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Essential role of monocyte chemoattractant protein-1 in gram-negative bacterial pneumonia

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ESSENTIAL ROLE OF MONOCYTE CHEMOATTRACTANT PROTEIN-1 IN GRAM-NEGATIVE BACTERIAL PNEUMONIA

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Dedicated to my parents, sister, husband and baby Kaitlyn

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Commonly Used Abbreviations

ALRTI	Acute lower respiratory tract infections
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
i.t.	Intratracheal
i.p.	Intraperitoneal
MCP-1	Monocyte chemoattractant protein-1
rMCP-1	Recombinant MCP-1
CCR2	Chemokine (C-C motif) receptor 2
CXCR-2	Chemokine (CXC motif) receptor 2
KC	Keratinocyte cell-derived chemokines
MIP-2	Macrophage inflammatory protein-2
G-CSF	Granulocyte colony stimulating factor
LTB4	Leukotriene B4
LIX	Lipopolysaccharide-induced CXC chemokines
ICAM-1	Intracellular cell-adhesion molecule-1
VCAM-1	Vascular cell-adhesion molecule-1
BALF	Bronchoalveolar lavage fluid
MPO	Myeloperoxidase
NF-κB	Nuclear Factor kappa B
MAPKs	Mitogen associated protein kinases
JNK	C-Jun N-terminal kinase
PRRs	Pattern recognition receptors
PAMPs	Pathogen associated molecular patterns
PYD	Pyrin domain
TLRs	Toll-like receptors
TIR	Toll-interleukin (IL)-1 receptor homology domain
MyD88	Myeloid differentiation primary response gene (88)
MD-2	Myeloid differentiation protein-2

TRIF	TIR-domain-containing adapter-inducing interferon-β
TIRAP	Toll-interleukin 1 receptor (TIR) domain-containing adapter protein
TRAM	TRIF related adaptor molecule
LRRs	Leucine rich repeats
NLRs	Nod like receptors
NOD1	Nucleotide oligomerisation domain 1
NOD2	Nucleotide oligomerisation domain 2
RIP2	Receptor interacting protein 2
NALPS	NACHT, LRR and PYD domain-containing proteins
NAIP5	NLR family apoptosis inhibitory protein 5
Birc1e	Baculoviral IAP repeat containing 1e
NACHT	Neuronal apoptosis inhibitor protein CIITA, HET-E and TP-1
TNF-α	Tumor necrotic factor alpha
IL-6	Interlukin 6
IL-17	Interlukin 17
LPS	Lipopolysaccharide
PMN	Polymorphonuclear leukocytes
AP-1	Activating protein-1
STAT	Signal transduction and transcription proteins
IRF	Interferon regulatory factor
IFN-γ	Interferon gamma
IRAKs	Interleukin-1 receptor-associated kinases
AM	Alveolar macrophages
WT	Wild type
CFU	Colony forming unit
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
RBC	Red blood cells
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
BSA	Bovine serum albumin

FBS   Fetal bovine serum

DMEM   Dulbecco's modified Eagle's medium

FACS   Flourescence activated cell sorting

PBS   Phosphate buffered saline

OD   Optical density

DAMPs   Danger associated molecular patterns

CLP   Cecal ligation and puncture

Abstract

Acute gram-negative bacterial infections are a leading cause of mortality among the nosocomial infections. Increasing numbers of immunosuppressed individuals and growing numbers of antibiotic resistant strains make antibiotic treatment difficult. Neutrophils are the first cells recruited to the site of infection and are critical players in the host defense against gram-negative bacterial pneumonia. Therefore, identification of targets that boost neutrophil-associated host defense in the lung is essential in designing better therapies to control pulmonary infections. Production of chemokines is an important step for neutrophil recruitment. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine that is important for monocyte and T-lymphocyte influx. It is important for the host defense during *Listeria monocytogenes* and *Streptococcus pneumoniae* infection. However, the role of MCP-1 during pulmonary gram-negative infections is not known. We hypothesized that MCP-1 is essential for the host defense during a gram-negative infection. To test the hypothesis, we infected MCP-1 gene-deficient (MCP-1$^{-/-}$) mice and controls intratracheally (i.t.) with *E. coli* (10^6 CFUs/mouse) and *Klebsiella pneumoniae* (10^3 CFUs/mouse). We found that MCP-1 is critical for host defense against gram-negative infections, mainly by recruiting neutrophils to the site of infection. MCP-1 utilizes it's receptor, CCR2, to recruit neutrophils directly and indirectly by regulating the expression of cytokines (IL-6, TNF-α) and chemokines (KC, MIP-2) through activation of NF-κB and MAPKs. We also observed that MCP-1 can regulate expression of G-CSF and thereby neutrophil numbers in circulation during Kp infection. In addition, exogenous administration of rG-CSF can restore the defects in host defense in MCP-1$^{-/-}$ mice following gram-negative Kp infection. This study demonstrates an unrecognized role of MCP-1 in host defense during gram negative bacterial pneumonia. These findings bolster pleiotropic effects of MCP-1 in the host defense and demonstrate a

potential role as a therapeutic agent to augment host defense during acute bacterial pneumonia.

Chapter 1

Introduction

Acute lower respiratory tract infections (ALRTI) are major causes of mortality throughout the world[1]. Unlike other infectious diseases that are common in poor communities, ALRTI cause significant morbidity and mortality across the socio-economic spectrum[2]. Although ALRTI can be caused by several infectious agents, bacterial infections claim thousands of lives every year[1, 3]. Medical and public health advances have been made in treating lung infections, yet there has been little or no improvement in death rates in the US and worldwide. The current treatment strategy for bacterial pneumonia is antibiotics. Wide spread antibiotic use has resulted in the emergence of multi-drug resistant bacterial strains [4, 5]. Moreover, growing populations of immunocompromised individuals make the treatment of bacterial pneumonia complicated. Therefore, novel approaches to treat bacterial pneumonia are warranted. Immunotherapy to modulate the immune response is an important therapeutic strategy [6, 7].

Pulmonary immune response against pathogens involves both innate and adaptive responses. Innate immune response is non-specific and occurs very quickly upon exposure to a pathogen. In contrast, the adaptive response is pathogen-specific, takes several days to develop and has a memory response. During acute infections, innate host defense plays a vital role in eliminating the pathogen [8, 9]. Neutrophils are the first cells recruited to the site to contain the infection. Neutrophil recruitment is a critical event during anti-bacterial host defense and involves a coordinated signal network that involves cytokines, chemokines, cellular adhesion molecules, leukocytes, epithelial cells and endothelial cells. In this regard, chemokines play a critical role [10, 11]. Monocyte chemoattractant protein 1 (MCP-1) is a member of the chemokine [C-C motif] family and a potent monocyte and T cell chemoattractant[12, 13]. MCP-1 has been very well studied for its role in monocyte recruitment. MCP-1 is produced by a variety of cells including epithelial cells [14], endothelial cells, fibroblasts [15] and monocytes/macrophages [16]. MCP-1 interacts with its

G-protein–coupled transmembrane receptor CCR-2 to bring about the biological effects [17, 18]. This chemokine is implicated in various acute and chronic disease conditions, including acute respiratory distress syndrome (ARDS) [19, 20]. Studies have shown that MCP-1 is important for the host defense during *Streptococcus pneumoniae[21]*, *Pseudomonas aeruginosa [22]* and *Cryptococcus neoformans* [13] infections in different models. MCP-1 has been shown to promote bacterial killing by macrophages during *S. pneumoniae*, *P. aeruginosa* and *S. typhimurium* infections alternatively it is important for the clearance of apoptotic cells and therefore helps in resolution and repair during *P. aeruginosa* infection of the lung [22]. Growing evidence suggests that MCP-1 can play a significant role in neutrophil chemotaxis [19, 23]. However, the role of MCP-1 in neutrophil recruitment during acute gram negative bacterial infection of the lung has not been studied.

We hypothesized that MCP-1 is important for neutrophil recruitment and host defense during gram-negative bacterial pneumonia. As MCP-1 has a pleiotropic role in immune mechanisms, it is a potential therapeutic target by boosting immune defense against bacterial infections. However, a more comprehensive understanding of the role of MCP-1 in the host defense is needed. Therefore, the major goals of this dissertation are to 1) determine the role of MCP-1 in neutrophil mediated host defense during gram-negative pneumonia and 2) determine the mechanism behind neutrophil recruitment following gram-negative bacterial infection. These goals were achieved by using MCP-1 gene deficient mice and their controls (C57BL6) and infecting them intratracheally (i.t.) either with *E. coli* ($1X10^6$ CFUs/animal) or *Klebsiella pneumoniae* ($1X10^3$ CFUs/animal).

The specific objectives of the first research goal were A) to use enzyme linked immunosorbent assay (ELISA) to determine the kinetics of MCP-1 in the bronchoalveolar lavage fluid (BALF) during gram-negative bacterial pneumonia; B) to use bone marrow transplantation to identify the cellular source of MCP-1 during infection; C) to assess

neutrophil numbers in BALF and in lung with a hemocytometer and myeloperoxidase assay and D) to characterize the expression of pro-inflammatory mediators (cytokine, chemokines, cellular adhesion molecules, NF-κB and MAPKs) in lungs of mice after i.t. infection with bacteria. Expression of cytokines and chemokines were measured by ELISA and expression and activation of NF-κB and MAPKs will be determined by immunoblot analysis.

The specific objectives of the second research goal were A) to determine the expression level of CCR2 after infection with gram-negative bacterial pneumonia; by flow cytometry and labelled antibody against the CCR2 receptor; B) to determine whether neutrophils can move by chemotaxis towards recombinant MCP-1 (rMCP-1) *in-vitro* as measured by the neutrophil transmigration assay using a Transwell system and bone marrow neutrophils after gram-negative bacterial infection [24], and *in-vivo* by administering rMCP-1 i.t and determining neutrophil numbers in the BALF following infection; C) to assess neutrophil numbers in the circulation of MCP-1 gene deficient mice post-bacterial infection by using flow cytometry and neutrophil specific antibody (Gr-1/Ly6G); and D) whether administration of exogenous MCP-1 can protect the mice after gram negative bacterial infection by determining the survival and bacterial burden in lungs, spleen, liver and blood.

This dissertation is organized and presented in five chapters. This general introduction is followed by a review of the literature pertinent to mechanisms of neutrophil recruitment during bacterial pneumonia. The third and fourth chapters detail the methods and results of the original research performed over the course of my graduate program. Each of these chapters are presented in the format required by the scientific journal in which it has been published or to which it has been submitted. The format of Chapter 2 conforms to that of *the American Journal of Respiratory Cell and Molecular Biology* in which the original article was published. The format of Chapter 3 conforms to that of *Infection and Immunity*, in which the original research was published. The format of Chapter 4 conforms to that of *The Journal*

of Immunology, to which the original research has been submitted. The fifth chapter is devoted to a general discussion of this research and its major conclusions.

