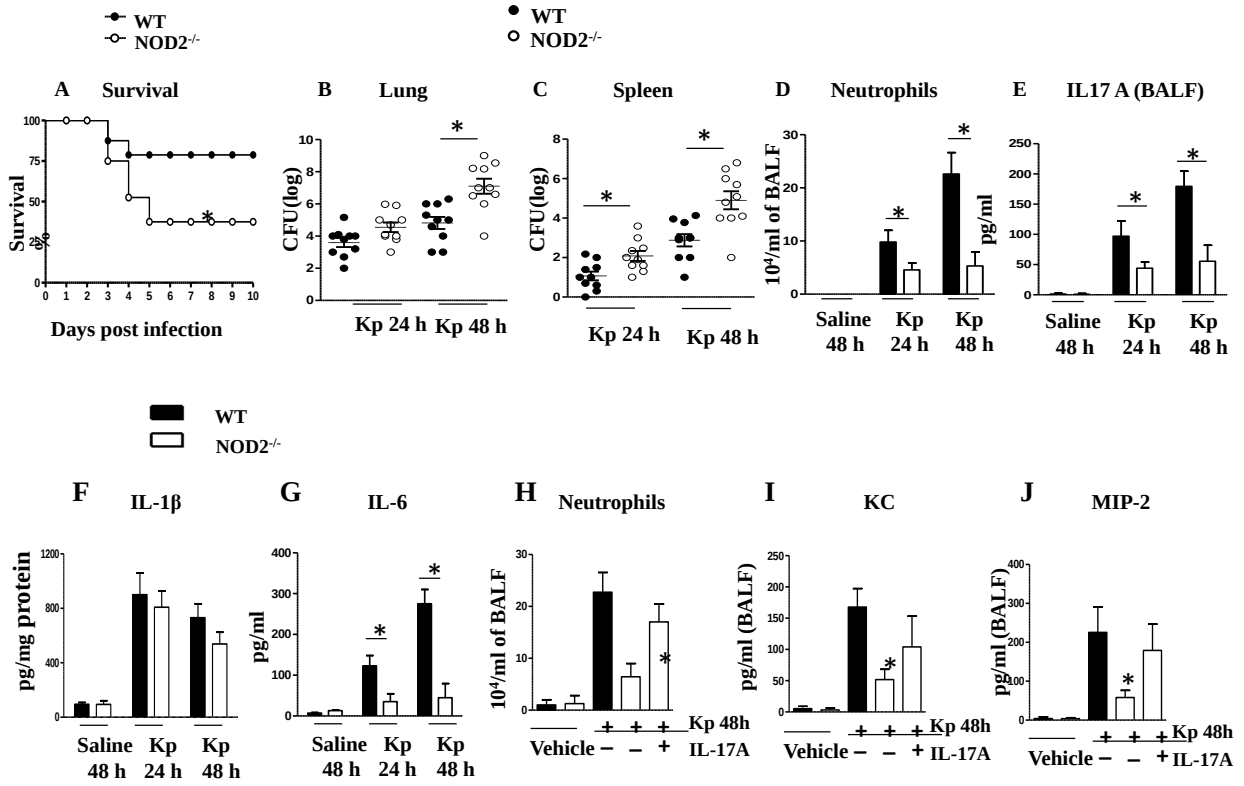

Figure 5.9: NOD2 is essential for neutrophil influx and IL-17A during Kp pneumonia.
A.NOD2$^{-/-}$ mice show higher mortality following i.t. Kp infection. NOD2$^{-/-}$ and WT (C57Bl/6) mice were i.t. inoculated with 10^3 CFUs/mouse of Kp, and survival was monitored up to 10 days. *n*=14 mice in each group. *, *P*<0.05 determined by Wilcoxon Rank Sign Test. **B**. Neutrophil numbers in the lungs of RIP2$^{-/-}$ mice after Kp inoculation Neutrophil numbers in BALF obtained from NOD2$^{-/-}$ and WT mice following 24 and 48h Kp post-infection (n=5-6 mice/group). *indicates significant difference between NOD2$^{-/-}$,WT mice; p<0.05.(**C-D).** Cytokines (IL-17A and IL-1b) levels in the BALF and lung of NOD2$^{-/-}$ and WT mice after Kp infection. Mice were infected by intratracheal instillation of Kp (10^3 CFUs/mouse), and BALF was collected from the lungs at designated time points. Concentrations (pg/ml) of IL-17A (C) and IL-1b (D) in BALF were quantified by sandwich ELISA. Asterisks indicate significant difference between NOD2$^{-/-}$ and WT mice (p<0.05; n = 4-6 mice in each group at each time-point). **E**. Enhanced neutrophil recruitment in airspaces **(F-G)** chemokine levels (KC and MIP2) are is a representation of 3 individual experiments where n=4-6 and *p<0.05.

Discussion

The aim of the current study is to determine the mechanism of IL-17A regulation by RIP-2 during Gram-negative pneumonia and pneumonia-induced sepsis. To accomplish this we infected RIP-2 gene deficient and WT mice i.t with Kp (10^3 CFU/ mouse) to study the host immune response in the lungs, the primary seat of infection, and in other vital organs. We used *Klebsiella* because it is a significant pulmonary pathogen that multiplies and disseminates from a small inoculum [37, 38].

Failure of early and effective host defense leads to bacterial dissemination and sepsis[39]. Gram-negative sepsis is a significant health care problem for critically ill patients [40, 41]. Neutrophils play a critical role in containing bacterial infection. Neutrophil recruitment to the site of infection is initiated by the activation of different PRRs [9, 42]. We have shown that a defect in NOD2 in neutrophils reduces bacterial killing through NADPH activation [22]. In addition, we showed that RIP-2 regulates neutrophil recruitment via IL-17A production[21]. In this study, we show that IL-17A regulation by RIP-2 occurs through IL-6 and RIP-2 is vital to controlling pneumonia and pneumonia induced sepsis.

Neutrophil recruitment is a multistep complex process that essentially requires CXC chemokines: MIP-2 and KC[43]. IL-17A regulates the chemokines MIP-2 and KC as well as the cytokine GCSF, and thus neutrophil- mediated host defense during Kp infection[44]. Our results also show remarkably reduced neutrophil numbers in lungs and in the blood of RIP-2 $^{-/-}$ mice accompanied by lower MIP-2, KC and GCSF levels in the lung compared to WT control. CXCR2 is a CXC chemokine receptor that is expressed on neutrophils, and it is shown to be down-regulated during sepsis [45]. Studies have shown that the CXCR2/CXCR4 ratio regulates neutrophil homeostasis in the blood [46]. Since we found lower neutrophil numbers in the blood and lung, we determined the expression of CXCR2 on neutrophils. Although we observed increased CXCR2 expression on both WT and RIP-2 deficient neutrophils after Kp infection, we

did not see any difference in CXCR2 expression on neutrophil between WT and RIP-2$^{-/-}$ mice. This shows that the defect in neutrophil recruitment to the lung in RIP2$^{-/-}$ mice is mainly due to reduced MIP-2, KC and G-CSF levels that are regulated by IL-17A.