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Chapter 2

Literature Review*

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The respiratory system continually encounters microorganisms. The pathogenicity of a microorganism not only depends on its virulence factors, but also on the host's immune defense and the environment. The myeloid and structural/resident cells of the respiratory system detect invading microorganisms by binding their pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) to bacterial ligands. This recognition triggers a cascade of events leading to the activation of transcription factors, production of chemokines/cytokines, upregulation of cell adhesion molecules, phagocytic cellular infiltration and subsequent clearance of the microorganisms. The major players of the innate immune system are neutrophils, macrophages and dendritic cells. Dendritic cells and macrophages not only produce proinflammatory mediators but also present antigens to induce an eventual adaptive immune response. The innate immune system in vertebrates confers immediate defense against invading microbes via mechanisms such as phagocytosis, whereas the adaptive immune system armed with T and B lymphocytes plays an important role in chronic or recurrent infections.

Pneumonia is an important cause of mortality both in developed and developing countries (1). When microbial infections overwhelm the innate immune mechanisms, the result is pneumonia, which is associated with extensive lung pathology (1, 2). Although pneumonia can be caused by a variety of microbes, such as bacteria, viruses and fungi, we have primarily focused upon bacterial pneumonia in this review. The initial phase of bacterial pneumonia is characterized by neutrophil-mediated inflammation (1, 2). While neutrophilic inflammation aids in removal of bacteria, it also induces bystander injury to the lung. When severe, this injury may lead to a clinical condition termed Acute Lung Injury (ALI)/Acute Respiratory Distress Syndrome (ARDS) (1, 2). It is therefore imperative to explore the molecular mechanisms that underlie pneumonia and ALI/ARDS in order to formulate

therapeutic strategies to augment host immune mechanisms to combat microorganisms while attenuating excessive lung damage.

This review addresses our current understanding of the innate immune responses of the host against different gram-positive and gram-negative bacteria during the acute phase of bacterial pneumonia with special emphasis on the role of PRRs such as TLRs and NLRs, transcription factors, cytokines and chemokines in neutrophil accumulation. Furthermore, we discuss emerging roles for cholesterol and alcohol in neutrophil recruitment to the lung during bacterial pneumonia.

Neutrophil Mobilization to the Lungs

A large pool of neutrophils is stored in the bone marrow. Granulocyte-colony stimulating factor (G-CSF) stimulates the proliferation and differentiation of the granulocytic lineage in the bone marrow into mature neutrophils. These neutrophils are mobilized to the bloodstream during bacterial infections and are believed to be the first line defenders of the innate immune system against microbes. Neutrophils in the bloodstream adhere to the vascular endothelium and migrate across the endothelium to reach sites of inflammation in various organs, including the lungs (3). This process involves multiple adhesion molecules from different families that are expressed on the surface of endothelial cells in response to different cytokines. As neutrophils migrate to inflammatory foci, they get activated, generate free radicals, release granule contents, and phagocytose and degrade microbes. Neutrophils are the first phagocytic cell recruited to a site of bacterial infection, but they have a very short life span (<6 h) after release from the bone marrow.

Neutrophils are one of the critical contributors to host defense in the lungs since 1) selective depletion of neutrophils results in substantial reduction in the clearance of *S.*

pneumoniae, K. pneumoniae and *L .pneumophila*, and 2) repletion of neutrophils in neutropenic mice improves host defense and survival in response to bacterial infection (4-6). Although the neutrophil is the critical cell type implicated in the pathogenesis of ALI/ARDS, its short life span renders it difficult to use genetic manipulations to investigate the signaling cascades in response to stimuli. In this context, a novel method has recently been developed to generate mature neutrophils from bone marrow derived progenitor cells via long term bone marrow culture system (7). These neutrophils are mature as determined by morphology, expression of surface markers (Gr1, CD11b, CD62L and CXCR2), and functional capabilities, including superoxide generation, exocytosis of granular contents, chemotaxis, phagocytosis, and bacterial killing. Furthermore, these *in vitro* mature neutrophils are capable of migrating to inflammatory sites *in vivo*. We anticipate that this system will serve as an important tool to advance our knowledge in neutrophil biology in the future.

Despite the fact that various animal model systems are used to delineate molecular mechanisms underlying neutrophil accumulation and lung injury, murine models have been used extensively because 1) bacteria can induce substantial neutrophil influx and subsequent damage to the lungs of mice during pneumonia; 2) several mouse strains with a variety of gene deletions or disruptions are available; and 3) there is ample availability of reagents and pharmacological agents to dissect out the signaling mechanisms in murine models.

Recognition of Microbes

The innate immune system is responsible for recognizing invading pathogens and subsequent initiating the inflammatory response and/or host defense. Distinguishing self from non-self is therefore an important hallmark of the immune system. The recognition of bacteria relies on PRRs that can recognize molecular moieties common to microbes. The discoveries of both membrane-bound and cytoplasmic PRRs, including TLRs, NLRs, mannose receptors, and

RNA helicases have stimulated investigations on the biology of the innate immune system. We have highlighted the roles of TLRs and NLRs in this review since these receptors have recently been identified and have important roles in host defense against bacterial infections.

A single microbe generally has a variety of molecules called pathogen associated molecular patterns (PAMPs) that can activate a single or multiple PRRs (8). The intracellular signaling cascades initiated by these receptors may lead to the activation of numerous transcription factors, such as those of the nuclear factor (NF)-κB, activating protein (AP)-1, signal transduction and transcription (STAT) proteins and interferon regulatory factor (IRF) families(8), thereby regulating the expression of pro-inflammatory mediators, such as cytokines/chemokines, and adhesion molecules. In turn, these proinflammatory mediators can induce infiltration of neutrophils via enhancing chemotaxis and activating neutrophils to release more cytokines/chemokines. While neutrophils combat pathogens in a nonspecific manner as innate immune cells, antigen presenting cells, such as dendritic cells and macrophages, present antigens to T lymphocytes to induce antigen specific adaptive immune response. However, recent reports have suggested that neutrophil-derived IL-18 together with dendritic cell-derived IL-12 can induce IFN-gamma synthesis in NK cells in response to i.v. *L. pneumophila* infection in mice (9). These recent findings reveal a new role for neutrophils in the control of bacterial infection, in addition to their classical phagocytosis/microbicidal functions.

TLRs

TLRs are type 1 transmembrane receptors, with an ectodomain composed of leucine rich repeats (LRRs), and are members of a larger superfamily of interleukin 1-receptors (IL-1Rs). Toll was initially identified in *Drosophila* as a receptor essential for dorsoventral polarity during embryogenesis and later was shown to also be important for antifungal host defense in

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insects. The members of this family share a conserved region of ~200 amino acids in the cytoplasmic region known as the Toll/IL-1R (TIR) domain (10) whereas the extracellular LRR region is diverse in nature and is directly involved in the recognition of microbes.

TLRs function as dimers, usually forming homodimers except for TLR2, which dimerizes with either TLR1 or TLR6 giving rise to different ligand specificities. To date, 12 TLRs in mice and 10 in humans have been identified (8). Several TLRs can recognize bacteria and/or their components. For example, TLR1, TLR2, and TLR6 recognize lipid and carbohydrate compounds, including lipoteichoic acid and lipoprotieins, from gram-positive bacteria. TLR4 recognizes lipopolysaccharide (LPS), a cell-wall component of gram-negative bacteria and myeloid differentiation protein-2 (MD-2) is a key molecule important for the TLR4 recognition of LPS, whereas TLR5 recognizes bacterial flagellin. TLR3,-7,-8, and -9 are receptors for nucleic acid and its derivatives. Furthermore, TLR11 has shown to be involved in the recognition of profilin and uropathogenic bacteria (Figure 2.1).

Several studies have unequivocally demonstrated the role of TLRs in pulmonary host defense (Table 1 and Figure 2.1). For instance, TLR2 has been shown to be essential for host defense against *Streptococcus pneumoniae* (11) and *Porphyromonas gingivalis* (12) , however TLR2 only mediates partial resistance to *L. pneumophila* (13-15). TLR4 contributes to a protective immune response against both *S. pneumoniae* (16) and *Klebsiella pneumoniae* (17), although its role is much more pronounced against *K. pneumoniae*. TLR4 also contributes to pulmonary host defense against *H. Influenza* (18). And MD-2 plays an important role during *E.coli* induced pneumonia (19). Both TLR2 and TLR4 have been shown to be significant for host defense against *Acinetobacter baumannii* (20) and *Pseudomonas aeruginosa* (21). TLR5 is an important regulator of neutrophil infiltration into the lung at early time-points (6 h), but not at late time points (24 h or beyond) during *L. pneumophila* infection (22). TLR9 is required for effective host defense not only against gram-negative pathogens, such as *L.*

pneumophila (23) and *K. pneumoniae* (24) but also gram-positive pathogens, such as *S. pneumoniae* (25). These observations reveal 1) the activation of multiple TLRs in response to bacterial infection; and 2) the time-dependent activation of TLRs in response to bacterial interaction.
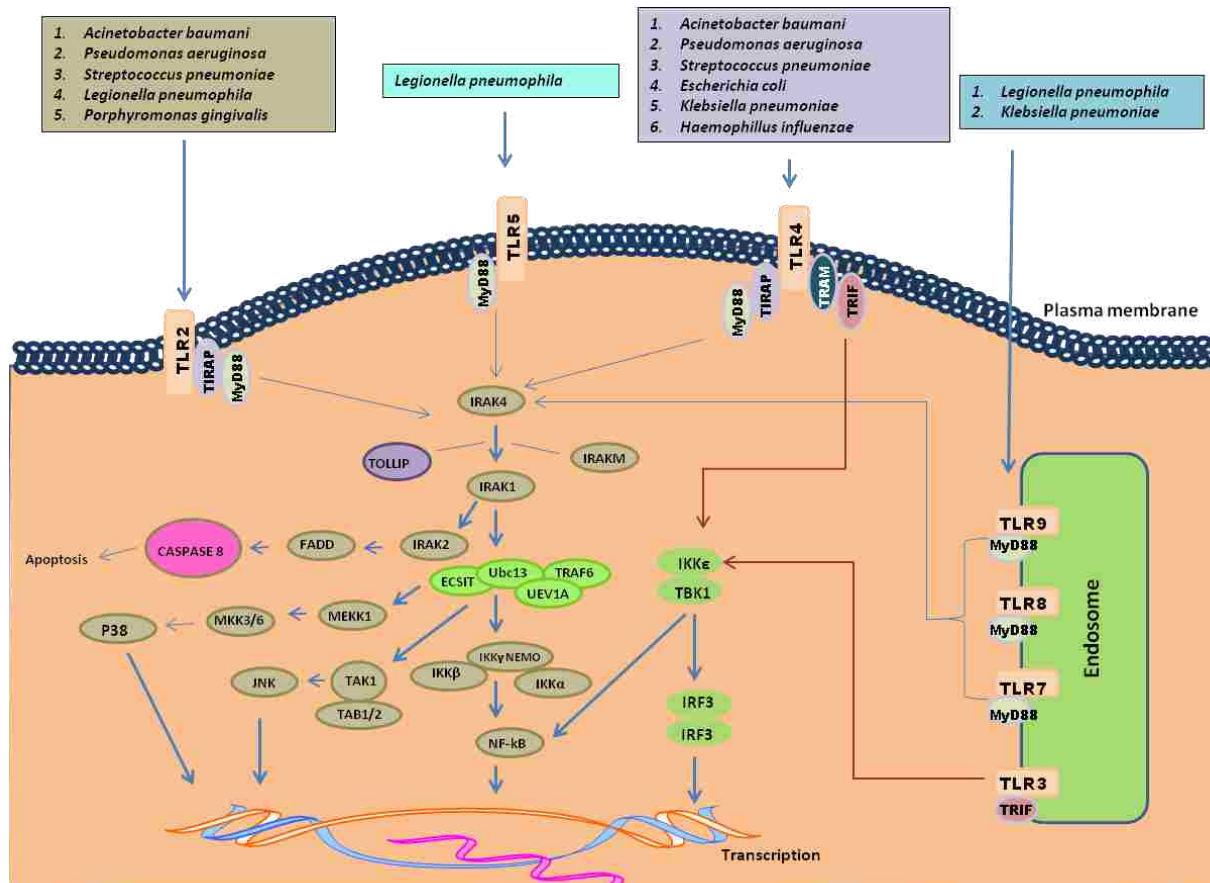
Figure 2.1: Respiratory pathogens are recognized by TLRs. Plasma membrane-bound TLRs (TLR2, TLR4 and TLR5) and endosome-bound TLRs (TLR3, TLR7, TLR8 and TLR9) recognize bacterial pathogens in the lungs. TLR2, TLR4, TLR5, TLR6, TLR7 and TLR9 recruit MyD88 whereas TLR2 and TLR4 recruit both TIRAP and MyD88. TLR3 and TLR4 recruit TRIF. Binding of pathogens and/or PAMPs to TLRs leads to complex signaling cascades, which results in transcription of proinflammatory mediators. In turn, these proinflammatory mediators, including chemokines, recruit neutrophils to the lungs during bacterial infection.

LRRs of TLRs -2,-4,-5 &-6 are located outside of the cell while the toll-interleukin (IL)-1 receptor homology (TIR) domain is located inside the cell in all TLRs (10). Distinct adaptor molecules, including Myeloid differentiation primary response gene (88) (MyD88), Toll-interleukin 1 receptor (TIR) domain-containing adapter protein (TIRAP), TRIF related

adaptor molecule (TRAM), TIR-domain-containing adapter-inducing interferon-β (TRIF) and Sterile-alpha and Armadillo motif containing protein (SARM), physically associate with the TIR domain of TLRs in order to transduce the signals. MyD88 is important for TLRs -1,-2,-4,-5,-6,-7, -8, -9, -10, and -11 mediated signaling network and it is recruited to the TLR complex by TIRAP in TLR2 and TLR4 initiated cascades. TRIF is involved in TLR3 and MyD88-independent TLR4 signaling. TRAM plays a key role in TRIF-dependent, MyD88-independent signaling through TLR4 (Figure 2.1).

It is important to note that individual TLRs can activate overlapping as well as distinct signaling cascades, ultimately providing diverse biological responses via activation of mitogen associated protein kinases (MAPKs), and transcription factors, in turn resulting in the expression of growth factors, cytokines/chemokines and cell adhesion molecules (Figure 1). For example, MyD88-dependent TLR signaling cascades lead to early NF-κB activation whereas MyD88-independent TLR signaling pathways result in delayed NF-κB activation. Of the TLR signaling cascades, the role of interleukin-1 receptor-associated kinases (IRAKs) in host defense has been well established. Four different IRAKs (IRAK-1, IRAK-2, IRAK-M, and IRAK-4) have been identified in mice and humans. Recently, patients with inherited IRAK-4 deficiency were reported who failed to respond to IL-1, IL-18, or to stimulation with TLR2, TLR3, TLR4, TLR5, and TLR9 agonists(26). In addition, findings with IRAK-M gene-deficient mice have shown that IRAK-M serves as a negative regulator of IL-1R/TLR signaling and therefore, IRAK-M knockout mice showed more neutrophils and augmented bacterial clearance to *P. aeruginos*a in the lungs in a sepsis model(27).

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

TLR	Infection (References)	Phenotype[a]			
		Survival	**Neutrophil Influx**[b]	**Bacterial Burden**[c]	**Bacterial Dissemination** [d]
TLR2	*Acinetobacter baumannii (20)*	↓	↓	**ND**	**ND**
	Legionella pneumophila (13-15)	↓	↓	↑	↑
	Porphyromonas gingivalis (12)	**ND**	**NS**	↑	**ND**
	Pseudomonas aeruginosa (21)	**ND**	**NS**	↑early	**ND**
	Streptococcus pneumoniae (11)	↓	↓	↑	↑
TLR4	*Acinetobacter baumannii (20)*	↓	↓	↑	↑
	Haemophillus influenzae (18)	↓	↓	↑	**ND**
	Klebsiella pneumoniae (17)	↓	**ND**	↑	**ND**
	Pseudomonas aeruginosa (21)	**ND**	↓late	**NS**	**ND**
	Streptococcus pneumoniae (16)	↓	↓	↑	**ND**
TLR5	*Legionella pneumophila (22)*	**ND**	↓early	**NS**	**ND**
TLR9	*Klebsiella pneumoniae (24)*	↓	**NS**	↑	↑
	Legionella pneumophila (23)	↓	**NS**	↑	**ND**
	Streptococcus pneumoniae (25)	↓	**NS**	↑	↑
TLR Adaptor					
MyD88	*Escherichia coli (35)*	↓	↓	**ND**	**ND**
	Haemophilus influenzae (31)	**ND**	**ND**	↓	**ND**
	Klebsiella pneumoniae (6)	**ND**	↓	**ND**	**ND**
	Legionella pneumophila (14,33,34)	↓	↓	↑	↑
	Pseudomonas aeruginosa (32,33)	↓	↓	↑	↑
	Staphylococcus aureus (32)	**ND**	↓	**ND**	**ND**
	Streptococcus pneumoniae (30)	↓	↓	↑	↑
TIRAP	*Klebsiella pneumoniae (6,36)*	↓	↓	↑	↑
	Escherichia coli (6,36)	**ND**	↓	↑	**ND**

(Table 1 Contd.)

TRIF	*Escherichia coli (36)*	↓	↓	↑	↑
	Pseudomonas aeruginosa (37)	**ND**	↓	↑	**ND**

Transcription Factor					
NF-κB p50	*Escherichia coli (48)*	↓	↓	**NS**	**ND**
	Streptococcus pneumoniae (49,50)	**ND**	↓	↑	**ND**
STAT3	*E. coli (53)*	**ND**	↓	↑	**ND**
STAT4	*K. pneumoniae (51)*	↓	**ND**	↑	↑
	Pseudomonas aeruginosa (52)	**ND**	**NS**	↑	**ND**
Cytokine					
IL-23	*Pseudomonas aeruginosa (57)*	**ND**	↓	**NS**	**NS**
	Klebsiella pneumoniae (56)	↓	**ND**	**ND**	**ND**
IL-17	*Klebsiella pneumoniae (59)*	↓	**ND**	**ND**	**ND**
TNFα	*Streptococcus pneumoniae (50)*	↓	↓	↑	**ND**
IL-1	*Streptococcus pneumoniae (50)*	↓	↓	↑	**ND**
CXC chemokine receptor					
CXCR2	*Pseudomonas aeruginosa (62)*	↓	↓	**ND**	**ND**
	Legionella pneumophila (64)	↓	↓	**NS**	**ND**
	Nocadia asteroids (63)#	↓	↓	↑	**ND**
CXC chemokine					
KC $	*Klebsiella pneumoniae (65)*	↓	↓	**ND**	**ND**
MIP2 #	*Klebsiella pneumoniae (65)*	↓	↓	↑	↑
	Nocadia asteroids (63)	**NS**	**NS**	**NS**	**NS**
Lungkine	*Klebsiella pneumoniae (67)*	↓	↓(24h) ** ↑(48h) **	↑	**ND**

[a]Phenotype was determined mainly by using gene-deficient or mutant mice after intrapulmonary infection and phenotypes established by transgenic mice are indicated seperately;

[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

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 (8), 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(28), 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 the IFN-α (29); these receptor agonists can be targeted to control bacterial infections.

Investigations have shown that TLR adaptor proteins play an important role in host defense against bacterial pathogens in the lung (Table 1). For instance, MyD88 is critical for host defense against numerous pathogens, including *S. pneumoniae* (25)*, H. influenzae* (30)*, P. aeruginosa* (31-33)*, S. aureus* (32)*, K. pneumoniae* (31)*, L. pneumophila (14, 33, 34)*, and *E coli (31)*. Furthermore, TIRAP is reported to be essential for host defense against *E. coli* and *K. pneumoniae* (6, 35). In addition, MyD88 gene-deficient mice show a more pronounced phenotype as compared to single or double TLR gene-deficient mice (14, 21, 34). Moreover, MyD88-independent, TRIF signaling has also been shown to be important for host defense in the lungs against *E. coli(35)* and *P. aeruginosa* (36). These observations demonstrate the 1) importance of MyD88 as an adaptor molecule for several TLRs; 2) importance of TRIF as an adaptor molecule for TLRs and/or 3) sequential activation of several TLRs during bacterial infection in the lungs. Our studies using a TRIF blocking peptide to attenuate the expression of IL-8, IL-6 and TNF-α, in response to *E. coli* demonstrate the importance of TRIF in humans (35). These results reveal the potential for

using cell permeable compounds to attenuate cytokine/chemokine production and thereby possibly be useful for reducing excessive neutrophil recruitment to the lungs.