It has been shown that innate IL-17A can be produced by NK cells. γδ cells, innate CD4 and LTi cells [21, 47]. Transformation of naïve T cells to IL-17 producing cells requires activation of the transcription factor RORγt [48]. Activation of another transcription factor, STAT3, is essential for the activation of RORγt. IL-6 initiates the activation of STAT3, which then activates RORγt and leading to IL-17A producing cells[36]. IL-1β and IL-23 induce innate IL-17 producing cells, mainly γζ cells [34, 49]. TLR4 has been shown to regulate IL-17A through IL-23.It has been shown that the NOD2/RIP-2 axis plays a role in regulation of IL-17A.The detailed mechanism of how NOD/RIP-2 regulates IL-17A during acute bacterial pneumonia has not been explored. Our data show that RIP-2 regulates IL-17A production and that exogenous IL-17A restores host defense during Kp pneumonia. This is consistent with our previous findings. We further observed that a RIP-2 deficiency in CD11c^{+} cells, not naïve CD4 T cells leads to reduced IL-17A production using an in-vitro co-culture system. Interestingly, we also observed lower levels of IL-6 and IL-1β in a RIP2$^{-/-}$ CD11c^{+} / CD4^{+} combination and in-vivo after Kp infection.We further determined the role of RIP-2 in regulation of IL-6 and IL-1β in in-vivo and in-vitro models. In our previous study, we have shown that the lack of RIP-2 impairs IL-6 production via reduced activation of NF-κB and MAPKs. Our current study also shows similar results with Kp infection in the lung. In addition, exogenous IL-6 substantially increased IL-17A level, STAT3 activation and neutrophil numbers in RIP-2$^{-/-}$ mice. This finding suggests that IL-6 may play a major role in RIP-2 mediated IL-17 regulation.

Production of IL-1β is mainly regulated by the activation of the inflammasome [50].RIP-2 mediated regulation of inflammasome activation and IL-1β production was our primary interest since we found reduced IL-1β level in the co-culture system . In this study, we found NOD2

independent, RIP-2 dependent IL-1β production. IL-1β production is regulated by inflammasome activation.A defect in RIP-2, not NOD2 show reduced caspase-1 activation in-vivo and in in-vitro models. This suggests RIP-2 may regulate host defense by regulating IL-1β combined with IL-17A.

Furthermore, we observed a high bacterial burden in the lung accompanied by extensive dissemination in RIP-$2^{-/-}$ mice at 48h post Kp infection. These findings clearly demonstrate the importance of RIP-2 in neutrophil-mediated host defense during Kp pneumonia. Very interestingly, we observed that RIP-$2^{-/-}$ mice show higher systemic inflammation compared to WT at 48h post Kp infection. On the contrary, we observed increased serum IL-10 levels in RIP-$2^{-/-}$ mice compared to WT after 48h *Klebsiella* infection. This increase in anti- inflammatory cytokine (IL-10) may be associated with an increased bacterial load in the RIP-2 knockout mice. Studies have shown that increased levels of IL-6 and IL-10 in the serum are associated with increased sepsis symptoms [51, 52]. We further observed an increase in pathological changes in histological sections and gross specimens of extra pulmonary organs in the RIP-2 knockout mice when compared to WT control. This could be mainly due to the presence of a higher bacterial burden and increased inflammation observed in RIP-$2^{-/-}$ mice after Kp infection. This finding clearly demonstrates that RIP-2 is essential to controlling sepsis induced by gram negative bacterial lung infection. Our data further suggests that RIP-2 dependent signaling mainly depends on NOD2 mediated signaling, which further confirms our previous report [22].

In conclusion, our study demonstrates that RIP-2 regulates neutrophil mediated host defense by IL-6 mediated IL-17A production and neutrophil release from bone marrow via IL-17A dependent G-CSF production during Kp pneumonia. In addition, our data also suggests that RIP-2 activates the inflammasome and thereby IL-1β production during Kp pneumonia. In summary, we found that the NOD/RIP-2 axis regulates IL-17A mediated host defense. Taken

together, these data demonstrate a therapeutic potential of RIP2 as a key molecule to augment the host defense during Gram negative pneumonia.

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Chapter 6: Role of RIP-2 in polymicrobial sepsis

Introduction

Sepsis is a life-threatening medical condition caused when a polymicrobial infection sets in the bloodstream triggering an uncontrolled inflammatory reaction that eventually causes multiple organ damage, immune suppression and other infections like pneumonia[1]. Sepsis still remains the leading cause of death in intensive care units. In the U.S. alone: more than 700,000 patients develop sepsis, around 40% of those die [2, 3]. Furthermore, more than 42,000 cases of pediatric sepsis are reported in the U.S. annually[4]. Specific therapies are generally unavailable because pathogenic mechanisms are still unclear [5]. Studies have shown that early neutrophil recruitment is essential to contain the bacterial infection associated with sepsis [1, 6, 7]. During experimental sepsis, neutrophils display reduced migration to the site of infectionas well as impaired function at the site of infection [8]. [9]. Studies suggest multiple TLRs play additive or synergistic roles during polymicrobial sepsis and their responses vary with the severity of the sepsis model [10, 11]. However, the role of NOD-like receptor signaling during sepsis has not been explored. In our *E. coli* pneumonia model, we have shown that RIP-2plays a critical role in neutrophil recruitment to the site of infection via IL-17A production. Furthermore, studies have shown that RIP-2 is highly up-regulated in human blood leucocytes during sepsis [12].

Nonetheless, the role of IL-17A in sepsis is not clear and seems to be dependent on the severity of sepsis [13, 14]. During moderate or mild sepsis, IL-17A has been shown to play a protective role by recruiting neutrophils to the site of infection[15]. In the current study, we determined the role of RIP-2 in local and systemic responses during polymicrobial sepsis. We used the cecal ligation puncture model because it is the highly relevant model which mimicshuman sepsis or septic peritonitis [16]. We provide evidence that RIP-2 plays a critical role in polymicrobial sepsis. We also found RIP-$2^{-/-}$ mice show reduced IL-17A in the peritoneum compared to their WT littermates. There was substantially lower neutrophil numbers in the blood of RIP-2 knockout mice as compared to WT controls. Furthermore, RIP-$2^{-/-}$ mice

show increased mortality combined with severe dissemination and excessive systemic inflammation.

Materials and Methods

Animals

Wild-type C57BL/6 mice and RIP-2 knockout (KO) mice, 6 to 11-week-old males weighing 18 to 25 g were used for the experiments[17]. Mice were kept under specific, pathogen-free conditions and had free access to standard rodent food and water ad libitum.

Cecal ligation and puncture (CLP)

Polymicrobial sepsis was induced by CLP as previously described [16]. Briefly mice were anesthetized with an intraperitoneal (i.p.) injection of a mixture of 10 mg/kg xylazine and 100 mg/kg ketamine hydrochloride. The abdomen was shaved and cleaned with alcohol. A 1 cm midline skin incision was made and the peritoneum was opened. Under sterile condition, the cecum was ligated and punctured once using a 21 gauge needle which causes around 30% mortality in the wild type [18]. The cecum was placed back into the abdomen and the two layers were closed with sutures. All mice were treated with 1ml warm saline for rehydration (s.c). Sham mice were treated identically, except for the ligation and puncture of the cecum[16].

Bacterial counts

For enumerating bacterial CFUs in the peritoneum and blood, peritoneal fluid and blood were plated on TSA agar after serial dilution. Blood was collected from the heart for bacterial counts. Whole lung, liver and spleen tissues were homogenized in 1 ml sterile saline and 20 µl of the resulting homogenates were plated by serial 10-fold dilutions on TSA plates [19]. In a similar manner, spleens were homogenized for bacterial culture. Bacterial colonies were counted after overnight incubation at 37^{o}C.

Leukocyte counts in the peritoneum

Total leukocytes in the peritoneum were determined using a hemocytometer. Cytospin samples were subsequently prepared from peritoneal fluid and stained with Diff-Quick (Fisher). Differential cell counts were determined by direct counting of stained slides.

Determination of blood neutrophil numbers and expression of CXCR2 on neutrophils

A total of 50 µl of whole blood from mice treated with saline or Kp was aliquoted into flow cytometry tubes and Fc blocked.10 µl of mouse-conjugated anti-mouse Gr-1(Ly6G), and CXCR-2 (R&D, Minneapolis,MN) antibodies were added to appropriate tubes. Samples were processed for flow cytometry and stored at 2-6 ^{o}C for FACS analysis[20, 21]

Cytokine and chemokine in the peritoneum and serum

Peritoneal lavage and serum were collected at 6 and 24 h time points after CLP surgical procedure.The level of cytokines (IL-1β,IL-6,TNF-α and IL-17A) and chemokines (KC and MIP-2) was measured by ELISA (eBiosciences and R&D).