NLRs

NLRs are the other types of PRRs involved in the innate immune system and are responsible to detect the intracellular pathogens and/or PAMPs. NLRs regulate both inflammation and apoptosis. To date, 22 NLR family members in humans have been reported (37). They have been further classified into subfamilies, including NODs and Neuronal apoptosis inhibitor protein CIITA, HET-E and TP-1(NACHT), LRR and PYD domains-containing proteins (NALPs), the MHC Class II transactivator (CIITA), IPAF and BIRC1. NLRs are characterized by the presence of a central NOD domain, a C-terminal LRR, and an N-terminal domain, including CARD or the putative protein-protein interaction (PYRIN) domain (37). The LRRs of NLRs are important for pattern recognition of microbial ligands.

Of the NODs, NOD1 and NOD2 are known to recognize bacteria and/or their components. NOD1 and NOD2 recognize the bacterial peptidoglycan components γ-D-glutamyl-mesodiaminopimelic acid and muramyl dipeptide, respectively(37). The NODs interact with microbial molecules by means of a C-terminal LRR region and activate downstream gene transduction events through N-terminal CARD domains, which leads to apoptosis. These receptors transmit the signals by the serine threonine kinase CARDIAK/RICK/RIP2 and activate NF-κB. Microbial molecules are detected by these proteins resulting in their oligomerization and activation of caspases (37). Active caspases play an important role in inflammation via cleavage and maturation of proinflammatory cytokines. Recent investigations have shown that NOD1 is important for bacteria-induced host response. For example, NOD1 detects *P. aeruginosa* peptidoglycan leading to NF-κB activation. Cytokine secretion kinetics and bacterial killing are altered in NOD1-deficient cells infected with *P. aeruginosa* in the early stages of infection (38). When NOD2 and NOD1 gene-deficient

mice were infected with *E. coli*, they showed reduced NF-κB activation, and attenuated cytokine/chemokines and neutrophil influx in the lungs (39). They also observed increased bacterial burden in the liver and spleen of NOD1 and NOD2 gene – deficient mice.

NACHT, LRR and PYD domains-containing proteins (NALPs) are the other members of the NLR family. NALPs have a PYRIN domain instead of a CARD domain at the N-terminus. This subfamily consists of 14 proteins but most of their functions have not yet been determined. NALP1, NALP2, and NALP3 have been involved in the cleavage of prointerleukin (IL)-1β and proIL-18 to mature forms via caspase-1, respectively (40). These NALPs form a complex of proteins called an "inflammasome" which comprises adaptor proteins and caspases. NALPs have emerged as mediators of antibacterial host defense in recent years. NALP1 has been shown to be important for host defense against *Bacillus anthracis(40)*. NALP3 is well studied and has been shown to be essential for host defense against *L. pneumophila* (41) and *Francisella tulerensis* (42). IPAF is another NLR family member. It contains CARD domains and has been shown to recognize *Salmonella* flagellin and induce IL-1β production (43). Flagellin of *L. pneumophila* is also responsible for IPAF-dependent caspase-1 activation (44). NLR family apoptosis inhibitory protein 5 (NAIP5) or baculoviral IAP repeat containing 1e (Birc1e) is an NLR protein that regulates host susceptibility to the intracellular pathogen *L. pneumophila*. NALP5 has been implicated in *L. pneumophila*-induced caspase-1 activation (44). Further understanding of the molecular pathways of NLRs in lung may lead to new strategies for controlling bacterial infectious diseases in the lungs.

Transcription Factors

Transcription factors are sequence-specific DNA-binding proteins that bind to the promoter or enhancer regions of specific genes and control the transfer of information from DNA to

RNA. Although several transcription factors, including NF-κB, AP-1, STAT and IRFs, play important roles in inflammation, NF-κB and STATs have been demonstrated to play important roles in bacterial pneumonia. NF-κB, a protein complex found in almost all mammalian cell types, is the most studied transcription factor to date. NF-κB exists in homodimeric and/or heterodimeric forms and consists of five family members, which include p50 (NF-κB1), p52 (NF-κB2), c-Rel, RelB, and RelA (p65) (45). All members of the family share the Rel homology domain. NF-κB acts as a transcription factor in response to stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens. In normal cells, NF-κB dimers are present in the cytoplasm and inhibited by IκBs (Inhibitor of κB), proteins that contain multiple copies of a sequence called an ankyrin repeat. The IκB proteins mask the nuclear localization signals of NF-κB proteins and keep them in an inactive state in the cytoplasm. Degradation of IκB leads to nuclear translocation of NF-κB and its subsequent binding to the promoters of and "turn on" expression of specific genes.

NF-κB can be activated by TLRs, NODs and NALPs and this triggers the expression of proinflammatory genes, including different cytokines and chemokines through MyD88 dependent and independent cascades. Recently, it was found that NLRs also activate NF-κB. Activation of NF-κB contributes to neutrophil accumulation elicited in the lungs by LPS, which is largely in part through the upregulation of several neutrophil chemokines (KC, MIP2, and CXCL5). Studies demonstrate that endogenous NF-κB protects the mice during *E. coli* (46, 47) and pneumococcal pneumonia and is essential for the survival during these infections (48). Neutrophil emigration to the alveoli during LPS-induced inflammation was severely reduced in TNF Receptor 1/RelA- double knockout mice, when compared to their control (TNFR1-deficient) mice (49). In a similar manner, it has been shown that deficiency of TNF-α and IL-1 receptor signaling reduces NF-κB activation to *S. pneumoniae (49)*.

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Furthermore, TRIF-deficient mice also showed reduced NF-κB activation and chemokine expression in the lungs during *P. aeruginosa* infection (36).

Although the role of NF-κB has been studied extensively in pneumonia, there are other transcription factors, such as STATs, which also play an important role in the innate immunity. STATS can be activated by cytokines, including IL-6. Cytokines bind to their homodimeric or heterodimeric receptors, which can bind to janus kinases (JAK). JAKs can be activated by transphosphorylation followed by phosphorylation of cytokine receptors, allowing STATs to bind cytokine receptors. The activated STATs translocate to the nucleus where they bind DNA and induce transcription of genes. Studies have shown that the STAT4 is an integral component of the host innate immune response leading to cytokine production and bacterial clearance during *K. pneumoniae* infection in the lung (50). STAT4 also contributes to *P. aeruginosa* induced inflammation but is not essential for bacterial clearance(51). Furthermore, STAT1 and STAT3 contribute to neutrophil emigration during *E. coli* pneumonia. During *E. coli* pneumonia, IL-6 family members specifically activate alveolar epithelial STAT3 in order to promote neutrophil infiltration into the lungs (52).

Cytokines

Cytokines are polypeptides produced by variety of cell types. They have autocrine, paracrine or endocrine functions to regulate inflammation and immune defense. Cytokines bind to their cognate receptor and signal via second messengers, thereby increasing or decreasing the expression of other membrane proteins and can be categorized as pro-inflammatory or anti-inflammatory. TNF-α, IL-1β, IL-6, IL-8 and IFN-γ are major pro-inflammatory cytokines which participate in acute inflammation; IL-8 acts as a chemoattractant whereas TNF-α and IL-1β stimulate antigen presentation and increase the expression of cell adhesion molecules. On the other hand, IL-10, TGF-β and IL-1Ra are major anti inflammatory cytokines that down regulate the inflammatory response in the lungs(53) .

The importance of interleukin (IL)-12 in antibacterial host defense has been widely established. The IL-12-IFN- γ axis is known to be involved in host immune defense against bacterial pathogens and induces cell-mediated immune responses to clear infection. However, the role of the IL-23-IL-17 axis has only recently been recognized, as mice which lack both IL-12 and IL-23 are more susceptible to bacterial infections (54). IL-23 is a newly described cytokine synthesized by macrophages and dendritic cells. It shares the p40 subunit with IL-12, an important cytokine in the induction of the Th1 response (e.g., IFN- γ), but has a distinct p19 subunit. In general, IL-23 produces more IL-17 from $CD4^+$ T cells than IL-12. Several recent studies suggest that bacterial infection can induce IL-23 expression by macrophages and/or dendritic cells. It has been documented that IL-23 is essential to mediate host defense in the lungs against *K. pneumoniae* and *P. aeruginosa* (55, 56).

IL-17, which is mainly produced by T cells via the Th17 immune cascade, has been proposed as a proinflammatory cytokine (57). Recently, five new IL-17 family members have been identified; IL-17B, IL-17C, IL-17D, IL-17E (also named IL-25) and IL-17F (57). Virtually all cells bear the IL-17 receptor, which has been shown to recruit neutrophils to the lung by stimulating the production of the ELR+CXC chemokines; IL-8 or KC (a mouse homolog of IL-8) (57), and G-CSF. In addition, IL-17A seems to be important for the early and the late phase of neutrophil accumulation in the lungs (52). Furthermore, IL-17 can induce the upregulation of other cytokines including IL-1β and IL-6, and ICAM-1 by several types of cells (e.g., endothelial cells, epithelial cells) (57). Using gene-deficient mice, it has been shown that IL-17 is important for host defense in the lungs against respiratory infection in mice caused by *K. pneumoniae* (58).

Chemokines

Cytokines that act as chemoattractants for other cells are known as chemokines. Chemokines are produced locally at sites of infection/inflammation and regulate the recruitment of

specific subpopulations of leukocytes from the bloodstream into tissues. Chemokines enhance neutrophil adhesion and extravasation across the post-capillary venules and direct migration neutrophils to sites of infection/inflammation. According to the composition of a cysteine motif located near the N-terminus of these molecules, they are categorized into the C, CC, CXC and CX3C subgroups. These chemokines have four cysteine molecules, the first two of which are separated by a non-conserved amino acid, and can be further categorized by the presence of the ELR (glutamic acid-leucine-arginine) motif immediately preceding the CXC sequence(59). All known ELR+CXC chemokines are neutrophil chemoattractants. Seven chemokines (IL-8; NAP-2; GRO α,β and γ; ENA-78 and GCP-2) have been identified in humans(59). Among these IL-8 is the most potent neutrophil chemoattractant in bacterial pneumonia. Although a real homolog of human IL-8 has not been identified in rodents, keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2, LPS-Induced CXC Chemokine (LIX; CXCL5), and lungkine are important chemoattractants in mice in response to bacterial lung infection (60).

While most chemokines are secreted by myeloid cells, lungkine and LIX are secreted by bronchial epithelial cells and type II alveolar epithelial cells respectively. There are two receptors identified for CXC chemokines: CXCR1 and CXCR2, which are expressed in both humans and mice. CXCR2 binds to all ELR+CXC chemokines and is essential for neutrophil infiltration into the lungs during bacterial infection. In this context, *in vivo* studies have shown that neutrophil recruitment induced by *P. aeruginosa* (61)*, Norcardia asteroids* (62) and *L. pneumophila* (63) involves the CXCR2 receptor (Table 1). Regarding the CXCR2 ligands, KC, and MIP2 are important to neutrophil influx during infection, as blocking these ligands impairs host defense (61, 64). Although lungkine is not important for neutrophil influx into the lung parenchyma, it is important for neutrophil accumulation in the airspaces (65) (Table 1). It has been reported that CXCL5 upregulation increases neutrophil trafficking

during infections with *P. aeruginosa*, *K. pneumoniae*, *L. pneumophila* and *Bordetella bronchiseptica*. Subsequent studies have shown that LIX is an important molecule for neutrophil influx in the lungs during LPS-induced inflammation (66). However, the role of LIX in neutrophil infiltration in the lungs during bacterial infections remains to be determined.

Emerging Roles for Cholesterol in PMN Recruitment to the Lung

Cholesterol exerts complex effects upon the pro-inflammatory functions of macrophages, PMNs, and other cell types. Cholesterol-overloading induces macrophages to produce cytokines in part through endoplasmic reticulum stress (67-69), though it may also either enhance or attenuate macrophage responses to LPS (70, 71). Hypercholesterolemia primes PMNs for oxidant and granule protein release (72, 73), induces PMN adhesion to and emigration from post-capillary venules (74), and promotes mononuclear cell accumulation in vascular lesions by inducing endothelial chemokines (75, 76). The net effect of systemic dyslipidemia on organ-localized inflammation is likely, however, to be complex, as cholesterol loading also impairs leukocyte chemotaxis (77), and lipoproteins, the particles that carry cholesterol in the bloodstream, bind to and neutralize bacterial lipopolysaccharide (LPS)(78) and inhibit leukocyte signaling responses to multiple Toll-like Receptor ligands (79). Further complicating expectations for the net effect of cholesterol upon inflammation in the lung in particular is the fact that the mechanism of, and requirements for PMN transmigration from the vascular compartment into the airspace differ substantially from those at work in other organs (3).

While little is known about either the sensitivity of lung-resident cells to systemic dyslipidemia (i.e., circulating lipoprotein cholesterol) or the lung's local regulatory mechanisms for cholesterol, emerging reports interestingly do suggest that cholesterol

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trafficking and inflammation may be coupled uniquely in the lung. For example, genetic deletion of the cellular cholesterol efflux pump ATP Binding Cassette Transporter G1 (ABCG1) induces recruitment of multiple leukocyte subtypes, including PMNs, to the unexposed murine lung (67, 69), responses that reflect cooperative pro-inflammatory contributions from cholesterol-overloaded alveolar epithelium and alveolar macrophages. Conversely, the potential for therapies that reduce cellular and serum cholesterol to reduce PMN recruitment to the airspace has also been reported. Three different hydroxy-methylglutaryl coenzyme A reductase inhibitors ('statins') used to treat clinical hypercholesterolemia have now been shown to reduce PMN recruitment to and microvascular injury within the rodent lung following LPS exposure (80-82), likely due to dual effects upon PMN chemotaxis and endothelium. Statins also enhance clearance of apoptotic PMNs by alveolar macrophages (83). While statins do exert some effects on leukocyte function by depleting cellular cholesterol (84), other effects stem from depletion of cellular isoprenoids and consequent inhibition of cellular protein prenylation (80). Systemic treatment of rodents with synthetic agonists of Liver X Receptor (LXR), a nuclear receptor that promotes cellular cholesterol efflux and reverse cholesterol transport, has also been shown to reduce PMN recruitment into the airspace induced by LPS and Gram-negative bacteria (85, 86). A potential untoward consequence of reduced PMN recruitment to the lung through cholesterol targeting is impaired antibacterial host defense, as both statins and LXR agonists also reduce clearance of bacteria deposited in the rodent lung (80, 86). In the case of statins, impaired bacterial clearance may also reflect drug-induced defects in PMN bactericidal function (80). This said, clinical expression of pneumonia and its complications reflect not only pathogen proliferation, but also bystander tissue injury from responding host cells.

In recent years, the relationship between statin use and risk of community-acquired pneumonia has been examined in observational studies. Several retrospective studies have

reported that statins are independently associated with either reduced risk of pneumonia (87, 88) or reduced pneumonia-associated mortality (89-93). Prospective, randomized, controlled trials will be necessary to resolve a potential role for statins in pneumonia therapy, however, as a prospective cohort study of patients hospitalized for community-acquired pneumonia reported no significant relationship between statins and a composite outcome of in-hospital mortality or admission to an intensive care unit (94). Given these findings, it may be difficult to conclusively rule out persistent confounding or a 'healthy user' effect (95). Moreover, publication bias cannot be excluded.

The effect of cholesterol upon PMN recruitment to the lung likely depends upon the cell types having dysregulated cholesterol, the specific molecular features of the dyslipidemic state, and perhaps the nature of the alveolar or hematogenous insult. Contrary to the effects of localized pulmonary cholesterol overload observed in ABCG1 deficiency, systemic dyslipidemia induced by either diet or genetic manipulation is associated with *reduced* recruitment of PMNs to the LPS-exposed airspace, reflecting both reduced airspace chemokine expression and impaired PMN chemotaxis; dyslipidemic rodents nevertheless have *increased* airspace fluid protein suggesting aggravated injury to the alveolocapillary barrier (Fessler, unpublished observations). Collectively, these data indicate that cholesterol has the potential to either promote or inhibit PMN recruitment to the airspaces, and may also impact lung injury through independent, direct effects on the integrity of the air-blood barrier. Given the high prevalence of dyslipidemia and its pharmacologic treatment in modern-day society, further investigation of the complex effects of cholesterol upon PMN recruitment to the lung is clearly urgently needed.

Alcohol

Alcohol abuse has been associated with increased risk for bacterial pneumonia. Observational data show that alcoholic men and women suffer increased mortality from bacterial pneumonia compared to controls (96). A key element to early host defenses against invading microbes is the robust recruitment of neutrophils into the infected lung. Extensive work has shown conclusively shown that alcohol intoxication interferes with both the recruitment and functional capacity of neutrophils during bacterial infection of the lower respiratory tract (97-100).

The early expression of "alarm" cytokines, such as TNF-α by alveolar macrophages is central to the development of the histologic hallmark of bacterial pneumonia: neutrophil infiltration (101, 102). Acute alcohol intoxication impairs lung TNF-α production in response to LPS at the post-transcriptional level (103, 104). By impairing the co-localization of TNF-α and the cell-surface bound TNF-α converting enzyme (TACE), acute ethanol exposure prevents cleavage of TNF-α from the producing cell's surface(105). The effect of chronic ethanol exposure on TNF-α release is controversial. While some work suggests chronic intoxication increases TNF-α release via augmented TACE activity (106) and stabilization of TNF-α mRNA(107), other studies show suppression of TNF- α production resulting from chronic alcohol (108, 109).

Although TNF-α is not itself a chemoattractant for neutrophils, it stimulates lung expression of CXC chemokines, which in turn promote the recruitment of neutrophils from the vasculature. Animal models of infection have shown that acute alcohol intoxication suppresses the lung's expression of the neutrophil chemokines MIP-2 and CINC, rodent orthologues of the human neutrophil chemokines IL-8 and Gro-α, which bind the CXCR2 receptor found on neutrophils (100, 110). This suppression is accompanied by decreased

neutrophil recruitment, and exogenous chemokine administration has shown promise in restoring intrapulmonary neutrophil influx (111). The expression of neutrophil chemokines comes from many cell types in the lung, including the alveolar epithelium (112). Taken together, this suggests that alveolar parenchymal cells may be susceptible to alcohol. We recently investigated this hypothesis by examining the effect of acute intoxication on pulmonary expression of LIX, a neutrophil chemoattractant whose expression is limited to alveolar type II epithelial cells(113). Ethanol exposure decreased LIX expression in response to airway endotoxin challenge *in vivo*, and ethanol exposure downregulated LIX in a dose-dependent manner in primary cultures of type II alveolar epithelium (114). These cells showed inhibition of NF-κB and p38 MAP kinase pathways in response to relatively low (25mM) ethanol concentrations, suggesting they are exquisitely sensitive targets of ethanol. In addition to the effects of ethanol on the lung epithelium, Zhang et al. have shown that acute ethanol intoxication profoundly suppresses the expression of the neutrophil chemokines S100A8 and S100A9 by leukocytes (115).

In addition to decreased chemokine expression, alcohol exposure results in intrinsic defects in neutrophils that impede migration. During pneumonia, circulating neutrophils up-regulate the β_2-integrin adhesion molecule, CD11b/CD18. CD11b/CD18 mediates neutrophil firm attachment to the pulmonary capillary endothelium and their subsequent trans-endothelial migration. Alcohol inhibits the up-regulation of CD18 expression on neutrophils in response to inflammatory stimuli (116) and suppresses neutrophil adhesion to the endothelium during appropriate stimulation (117). Furthermore, studies of neutrophils taken from chronic alcohol abusers show that these cells are hypo-responsive to chemotactic stimuli (118, 119). In alcoholics with liver disease, it has been proposed that systemic LPS exposure (through increased intestinal permeability and portal blood LPS content) induces a chronic inflammatory state. Neutrophil chemokines (IL-8) and chemotactic complement fragments

(such as C5a) are elevated in the peripheral circulation of patients with alcoholic liver diseases (120, 121). The chronic *in vivo* activation of neutrophils has been postulated to account for the blunted response of neutrophils to chemoattractants in these hosts. Compounding this recruitment defect, alcohol-exposed neutrophils are less capable of killing bacteria owing to abnormalities in phagocytosis, degranulation, and superoxide generation (122, 123).

Alcohol abusing patients are frequently leukopenic and they often do not mount an appropriate leukocytosis during pneumonia (124). Given the importance of neutrophils in pathogen clearance, it is not surprising that neutropenia in the alcoholic increases mortality from pulmonary infection, particularly bacteremic pneumococcal pneumonia (125). During bacterial pneumonia, the lung expresses G-CSF (126, 127). In contrast to most cytokines, which are "compartmentalized" within the lung during infection, G-CSF readily exits the pulmonary tissue and enters the circulation during infection (126, 128). G-CSF thus serves as a means of communication between the infected lung and bone marrow (129). Studies of systemic and pulmonary infection have shown that acute alcohol inhibits the expression of G-CSF (130), and animal studies have shown that recombinant G-CSF treatment can partially restore lung neutrophil recruitment in response to a bacterial stimulus (131). Examination of bone marrow from chronic alcoholics often shows hypocellularity, maturation arrest, and vacuolization of myeloid progenitor cells (132, 133). Bone marrow cells treated with clinically relevant ethanol concentrations show impaired granulocyte colony formation, also suggesting a direct toxic effect on these tissues (134, 135). In the context of bacterial infection, we have recently shown that alcohol inhibits the increase in granulopoietic progenitor cell proliferation in mice challenged with *E. coli* (136). This effect is at least partially due to alcohol preventing the phenotypic reversion of hematopoetic precursors to a

less-differentiated state, a phenomenon described in hematopoetic precursors (129) which appears requisite for optimal granulocyte production.