Bronchoalveolar lavage fluid (BALF) collection

Total and differential cell counts, along with cytokine/chemokine levels were determined from BALF. Approximately 3 ml of lavage fluid was retrieved per mouse. Total leukocytes in BALF were determined using a hemocytometer. Cytospin samples were subsequently prepared from BALF cells and stained with Diff-Quick (Fisher). Differential cell counts were determined by direct counting of stained slides. For examination of cytokines/chemokines, the remainder (2 ml) of the undiluted cell-free BALF was passed via a 0.22-µm filter and used immediately or stored at -80^{o}C[20, 22-24].Protein was measured using a protein assay kit from Sigma.

Measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity

The levels of AST and ALT (U/ml) were measured in the serum of WT and RIP-2 knockout mice after 24 h of CLP as markers of liver injury. using commercial colorimetric kits (Thermo scientific). The results are expressed as means ± SEM.

Statistical analysis

Data were expressed as means ± SE. Data were analyzed by one-way or two-way ANOVA followed by Bonferroni's post hoc correction for multiple comparisons. Statistical calculations were performed using InStat software and GraphPad Prizm 4.0 (San Diego, CA). Differences were considered statistically significant when *P<0.05 between WT and KO mice.

Result

Mice deficient in RIP-2 are more susceptible to CLP induced sepsis.

To explore the role of RIP-2 in polymicrobial sepsis, we developed a non-severe septic peritonitis model using CLP. We observed 80% mortality in RIP-$2^{-/-}$ mice while WT control mice showed 30% mortality (Fig 6.1A). Higher bacterial burden in the peritoneum and systemic organs was observed in RIP-$2^{-/-}$ mice(Fig6.1 B,C) . Early neutrophil recruitment is essential for an effective host protective response. We found RIP-2 knockout mice have a significantly lower number of neutrophils in the peritoneum at 6 and 24h post CLP(Fig6.1D, E). In addition, a cytospin of peritoneal fluid from RIP-$2^{-/-}$ mice show lower neutrophil numbers accompanied by higher bacterial counts when compared to WT controls (Fig 6.1 F).

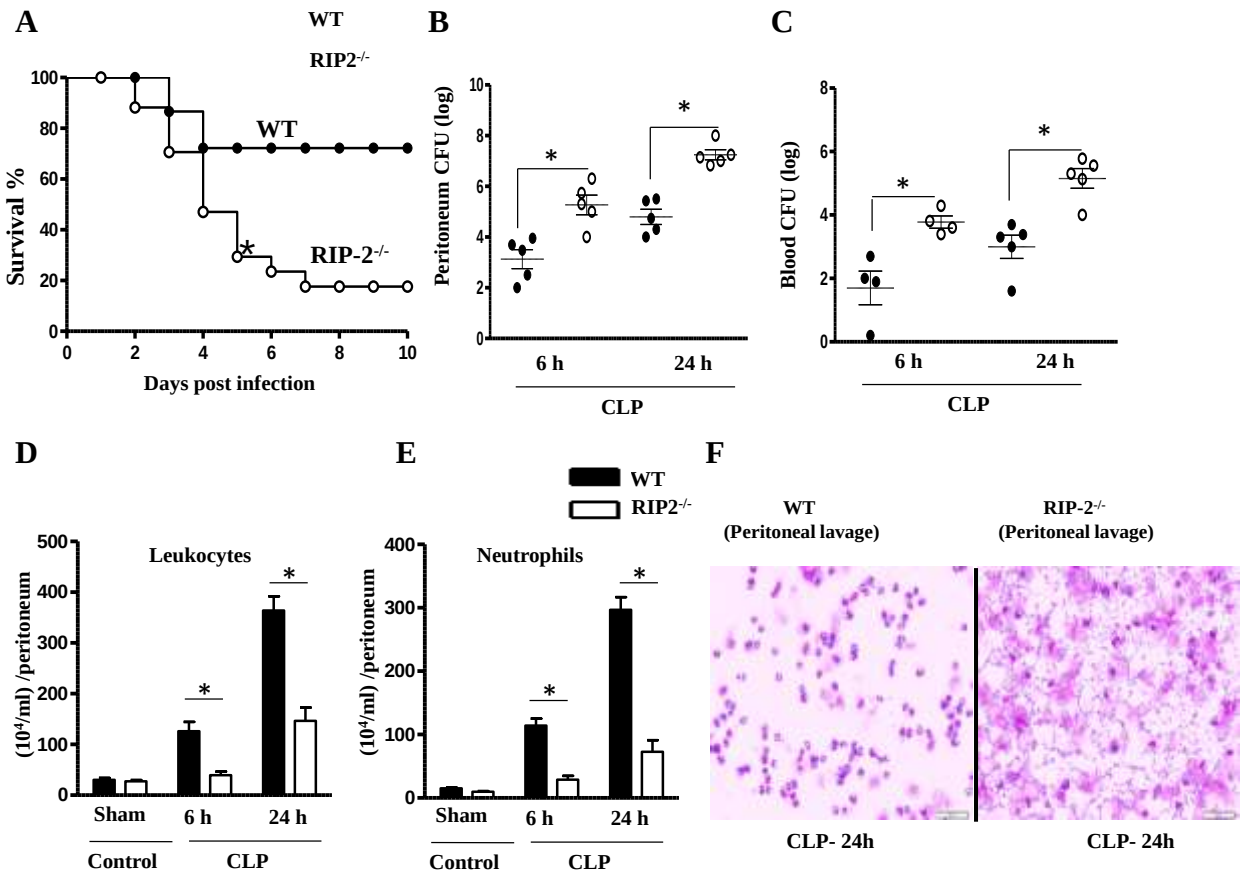

Figure 6.1. RIP2 is indispensable for host defense to polymicrobial sepsis. A, Enhanced mortality in RIP-2$^{-/-}$ mice after CLP. WT and RIP-2 $^{-/-}$ Mice were subjected to sham surgery and moderate sepsis using the CLP model and survival was assessed up to 15 d. Data are from two separate experiments (n = 10 mice/ group). * indicates the difference between RIP2$^{-/-}$ and WT mice ($p<0.05$, Wilcoxon rank sign test). **B and C**, Impaired bacterial clearance in the peritoneum and blood in RIP2$^{-/-}$ mice after CLP. The CFUs were examined in peritoneal lavage fluid and in whole blood at 6 (n=4) and 24 h post CLP (n = 5/group from 3 independent experiments; *$p<0.05$; **D and E**, RIP2$^{-/-}$ mice have reduced cellular/neutrophil accumulation in the peritoneum after CLP. **F,** Attenuated neutrophil recruitment to the peritoneum.Cytospin section from peritoneal lavage fluid of RIP2 $^{-/-}$ mice and WT 24h after CLP. D–F, n = 6/group from three separate experiments; *$p<0.05$.

Effect of RIP-2 deficiency in cytokine/chemokine regulation in the peritoneum after CLP

Since we found reduced neutrophil numbers in peritoneum of RIP-2$^{-/-}$ mice, we wished to determine whether this may be attributed to a lowered inflammatory response in the peritoneum. During moderate sepsis, it has been demonstrated that IL-17A induces neutrophils to peritoneum [15]. We previously reported that RIP-2 regulates IL-17A in the lungs during acute

E. coli infection [25]. Thus, we determined the levels of cytokines, including IL-17A, in the peritoneum of RIP-2$^{-/-}$ mice and WT controls. We found reduced levels of IL17A in RIP-2 deficient mice compared to WT at 6h post CLP (Fig 6.2A). In a similar fashion, other cytokines (IL-1 β, IL-6 and TNFα) and chemokines (KC and MIP-2) show significant reduction in RIP-2 $^{-/-}$ mice when compared to WT mice at 6h post-CLP (Fig 6.2B-F). However, at 24h post-CLP, all pro-inflammatory cytokines and chemokines (only KC, not MIP2) were increased in RIP-2$^{-/-}$ mice as compared to their controls.

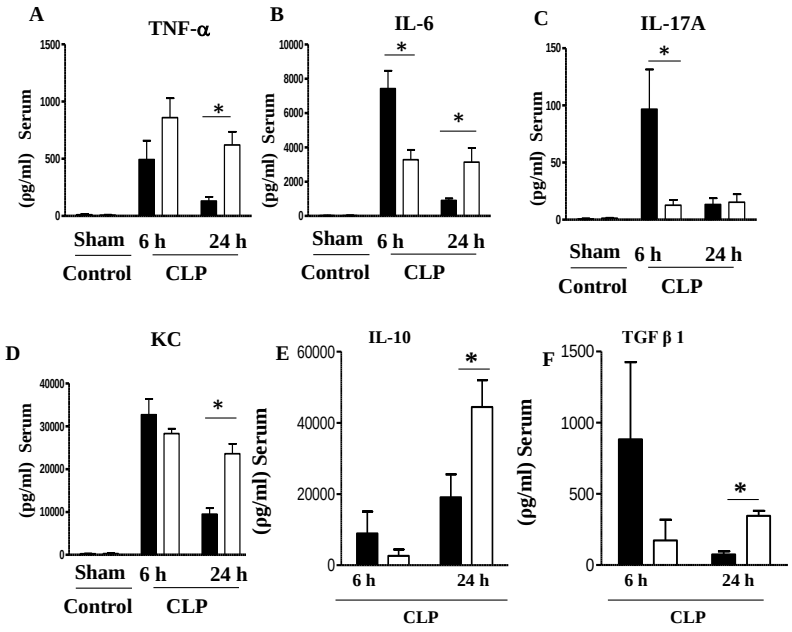

Figure 6.2. Cytokine and chemokine levels in the peritoneum in the absence of RIP-2 signaling. RIP-2 $^{-/-}$ mice and WT controls were subjected to sham surgery or moderate sepsis induced by the CLP model. IL-17A, IL-6, TNF-a, IL-1b, MIP-2, and KC concentrations (pg/ml) were quantified in the peritoneal exudate (A, B, C, D, E and F, respectively) 6 and 24 h after CLP as described in Materials and Methods. The results are expressed as means ± n=6-8 mice/group. (* indicates $p<0.05$ compared with RIP-2$^{-/-}$ mice).

Lack of functional RIP-2 results in lower blood neutrophil numbers and CXCR2 expression in neutrophils.

Studies have shown that defective neutrophil release into the circulation from the bone marrow leads to higher mortality in sepsis [4, 6]. In humans, neutropenic patients are susceptible to both

Gram-positive and Gram-negative bacterial infections [6]. In previous studies, CXCR2 was down-regulated in neutrophils, leading toreduced neutrophil migration to the site of infection [26, 27]. Thus, we determined the neutrophil numbers in circulation and CXCR2 expression on these cells after CLP. RIP-2$^{-/-}$ showed attenuated blood neutrophil numbers both at 6 and 24 h after CLP as compared to WT controls (Fig 6.3A, B). Although CXCR2 expression is reduced on neutrophils of both WT and RIP-2$^{-/-}$ mice at 6h post CLP, CXCR2 expression was downregulated on RIP-2$^{-/-}$ neutrophils as compared to WT at 24h (Fig 6.3 C,D). The cytokine G-CSF plays a critical role in neutrophil release from bone marrow by regulating the ratio of CXCR2/CXCR4 [28]. Thus, we also determined G-CSF levels in serum. Our results show reduced G-CSF levels in RIP-2 deficient mice at 6h post CLP whereas no difference at 24h as compared to the WT mice (Fig 6.3E). Reduced neutrophil numbers in the peritoneum and blood at 6h could be due to lower G-CSF levels and at 24h may be attributed to reduced CXCR2 expression on neutrophils in the RIP-2$^{-/-}$ mice. Therefore, we determined the CXCR2 expression on neutrophils. Although we did not see any difference in CXCR2 expression at 6h, it was remarkably reduced at 24h post CLP. This suggests that RIP2 does not directly regulate CXCR2 expression but increased pro inflammatory cytokines at 24 h post CLP could have down regulate CXCR2 expression.

RIP-2 deficiency leads to increased systemic inflammation during sepsis.

During sepsis, inflammatory responses in the tissues are complicated. Increased pro-inflammatory responses are observed in early time-points, followed by anti-inflammatory responses at later timepoint, Studies have also shown pro- and anti-inflammatory responses overlap each other and an increased level of TNF-α,IL-6 and IL-10 in the serum is an indicator of a poor outcome of human sepsis and experimental polymicrobial sepsis[1, 29]. Our data shows that in serum at 6h-post CLP, RIP-2 knockout mice show (Fig6.4A-F) reduced pro-inflammatory cytokines (IL-6 and IL-17A) while at 24h, all pro inflammatory cytokines (TNF-α,

IL-6, and IL-1β) were increased in RIP-2$^{-/-}$ mice compared to the WT control. Similarly, the chemokine KC is high at 24h post-CLP in RIP-2 knockout mice.

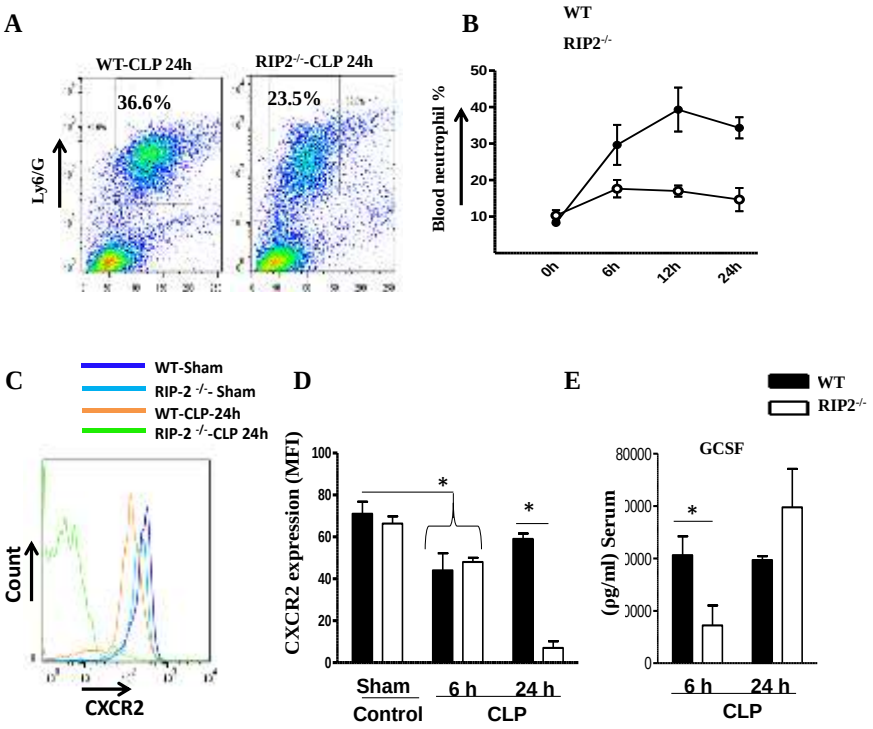

Figure 6.3. Role of RIP2 deficiency on blood neutrophil numbers, CXCR2 expression on neutrophils and G-CSF levels in serum. Blood neutrophil percentage was determined following CLP. Neutrophils were gated and the percentage of Gr-1/Ly6G positive cells were determined **(A-B). C-D.** Flow cytometric analysis of blood from RIP-2 $^{-/-}$ and WT mice at 6 and 24 h after CLP induced sepsis after using tagged antibodies against CXCR2. This is representative of 3 independent experiments with comparable results**. E.** G-CSF protein concentration in serum was determined by using a sandwich ELISA. Data shown here are a representation of 3 individual experiments where n=4-6 and *p<0.05.