The discovery of IL-17 has broadened our understanding of how the lung solicits neutrophil influx during infection. A product of T lymphocytes and related cell types, IL-17 induces CXC chemokine production which promote neutrophilic inflammation (137). In animal studies, inoculation with *K. pneumoniae* induces a TLR4- dependent pulmonary IL-17 expression within 12 hours, and animals deficient in the receptor for IL-17 show enhanced mortality from infection (138). Chronic alcohol intoxication inhibits IL-17 secretion in the lungs in response to *K. pneumoniae*, and in vitro stimulation of T cells confirms a dose-dependent inhibition of IL-17 by ethanol (139). IL-23 is induced in alveolar macrophages and myeloid dendritic cells during challenge and is the dominant trigger for IL-17 (140). The expression of IL-23 in the lung during bacterial challenge is strongly suppressed by acute ethanol intoxication (55). By pre-treating animals with an adenoviral vector encoding IL-17 prior to *K. pneumoniae* inoculation, neutrophil recruitment is restored and survival improved in alcohol-treated animals, suggesting transient pharmacologic gene expression therapies may offer promise in the treatment of immunosuppressed patients with bacterial pneumonia (55). A product of NK T cells, invariant NK T (iNKT) cells, gamma delta T cells, and CD4+ T cells, IL-17 induces CXC chemokine production, which promote neutrophilic inflammation (58, 137, 141, 142). While the precise source(s) of lung IL-17 expression during acute bacterial infection are not known, work has shown that depletion of CD4+ or CD8+ cells results in a decrease in lung IL-17 expression as expressed during *Klebsiella* infection. It is likely that several of these cell populations each contribute to the IL-17 response to infection.

Concluding Remarks

Bacterial pneumonia is an important disease. Neutrophils are one of the first cells to reach the site of bacterial infection. An ideal therapeutic strategy would be to attenuate the tissue destructive potential of neutrophils without reducing their efficacy in antibacterial defense. Theoretically, one-way to do this may be to identify therapeutic targets to attenuate excessive neutrophil accumulation during bacterial pneumonia. 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 cholesterol in neutrophil recruitment and it has also been shown that alcohol affects neutrophil recruitment to the lungs 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 increasingly difficult. The future challenge will be to apply our current understanding of neutrophil function to design therapeutic methods to maintain the host defense potential of neutrophils while modulating their destructive potential. In this context, therapeutic potential of TLRs and NLRs remains to be explored.

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Chapter 3

Monocyte Chemoattractant Protein 1 Regulates Pulmonary Host Defense via Neutrophil Recruitment during *Escherichia coli* Infection **

Introduction

Bacterial pneumonia is a leading cause of death in both immunocompromised and immunocompetent individuals (12, 25, 33). Successful host defense against bacterial infection in the lung is dependent on effective recruitment of phagocytes into the alveolar space. During cellular recruitment, neutrophil infiltration is the first event followed by monocyte/macrophage accumulation at the site of infection. Several lines of evidence suggest that cytokines/chemokines regulate leukocyte migration in concert with cell adhesion molecules, including ICAM-1 and VCAM-1 (1, 11, 32). In this context, monocyte chemoattractant protein 1 (MCP-1/ chemokine [C-C motif] ligand 2 [CCL2]) has been shown to be a potent monocyte/macrophage and T cell chemoattractant in lung infection against bacteria (10, 31, 34, 35, 39, 49). MCP-1 is produced by a variety of cells, including epithelial cells (41), endothelial cells, fibroblasts (42), and monocytes/macrophages(48) and it binds to the G-protein–coupled transmembrane receptor CCR2.

Prior studies have unequivocally demonstrated the important role of MCP-1 in monocyte/macrophage-mediated host defense against bacterial infection. During *Streptococcus pneumoniae* infection, overexpression of MCP-1 has shown to improve bacterial clearance (46). Using MCP-1$^{-/-}$ mice, Winter *et al.* (45) have demonstrated that MCP-1-mediated macrophage recruitment is important to prevent bacterial dissemination following pulmonary *Streptococcus pneumoniae* infection. During *Pseudomonas aeruginosa* infection, MCP-1 promoted resolution and repair of the lung by enhancing the uptake of apoptotic neutrophils by alveolar macrophages (2). In addition, mice deficient in CCR2 showed impairment in macrophage migration and clearance of bacteria from the lungs and extrapulmonary organs after i.v. challenge with *Listeria monocytogenes* (24). MCP-1 is also shown to be important for the survival of mice during *P. aeruginosa* and *Salmonella typhimurium* infections and MCP-1 promotes bacterial killing by macrophages (35).

MCP-1 is further implicated in cellular trafficking to the inflamed lung via multiple mechanisms. MCP-1 modulates the expression of β2 integrin and thereby triggers firm monocyte adhesion to the inflamed endothelium (28). Studies have shown that CCR2 plays an essential role in the induction of adaptive arms of the immune system via the induction of Th1 cytokines (10). However, the role of MCP-1 in neutrophil recruitment is debatable and the role of MCP-1 in neutrophil recruitment during *E. coli* infection of the lungs is not studied. In one study, it has been shown that administration of exogenous MCP-1 alone did not cause neutrophil influx into the lungs whereas administration of MCP-1 along with LPS did cause excessive neutrophil recruitment (29). In another investigation, it has been demonstrated that neutrophils express CCR2 and chemotax towards MCP-1 (21). However, the role of MCP-1 in neutrophil trafficking and activation in the lung in acute bacterial pneumonia has not been studied.

The objective of the current investigation was to determine the role of MCP-1 in neutrophil-mediated host defense following *E. coli* infection. In this regard, we used MCP-1 gene-deficient (MCP-1$^{-/-}$) mice. Our findings demonstrate MCP-1 is induced upon *E. coli* infection and can be produced by both myeloid and non myeloid cells. MCP-1 contributes to bacterial clearance in the lungs via neutrophil recruitment in a murine model. Our findings indicate that MCP-1 causes neutrophil recruitment to the lungs directly via chemotaxis as well as indirectly via modulating the levels of KC and MIP-2 following *E. coli* infection. Furthermore we show that neutrophils from blood and BALF express CCR2 and it is increased upon *E. coli* infection.

Materials and Methods

Mice: Eight to 10-wk-old female mice genetically deficient in MCP-1 (16) or MD-2 (9) were used while age- and gender-matched C57Bl/6 mice were used as controls. All animal

studies were approved by the Louisiana State University and National Jewish Health Animal Care and Use Committees. The mice ranged from 19 to 25 g in weight.

Infection Model: Bacteria were prepared for mouse inoculation, as described in previous studies (8, 22). *E. coli* (American Type Culture Collection (ATCC) 25922) 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 20×10^6 CFU/ml. Mouse strains were anesthetized with i.p. ketamine/xylazine (250 mg/kg), followed by intratracheal (i.t.) inoculation of 50 μl of bacteria (10^6 CFU/mouse) whereas control mice were i.t. inoculated with 50 μl of saline. The initial mouse inoculums 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 for 30 s, 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 15 s for bacterial culture. Bacterial colonies were counted after incubation overnight at 37°C. For CFU studies, we used a higher dose of *E. coli* (5×10^6 CFU/50 μl/mouse) since low dose (10^6 CFU/mouse) did not induce substantial mortality either in MCP-1$^{-/-}$ or WT mice.

Lung Pathology: The lungs were perfused from the right ventricle of heart with 10 ml isotonic saline at 24 h post infection from WT and MCP-1$^{-/-}$ mice. Lungs were then removed and fixed in 4% phosphate-buffered formalin. Fixed tissue samples were processed in paraffin blocks, and 5 μm sections were cut with a microtome and stained with hematoxylin and eosin (H&E). Analysis of histopathology was 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.

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 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°C.

Bone Marrow Transplantation : Bone marrow chimeras were generated as described earlier (7, 9) . Recipient mice were γ irradiated from a cesium source in two 525-rad doses 3 hours apart. Bone marrow cells (8X10^{6}/mouse) from donor mice were injected into the tail vein of the irradiated recipients. Transplanted mice were maintained on 0.2% neomycin sulfate for the first 2 weeks. The reconstituted mice were used 2 months after the transplantation. In parallel experiments, we used green fluorescent protein–expressing donor cells. Sample blood was collected from these recipients between 6 and 8 weeks after transplantation, and hematological parameters (differential counts) were assessed. Greater than 90% of blood leukocytes were derived from donor mice at the time the mice were used for experiments (8 wk after transplantation; data not shown). Irradiated mice that were not transplanted with donor cells died between Days 20 and 22 after transplantation (data not shown).

Cytokine, Chemokine and LTB4 Determination: We used BALF and lungs that were obtained from animals after *E. coli* infection or saline instillation. ELISA kits for TNF-α, IL-6, LTB$_4$ and MCP-1 were obtained from eBiosciences, PA whereas kits for KC and MIP-2

were obtained from R&D systems, MN. The minimum detection limit is 8 pg/ml cytokine protein whereas the detection limit for LTB$_4$ is 13.7 pg/mg/ml.

Western Blotting: The lungs were collected at the designated time points and used for Immunoblotting as described in our previous publications (8). The primary 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), p47[phox], p67[phox] and iNOs were obtained from Cell Signalling, Boston, MA, and were added at a 1:1,000 dilution. The primary Abs to total p38 and GAPDH (Santa Cruz Biotechnology, CA) were added at 1:5,000 dilution. Immunostaining was performed using 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.

NF-κB DNA Binding Assay: Nuclear proteins were extracted from 50-80 mg lung tissue collected at 6 and 24 h post-*E. coli* or saline administration. 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 from Active Motiff, Carlsbad, CA). Wells were then washed and plates were incubated with NF-κB/p65 antibody for 1 h. Plates were then washed three times 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. This method has been described in our earlier publications (8, 9, 22).