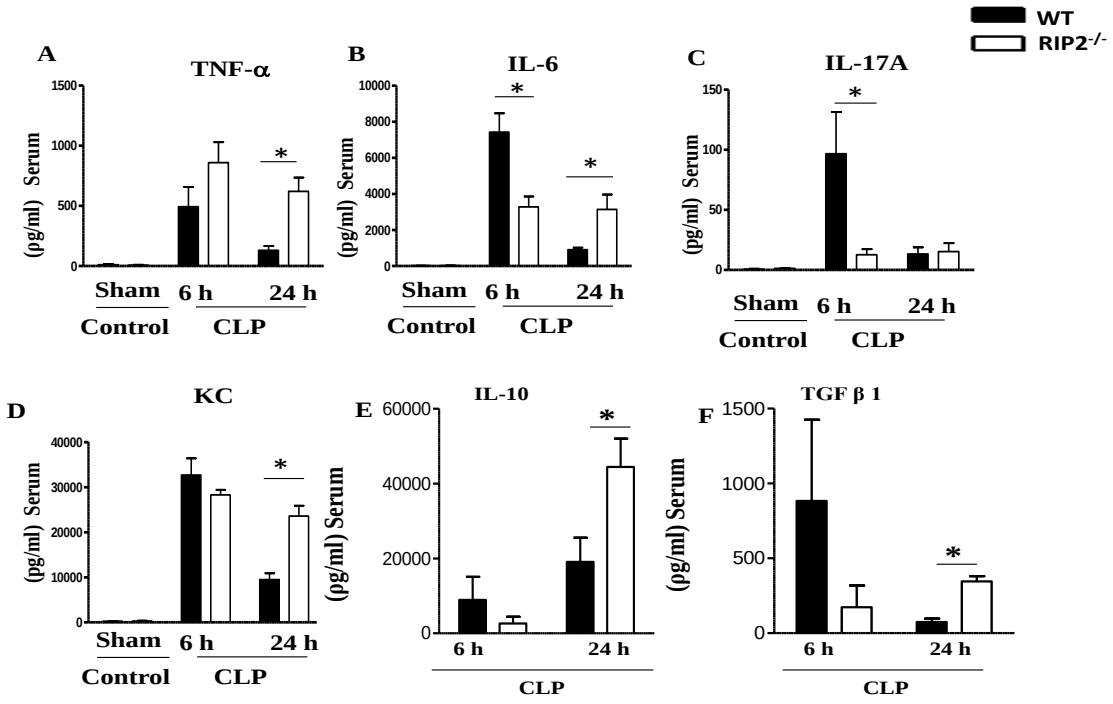

Figure 6.4. Cytokine and chemokine levels in serum of RIP-2$^{-/-}$ mice. RIP-2 $^{-/-}$ mice and WT controls were subjected to sham surgery or moderate sepsis induced by the CLP model. IL-17A, IL-6, TNF-a, KC, IL-10 and TGF-β concentrations (pg/ml) were quantified in serum (A, B, C, D, E and F respectively) 6 and 24 h after CLP. Data shown here is a representation of 3 individual experiments where n=4-6 and *p<0.05.

RIP-2 knockout mice display increased anti-inflammatory cytokines (IL-10 and TGF-β) during sepsis.

IL-10 and TGF-β are considered anti-inflammatory cytokines. However, early levels of IL-10 has been shown to be critical for the host defense during sepsis [30], although some studies have suggested inhibition of IL-10 at later time-points during sepsis is a therapeutic strategy to improve survival [31]. IL-10 and TGF-β are increased in serum of human sepsis patients and in animal sepsis models [30]. In our study, we found that IL-10 and TGFβ levels were increased in RIP-2$^{-/-}$ mice in the later time-point (Fig 6.4E and F).

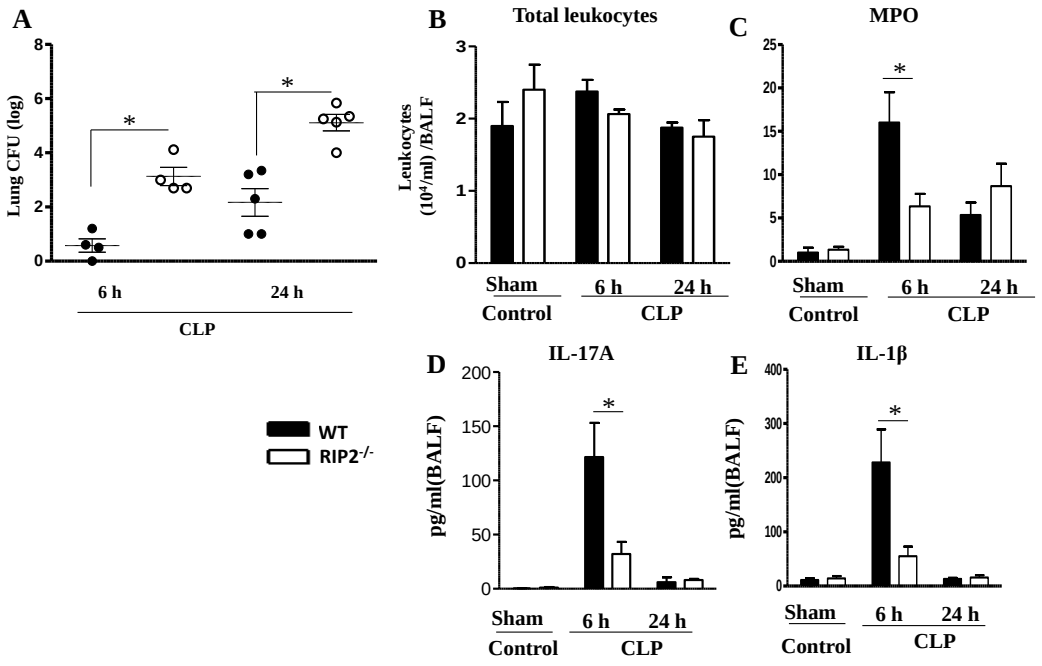

Figure 6.5. Lack of functional RIP-2 affects lung inflammation induced by sepsis. WT and RIP-2 $^{-/-}$ Mice were subjected to sham surgery and moderate sepsis using CLP model. **A**, Impaired bacterial clearance in the lung of RIP-2$^{-/-}$ mice after CLP. The CFUs were determined in lung homogenates at 6 and 24 h post CLP n = 5/group from 3 independent experiments; *p<0.05; **B**, WT and RIP-2$^{-/-}$ mice have a comparable number of total leukocytes count in BALF after CLP. **C,** Attenuated neutrophil numbers in the lung parenchyma as measured by myeloperoxidase (MPO) activity in RIP-2 $^{-/-}$ mice and WT 24h after CLP.IL-17A and IL-1β were measured in the BALF of WT RIP-2 $^{-/-}$ mice 6 and 24 h post CLP and n = 6/group from three separate experiments; *p<0.05.

Importance of RIP-2 in lung inflammatory responses during sepsis.

Sepsis induces lung inflammation and damage [32]. Therefore, we investigated the role of RIP-2 in lung inflammation during CLP. We observed significantly higher CFU (Fig 6.5A) in lungs of RIP-2-/- mice as compared to WT mice. In addition, we found reduced pro-inflammatory cytokines levels (IL-1β and IL-17A) in BALF of RIP-2 knockout mice (Figs 6.5D and E). However, we did not see any difference in total leukocyte numbers in BALF of WT and RIP-2$^{-/-}$ mice, where almost all BALF cells were mononuclear cells (Fig 6.5B). However, we observed reduced MPO levels in lungs at 6h post CLP, suggesting less neutrophils in lung paranchyma. However, there was no difference in MPO levels at 24 h post-CLP (Fig 6.5C).

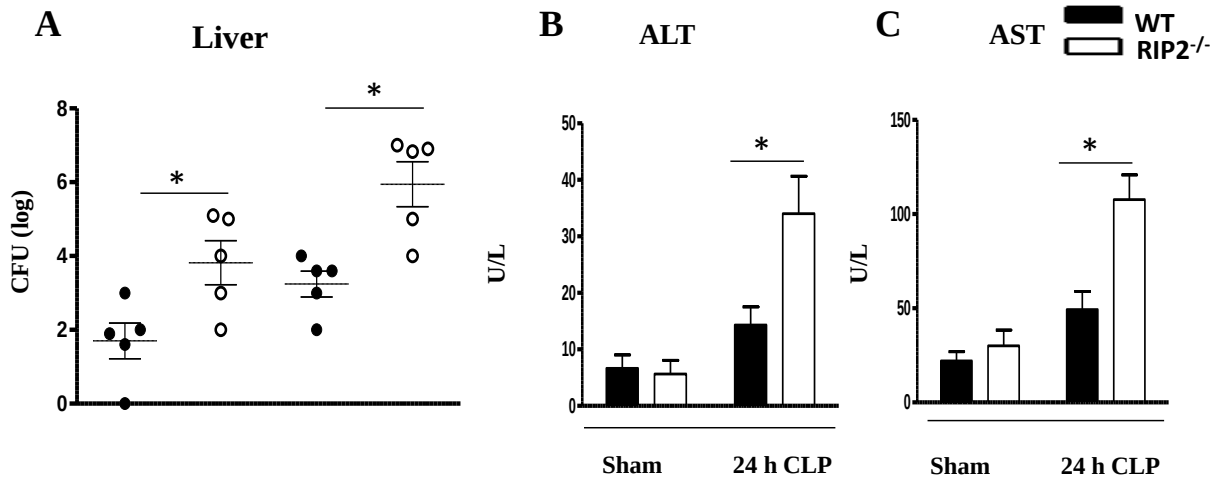

Figure 6.6. Lack of RIP-2 causes severe liver damage during CLP induced sepsis

WT and RIP2 $^{-/-}$ Mice were subjected to sham surgery and moderate sepsis using a CLP model. **A**, Impaired bacterial clearance in the liver of RIP-2$^{-/-}$ mice after CLP. The CFUs were examined in liver homogenates at 6 and 24 h post CLP, n = 5/group from 3 independent experiments; *p<0.05; **B-C**, RIP-2$^{-/-}$ mice have increased ALT and AST enzymes in the serum 24h post CLP, n = 6/group from three separate experiments; *p<0.05.

RIP-2$^{-/-}$ mice display enhanced liver injury during sepsis.

Since we found high levels of cytokines in RIP-2$^{-/-}$ mice, we further determined the level of liver injury by measuring the liver enzymes both ALT and AST. We also found that RIP-2$^{-/-}$ mice have a higher bacterial burden in liver, combined with an increased level of ALT and AST in the serum (Fig 6.6 A-C), suggesting a lack of RIP-2 signaling causes increased pathological changes in the liverduring CLP induced sepsis.

Discussion

Early neutrophil recruitment to the site of infection is critical for host defense against invading pathogens [33].This prevents the subsequent dissemination and excessive systemic inflammatory responses. Previous studies have shown that the severity of sepsis induced by CLP is closely associated with reduced neutrophil migration to the infection site [34].

Furthermore, neutrophils from septic patients and animals which are subjected to polymicrobial sepsis display a reduced chemotactic response to chemokines[35, 36].

To understand the mechanisms involved in neutrophil migration to the site of and in confining the infection locally, we determined the role of RIP-2 in neutrophil mediated host defense during CLP-induced sepsis. Further, we sought to determine the role of RIP-2 in IL-17A regulation and host defense during CLP- induced sepsis. First, we have demonstrated the importance of RIP-2 signaling in host defense in a clinically relevant sepsis model. We found that RIP-$2^{-/-}$ mice show increased mortality as compared to WT mice. This was associated with higher bacterial burden in the peritoneum and blood of the RIP-2 mice. Early neutrophil recruitment is shown to be important for the clearance of bacteria from the site of infection [26, 27]. We found a reduced number of neutrophils in the peritoneum, which correlated with less cytokine (IL-17A and chemokine (KC and MIP-2) levels in RIP-$2^{-/-}$ mice compared to WT. In addition, we also observed that RIP-2 regulates neutrophil numbers in circulation by regulating G-CSF and CXCR2 expression during polymicrobial sepsis. We also observed that a lack of RIP-2 causes increased systemic inflammation and vital organ damage. We also found evidence that RIP-2 possibly plays a role in neutrophil mediated inflammation in the lungs during polymicrobial sepsis.

IL-17A is an important pro inflammatory cytokine that is essential for neutrophil recruitment [37, 38].Pro inflammatory cytokines, including IL-17A, are produced upon bacterial infection by various immune signaling pathways which are initiated by the recognition of PRRs[39].Toll-like receptors have been shown to regulate neutrophil influx during polymicrobial sepsis[40] while the Role of NOD signaling in CLP-induced sepsis has not been explored. We have shown that RIP-2, an adaptor for NOD2 signaling, plays a critical in regulating IL-17A and subsequent neutrophil-mediated host defense during bacterial lung infection [41]. It has been shown that IL-17 induces the release of several inflammatory mediators, including chemokines (e.g., KC, MIP-

2, and LIX), which induce neutrophil migration [37]. However, the role of IL-17A signaling in sepsis is different, depending on the sepsis model. Some studies show that IL-17A is protective in moderate sepsis[15, 42], while others showed that IL-17A plays a detrimental role in severe sepsis by increasing systemic inflammation [14].

We used a moderate/mild sepsis model where WT mice show 30% mortality. RIP-2 $^{-/-}$ mice show reduced IL-17A levels resulting in reduced chemokine (KC and MIP-2) production and neutrophil numbers at the site of infection. We observed that RIP-2 mice showed lower neutrophil numbers in the blood. This could explain why we observed significant dissemination in the knockout mice, as blood neutrophil numbers are important in protecting the host during sepsis [18].G-CSF levels regulate neutrophil release from bone marrow during bacterial infection [43]. We also observed that RIP-2 can regulate G-CSF level in serum. Reduced IL-17A levels can be the cause of reduced G-CSF levels in the RIP2$^{-/-}$ mice during sepsis[44]. We found substantially lower numbers of neutrophils in the peritoneum of RIP-2 knockout mice despite increased KC level in the peritoneum. This led us to determine CXCR2 expression on neutrophils, because studies have shown that the human and murine chemokine receptor CXCR2 is down-regulated in neutrophils during severe sepsis [26]. Furthermore, a previous study demonstrated that the expression of CXCR2, but not CXCR1, is down-regulated on the membrane of neutrophils in the septic patients [27]. Although we did not see any difference in CXCR2 expression in either group of mice at 6h, there was a remarkable reduction in CXCR2 in RIP2KO mice at 24h post CLP. This could be due to increased levels of cytokines in the serum of RIP-2 knockout mice at 24h post-CLP, because previous reports indicate that high level of cytokines (TNFα and IL-1β) in the serum down-regulates CXCR2 expression on neutrophils and reduce neutrophil recruitment to the site of infection [45]. This may be the reason for lower neutrophil numbers in the peritoneum of RIP-2 $^{-/-}$ mice at 24h post- CLP.