Neutrophil Transmigration Assay. Chemotaxis assays in a Transwell system were performed using 24-well tissue culture plates treated polycarbonate membrane polystyrene

plates (3415, Costar, Corning Inc. NY) with a pore size of 3.0 µm. Chemoattractants, either KC (2µg/ml, 1 µg/ml) or MCP-1(2 µg/ml) (R&D, Minnestota, MN), and PBS supplemented with bovine serum albumin (2 µg/ml) were added to each of the lower wells (500µl final volume). A 100-µl suspension of $1X 10^6$ LPS activated PMN in DMEM 0.1%BSA was added to each well of the upper filter plate. After incubation at 5% CO_2 at 37 °C for 3h, the upper plate was removed, and cells in the lower plate were counted from 20 non overlapping fields by using inverted microscope (18, 19).

Examination of CCR2 Expression on Neutrophils. A total of 50 µl of whole blood or BALF of WT and MCP-1^{-/-} mice treated with saline or *E .coli* was aliquoted into flow cytometry tubes and Fc blocked and10 µl of mouse conjugated anti-mouse Gr-1, CCR2, and CXCR-2 (R&D, Minnestota,MN) antibodies were added to appropriate tubes. Samples were vortexed and incubated for 30 min at room temperature in dark. Then cells were washed by adding 2 ml of 1X PBS and centrifuged at 1000 rpm (200 x g)/8 min. RBC was 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 1000 rpm (200 x g)/8 min and the supernatant was removed. Cells washed twice with PBS. Cells were fixed by adding 200 µl of cold 1% formaldehyde-PBS and stored at 1-6 °C for FACS analysis.

Neutrophil Depletion: The protocol used to deplete neutrophils (Gr1^{+}) in mice has been described in our earlier reports with minor modifications (7). A total of 50 µg anti-Gr1/Ly6G mAb (Sodium azide- free and low LPS content; 1A8 BD pharmingen, San Diego, CA) in 250 µl was administered i.p. at 12 and 2 h before bacterial infection. In control experiments, 50µg of isotype control mAb in equal volumes was administered at the same time points prior to bacterial infection. To validate the efficiency of neutrophil depletion by the antibody, we

obtained differential WBC counts in blood every 12 h up to 3 days, and ≤ 3% neutrophils were found up to 3 d after depletion.

AM Culture: AMs were isolated as described in earlier publications (7). Positive selection by CD11b MicroBeads (stem Cell Technologies, Vancouver, British Columbia, Canada) was applied to isolate AMs from BAL cells. Pooled BAL cells were resuspended in RoboSep buffer, and CD 11b PE labelling reagent was mixed with cell suspension, followed by incubation at room temperature for 15 min. EasySep PE selection mixture was thereafter added to the cell suspension, and the suspension was further incubated for 15 min at room temperature. EasySep Magnetic nanoparticles were lastly mixed with the cell suspension, followed by magnetic separation of $CD11b^+$ cells. Cells were washed with PBS, centrifuged at 1200rpm for 10 min, and resuspended in DMEM containing 5% FBS. Cells were infected with *E.coli* (MOI of 1) and harvested at designated time points using urea/CHAPS/Tris buffer. Samples were centrifuged and supernatants were used for western blotting. Finally, the total number of live cells and the percentage of purified AMs were determined by utilising trypan blue exclusion and cytospin preparations stained with Wright-Giemsa, respectively. Percentage recovery of AMs was calculated as the ratio of AMs to the total macrophages isolated from the BALF. Using this technique, we obtained ~85-90% AMs.

Statistical Analysis: Data are expressed as mean $\pm$ SE. The intensity of immunoreactive bands was determined using a Gel Digitizing Software (UN-SCAN-IT gel**TM**) from Silk Scientific, Inc, Utah, USA. Data were analyzed by ANOVA followed by Bonferroni's post hoc analysis for multiple comparisons. All statistical calculations were performed using InStat software and GraphPad Prizm 4.0. Differences were considered statistically significant at *P<0.05 when compared with control.

Results

MCP-1 is Essential for Bacterial Clearance Following *E. coli* Infection. We used an experimental model of pulmonary *E. coli* infection to demonstrate the MCP-1 protein expression in BALF and lung homogenates. MCP-1 levels were increased at 6 and 24 h in BALF (Fig. 3.1A) and the lungs of WT (C57Bl/6) mice (Fig: 3.1B) compared to their saline-challenged animals after i.t. *E. coli* (10^6/mouse) infection (Fig: 3.1A-B).
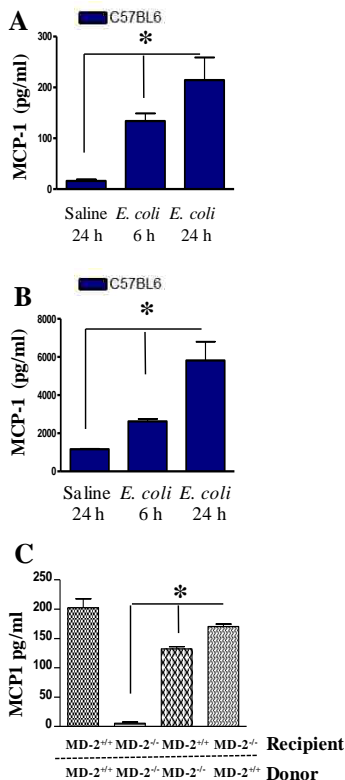
Figure 3.1. A-B. Kinetics of MCP-1 production. MCP-1 concentration was measured in BALF (A) and lung homogenate (B) from WT mice by ELISA after i.t. infection with *E. coli* (10^6/mouse). Asterisks indicate significant difference between MCP-$1^{-/-}$ and WT mice ($p < 0.005$; n = 4-5 mice/group/time-point and the figure is a representation of 3 individual experiments). MCP-1 expression in mice transplanted with bone marrow following *E. coli* infection (C). *indicates significant difference between KO and WT mice; $p < 0.005$. Data shown is a representation of 3 individual experiments.

In order to determine the cell type that can produce MCP-1 during *E. coli* infection, we used chimeric MD-$2^{-/-}$ mice and infected with *E. coli*. 24 h post infection. We found that both myeloid and non myeloid cell-derived MD-2 is important to produce MCP-1 following *E. coli* infection (Fig: 3.1C). To determine whether a deficiency in MCP-1 compromised host defense against *E .coli* in the lung, mice were infected with i.t. *E. coli* (1×10^6 CFU/mouse) and sacrificed at 6 and 24 h post-infection. The lungs and spleens were isolated to determine the bacterial CFU. MCP-$1^{-/-}$ mice had greater numbers of CFU in the lung at 6 and 24 h post-infection (Fig: 3.2A). However, we did not observe bacterial dissemination in the bloodstream in either MCP-$1^{-/-}$ mice or their controls at 6 or 24 h (data not shown). Since we observed higher bacterial burden in MCP-$1^{-/-}$ mice both at 6 and 24 h

following regular dose of *E. coli* (1×10^6/mouse) infection (Fig. 3.2A), we chose to use the regular dose in the rest of our experiments.

MCP-1$^{-/-}$ Mice Show Attenuated Cellular Recruitment to the Lung following *E. coli* Infection. Leukocyte recruitment to the site of infection is a critical step to clear bacteria. Therefore, we determined the total as well as differential leukocyte count in BALF at 6 and 24 h post-infection with the regular dose (1×10^6 CFU/mice) (Fig. 3.2B-C). We observed reduced leukocyte influx at 24 h post-infection in MCP-1$^{-/-}$ mice and this reduction was primarily due to reduced neutrophil influx although macrophage numbers were reduced at this time (Figs. 3.2B-C). Furthermore, attenuated leukocyte numbers and alveolar edema was noted in lung histology of MCP-1$^{-/-}$ mice at 24 h. i.e., lungs from WT mice infected with *E. coli* show a score of 2 (average of 3 mice) whereas lungs from MCP-1$^{-/-}$ mice infected with bacteria show a score of 1 (average of 3 mice) (Fig. 3.2D). These findings show that MCP-1 is important for the neutrophil accumulation in addition to macrophage influx during *E. coli* infection.

MCP-1 Regulates the Production of Cytokine/chemokines and Expression of Cell Adhesion Molecules. Leukocyte recruitment during infection is a multistep process in which cytokine/chemokine production along with up regulation of cell adhesion molecules plays an important role. Hence, we used BALF to determine cytokine/chemokine levels following *E. coli* infection. Our data demonstrate that levels of cytokines, such as TNF-α and IL-6 (Figs. 3.3A-B) and neutrophil chemoattractants, such as KC and MIP-2 (Figs. 3.3C-D) were reduced at 24 h following *E. coli* infection in MCP-1$^{-/-}$ mice. We also examined the expression of the neutrophil chemotactic lipid LTB$_4$ in the lung of MCP-1$^{-/-}$ mice following *E. coli* administration and found that the levels of LTB4 were decreased in MCP-1$^{-/-}$ mice at 6

h, but not at 24 h (Fig. 3.3E). Although ICAM-1 expression in the lung remained unchanged, the VCAM-1 expression was substantially reduced in MCP-1$^{-/-}$ mice at 24 h after infection with *E. coli* (Fig. 3.3F-H). Our data suggest that MCP-1 controls the expression of cytokines, neutrophil chemoattractants and cellular adhesion molecules.
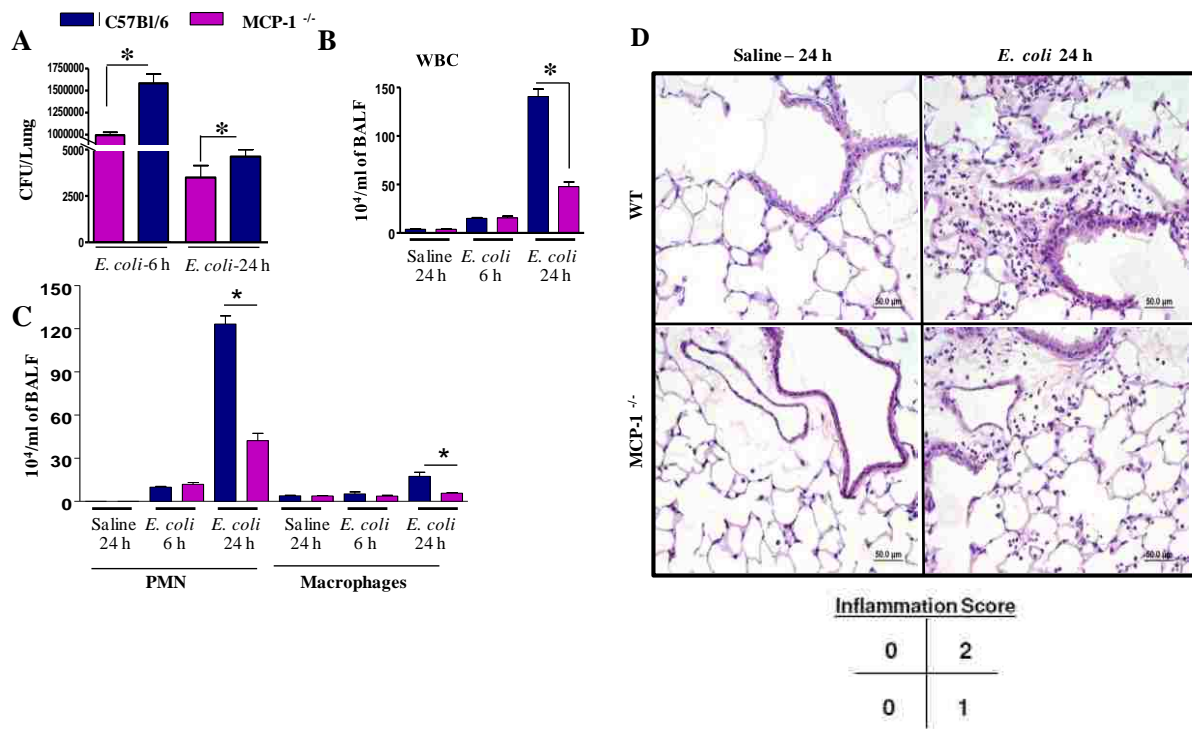