Systemic inflammatory responses cause deleterious pathogenic events in severe sepsis. High levels of pro-inflammatory cytokines (TNF α and IL-6) in serum are accompanied by

multiple organ damage [29, 46]. We also observed that RIP-2 knockout mice show increased systemic levels of IL-6 and TNFα, along with ALT and AST, the two biochemical markers of liver injury compared to WT controls. This is mainly due to extensive dissemination in RIP-$2^{-/-}$ mice and the inability to clear the bacteria from the site of infection due to defective host defense at early time points. It is notable that the dynamics of anti-inflammatory cytokines IL-10 and TGFβ are similar to the pro-inflammatory cytokine dynamics, suggesting that RIP-2 may have a central role in balancing inflammation, both locally and systemically. Although reports show that IL-10 antagonizes pro-inflammatory responses, during sepsis it has shown to be elevated [47]. It may be a negative feedback mechanism to reduce inflammation. Recent studies also indicate the protective role of IL-10 in early time periods of polymicrobial sepsis [48]. According to our findings, IL-10 and TGFβ may be positively involved in inflammation and IL-10 and TFGβ is regulated by RIP-2 at an early time point in CLP. However, at a later time point pro and anti inflammatory responses are increased in the RIP-$2^{-/-}$ mice despite lack of RIP-2 signaling suggesting an involvement of a phenotypic shift in inflammatory cells in this process. However, the precise mechanism needs to be investigated by future studies.

Studies have shown that sepsis induces lung injury and organ damage by excessive neutrophil recruitment to the lung[49], but some studies indicated that lung injury is minimal during sepsis [47]. Our data regarding lung inflammation during CLP induced sepsis did not show any difference in total leukocytes in BALF. This may be explained by the sepsis model we used, which causes mild or moderate sepsis that may result in less/no lung inflammation. We observed increased MPO in the WT which suggests neutrophil sequestration in the lung parenchyma was consistent with previous studies [32, 34]. But, RIP-$2^{-/-}$ mice showed less MPO, along with increased bacterial counts in the lung of compared to WT mice. This could be due to lower cytokine levels and neutrophil numbers in RIP-$2^{-/-}$ mice resulting in reduced bacterial clearance. Even though the mechanism of sepsis is very complex, here we have shown the

protective role of RIP-2 signaling in polymicrobial sepsis by regulating IL-17A and neutrophil influx to site of infection to prevent dissemination.

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Chapter 7: Conclusions

NOD-like receptor mediated immune responses during acute bacterial pneumonia and polymicrobial sepsis

Pneumonia and sepsis are frequently interrelated, as pneumonia can lead to sepsis while sepsis can cause lung injury [1-3]. Bacterial pneumonia can affect individuals regardless of their age or health status and can occur in both community and hospital settings. Bacterial pneumonia is a main cause of childhood mortality in the world, where more than 1.6 million children die annually [4-6]. It is not only a problem in developing countries but also a problem in developed countries. In addition, treatments such as organ transplantation and chemotherapy that result in immunosuppression make the individuals with pneumonia difficult to treat[7]. Gram- positive bacteria such as *Streptococcus pneumoniae and Staphylococcus aureus,* along with Gram negative *Klebsiella pneumonia* (Kp), *Pseudomonas aerogenousa* and *Legionella pneumophilia* are the major causative agents of bacterial pneumonia[8, 9]. Among them, Gram-negative bacterial pneumonia is becoming a potentially difficult problem to treat because of the emergence of antibiotic resistant strains, such as carbapenam-resistant Kp, popularly known as ” superbug”[10, 11].

Sepsis is a complex clinical syndrome resulting from various etiological reasons. During polymicrobial sepsis, lack of early host defense leads to excessive bacterial loads. In the United States alone, more than 700,000 patients annually develop sepsis, and mortality rates are on the rise despite current advanced medical facilities[12]. When we consider the host defence mechanism acting against acute bacterial infection, innate immunity is the first line of defence [13, 14]. The innate defence consists of a physical barriers, antimicrobial agents, and phagocytosis by resident alveolar macrophages (AMs) and recruitment of polymorphonuclear cells. According to studies with bacterial infections in various models, neutrophils play a predominant role in the clearance of infection [13, 15]. Neutrophils are recruited to the site of infection, mainly by the action of chemokines[8]. IL-17A, a recently identified cytokine, plays a critical role in the host defense during bacterial pneumonia and sepsis via modulating neutrophil

chemokines. IL-17A drives neutrophils to the site of infection from the blood and to the blood from the bone marrow by regulating KC,MIP-2 and GCSF[16, 17]. Studying the regulation of IL-17A is of particular importance because identifying the mechanism of regulation of IL-17 may lead to important therapies for many diseases.

Production of cytokines and chemokines begins with the recognition of PAMPs by the host PRRs resulting in immune signalling to recruit immune cells and to initiate production of antimicrobial agents that eventually clear the bacteria[9]. Toll-like receptors which are mainly located on the cell membrane and NOD-like receptors located in the cytosol are the two important PRRs. NLRs consist of a group of 23 cytosolic PRRs that have a conserved tripartite structure wherein the central nucleotide oligomerisation domain (NOD) is flanked by caspase recruitment domain (CARD), or pyrin (PYR) domain, or baculovirus- inhibitor repeat domains (BIR) at the N-terminus, and LRR at the C terminus[9, 18]. The NOD is important for nucleotide binding and self-oligomerisation, while LRRs detect specific PAMPs. The amino terminal CARD domain is important for downstream signaling; similarly PYD domain that is homologous to the CARD domain is also important for protein- protein interaction and downstream signaling. Both CARD and PYD domains are associated with proteins involved in apoptosis and inflammation[9, 19]. In this study, we mainly focused on studying role of the NLR (NOD2 and NOD1), and its adaptor RIP-2 in regulation of IL-17A during acute bacterial pneumonia and polymicrobial sepsis.

In chapter 2 we have studied the role of RIP-2, a common adaptor molecule of NLRs, in an *E. coli* model of lung infection. We found that RIP-2 controls neutrophil recruitment to the lung. We also found that RIP-2 knockout mice show reduced levels of cytokine, IL-6, and chemokine, MIP-2.Interestingly, we found that RIP-2 mediated signaling regulates IL-17A, and we further showed that exogenous administration of IL-17A restores neutrophil numbers and host defence during *E. coli* lung infection. However, we did not observe any difference in the expression of

cellular adhesion molecules on neutrophil of both WT and RIP-2$^{-/-}$ mice. This led to the question regarding the upstream molecules of RIP-2.

We performed the study outlined in chapter 3 to determine the upstream molecules of RIP2. We found that NOD2, but not NOD1, is important for neutrophil recruitment in the lungs *during E. coli* pneumonia. We also observed that NOD-2 is expressed on neutrophils, and defects in NOD2 affect bactericidal activity of neutrophils against of *E. coli*. In addition, we observed NOD2 mediated activation of NADPH complex, through p47^{phox} in neutrophils. Using NOD2/RIP2$^{-/-}$ mice we found that the pro-inflammatory parameters IL-6 and MIP-2 are substantially reduced in NOD-2/RIP-2$^{-/-}$ mice, when compared to WT and are comparable to NOD-2 or RIP2 single gene deficient mice, suggesting that NOD2 dependent RIP-2 signaling plays a key role in neutrophil mediated host defence during *E. coli* pneumonia.

The limitations of the two studies from chapters 3 and 4 are 1) *E. coli* gets cleared from both WT and knockout mice, and 2) doesn't disseminate to distal organs to induce mortality. Because of these limitations, we chose Kp pneumonia and a CLP model to dissect the role of NOD2/RIP2 mediated signalling in local (chapter 5) and systemic responses (chapter 6).

In the study outlined in chapter 5, we found that RIP-2 gene-deficient mice are more susceptible to Kp, as a result of impaired bacterial clearance and extensive bacterial dissemination. We also observed increased systemic responses, such as higher pro-inflammatory cytokine levels, in serum that is accompanied with increased pathological changes in the liver of RIP-2$^{-/-}$ mice. These findings suggest that mortality in RIP2$^{-/-}$ mice upon Kp infection could be due to extensive bacterial dissemination leading to a systemic inflammatory response. In addition, we also show that RIP2 regulates IL-17A through IL-6- mediated STAT3 activation. This was further confirmed by administering exogenous IL-17A and IL-6 which restored the host defense. It was interesting to note that while rIL-6 and rIL-17A treatment but not completely restored neutrophil numbers

and host defense. These data suggest that there are additional mechanisms that regulate neutrophil recruitment. In this regard, we found that RIP2 can regulate the activation of the inflammasome cascades. It is possible that IL-1β is the second mechanism by which RIP2 regulates neutrophil recruitment and host defense. However, these possibilities need to be explored by future experiments.

In chapter 6, our data show higher mortality in RIP-2$^{-/-}$ mice when compared to their WT counterparts after CLP- induced polymicrobial sepsis. We also observed reduced neutrophil recruitment along with less cytokine (IL-1β and IL-6) and chemokine (KC and MIP-2) levels in the peritoneum of RIP-2 $^{-/-}$ compared to WT mice. We also identified that IL-17A is significantly reduced in the knockout mice, which may explain the defective host defense in RIP-2 knockout mice during CLP. Intriguingly, we observed reduced expression of CXCR-2 on neutrophil in RIP-2 knockout mice at the later time point of CLP, this may be due to the presence of increased systemic chemokine levels (KC and MIP-2) in the serum of RIP-2 knockout mice. Although we haven't seen neutrophil recruitment in the BALF of WT and RIP-2 knockout mice, we observed reduced MPO level in the lung of RIP-2 knockout mice along with a higher bacterial burden in the RIP-2 knockout mice.

In summary, this dissertation work reveals the critical role of NOD2/RIP2 signalling during acute bacterial pneumonia and sepsis. Early neutrophil mediated host defense is regulated by the NOD2/RIP2, axis mainly by producing IL-6, IL-17A and IL-1β. In addition to our findings, previous reports also show the essential role of NOD2/RIP2 signaling in different disease conditions such as tuberculosis[20], Crohn's disease[21] and inflammatory bowel disease[22]. Therefore, targeting NOD2/RIP2 signaling to augment the host defense during acute bacterial infection is a viable treatment strategy.

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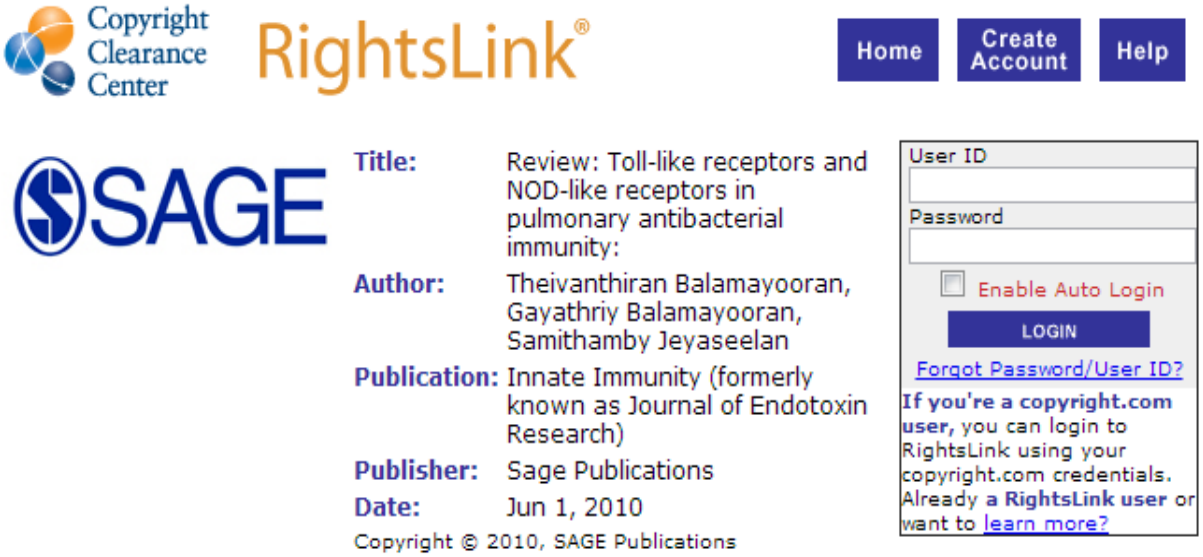

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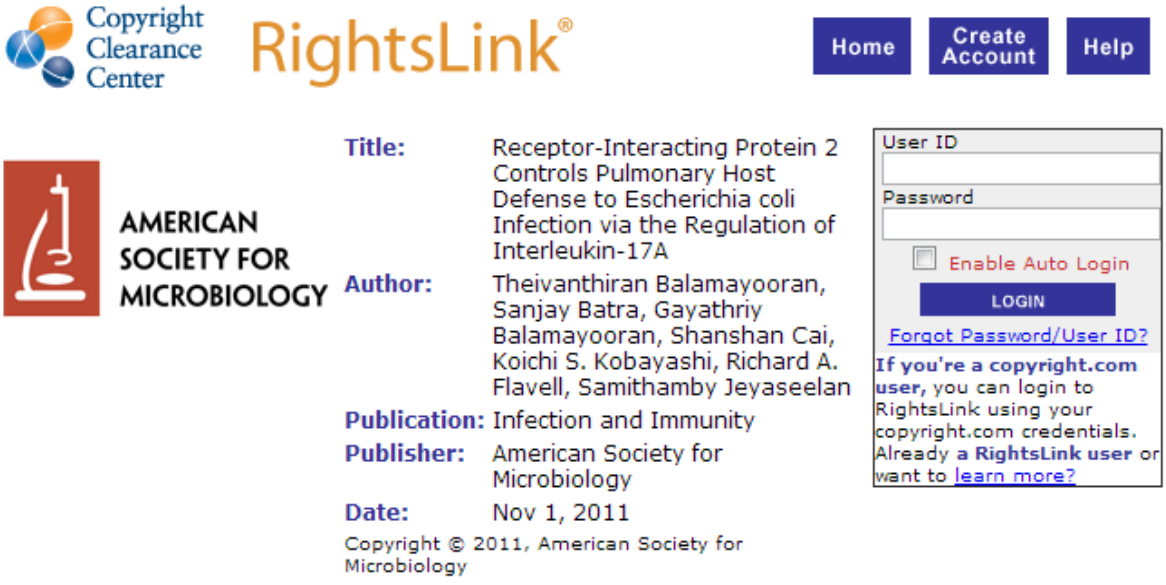

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Vita

Balamayooran Theivanthiran was born in Jaffna, Sri Lanka, to Karthigesu Theivanthiran and Sakunthaladevy Theivanthiran. He has four older brothers, loving wife and a beautiful daughter Kaitlyn. Balamayooran has graduated with the Bachelor of Veterinary Science (B.V.Sc; DVM equivalent) from the Faculty of Veterinary Medicine and Animal Science at the University of Peradeniya. After graduation, he was a veterinarian at Royal Veterinary Clinic, Colombo. His interest in immunology of infectious diseases urged him to join the Lung Biology Laboratory at the Department of Pathobiological Sciences, Louisiana State University in 2009 Summer, to pursue his doctoral degree under the guidance of Dr. Samithamby Jeyaseelan. In lung biology laboratory, he has learned pulmonary innate immune mechanisms involved with bacterial infections. Balamayooran will graduate in May 2013 with his Doctor of Philosophy degree. After graduation, Balamayooran is interested in pursuing his research interests in the field of immunology of infectious diseases.