Figure 3.2. Bacterial clearance and leukocyte recruitment in the lungs of MCP-1$^{-/-}$ mice following *E.coli* infection. A. Bacterial burden in the lung of MCP-1$^{-/-}$ mice following *E. coli* infection (10^6/mouse). Lungs were collected from control and infected group of mice at the designated times, homogenized and the number of bacteria were enumerated (n=5-6 mice/group). *indicates significant difference between KO and WT mice; p<0.005. Data shown is a representation of 3 individual experiments. B-C. Cellular infiltration in the lung in MCP-1$^{-/-}$ mice against *E. coli*. 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 infiltration to the lung (n=4-5 mice/group; p<0.005, data are a representation of 3 individual experiments). D. Lung histology in MCP-1$^{-/-}$ mice following *E. coli* infection. Mice were inoculated with *E. coli* (10^6 CFU/mouse), lungs were obtained at 24 h post-infection. This picture is a representative of 3 separate mice with comparable results.

MCP-1 Activates NF-κB, MAPKs in the Lung following *E. coli* Infection. NF-κB and MAPK regulates the expression of multiple proinflammatory genes in the inflammatory setting. We therefore investigated the activation of NF-κB and MAPKs in the lungs after *E.*

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coli infection. Our findings demonstrate that NF-κB activation was reduced in MCP-1 $^{-/-}$ mice
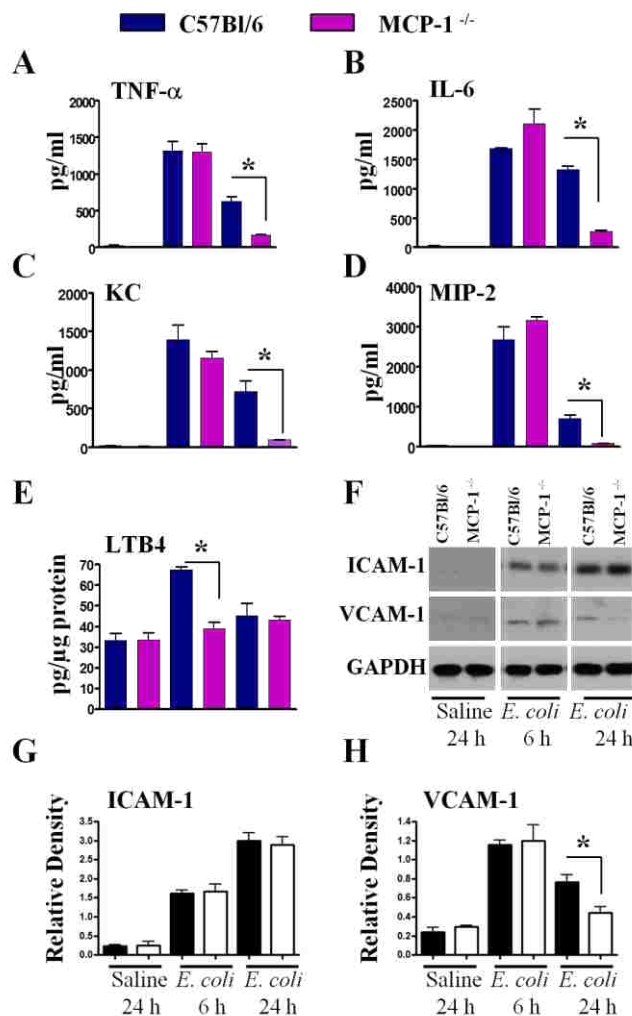
at 24 h post-infection (Figs. 3.4A-G). In addition, MCP-1 $^{-/-}$ mice infected with *E. coli* showe d reduced activation of p38 and ERK kinases at 24 h (Figs. 3.5A-B) whereas JNK activation is reduced at earlier time point (6 h) in MCP-1 $^{-/-}$ mice (Figs. 3.5A-B).

Figure 3.3. A-E. Cytokine, chemokine and chemotactic lipid/LTB4 levels in the lung following *E. coli* infection. Mice were infected by intratracheal instillation of *E.* coli (10^6 CFU/mouse), and BALF was collected from the lungs at designated time points. Concentrations (pg/ml) of TNF-α (A), IL-6 (B), KC (C) and MIP-2 (D) in BALF were quantified by sandwich ELISA. Asterisks indicate significant difference between MCP-1 $^{-/-}$ and WT mice (p<0.005; n = 4-6 mice in each group at each time-point and the data are a representation of 3 separate experiments). E. Expression of LTB$_4$ in the lung following *E. coli* infection. Lung homogenates were prepared after *E. coli* infection, the levels of LTB4 were measured in homogenates and were normalized against total protein concentration. F. Expression of ICAM-1 and VCAM-1 in the lung 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 for ICAM-1, VCAM-1 and GAPDH. This is a representative blot of 3 independent experiments with similar results. G-H. Densitometric analysis was performed in 3 blots to demonstrate the expression of ICAM-1 and VCAM-1 in the lung following *E. coli* infection
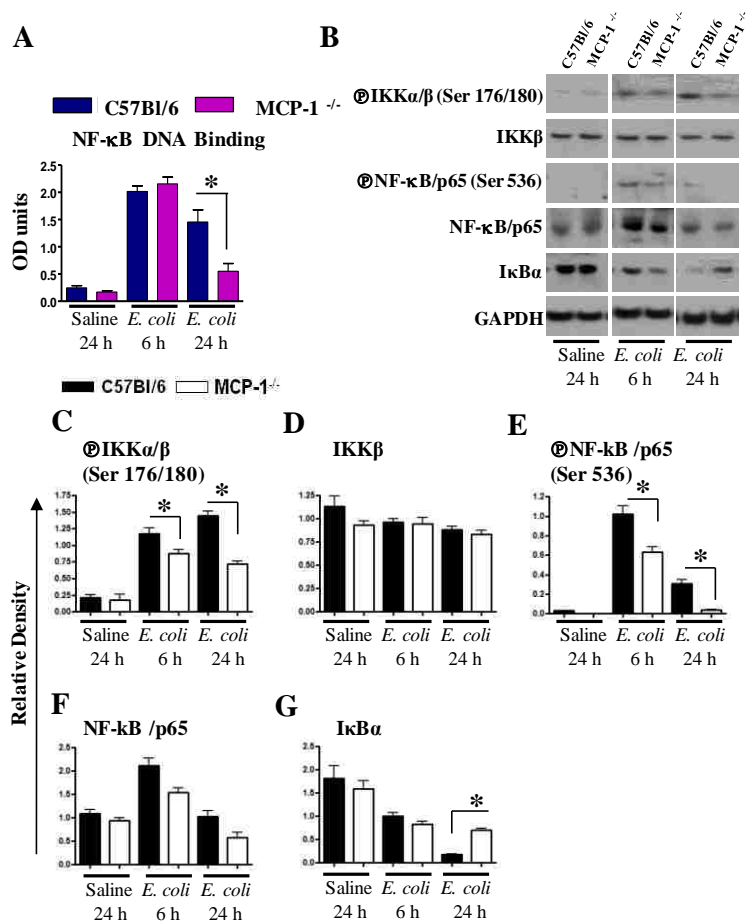
Figure 3.4. **A-B. Activation of NF-κB in the lung following infection with *E. coli*.** Lung homogenates and nuclear lysates from MCP-1^{-/-} mice and their controls were prepared at 6 and 24 h after infection with *E coli*. NF-κB binding assay was performed in nuclear lysates from lung (A) and expression and phosphorylation of NF-κB pathway members were determined using Western blots of lung homogenates (B). The blots are representative of 3 independent experiments with comparable results. C-G. Relative densities normalized against GAPDH are representatives of 3 independent experiments. OD, optical density; encircled P, phosphorylated form; *, $P < 0.005$.

A
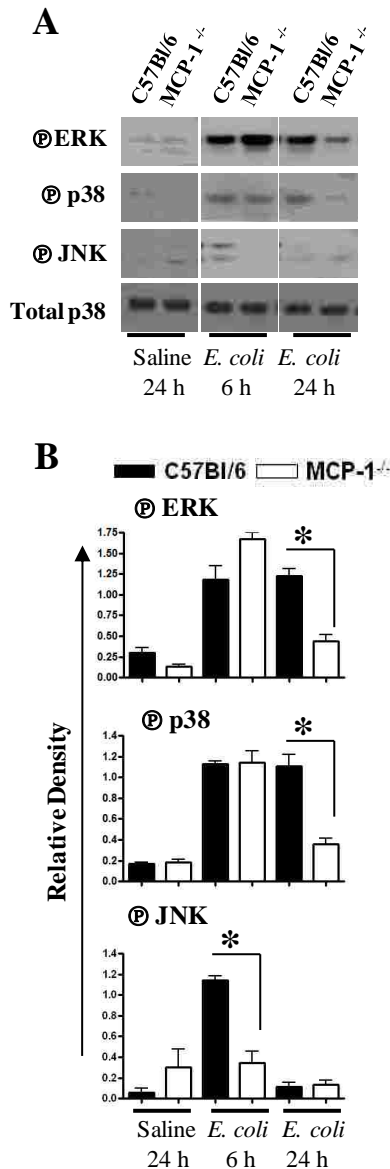
gure 3.5. A. **Activation of MAPKs in the lung following *E. coli* infection**. Total proteins in the lung were isolated from MCP-1$^{-/-}$ and control mice at 6 and 24 h after infection with *E. coli*, resolved on an SDS-PAGE and the membrane was blotted with the Abs against activated/phosphorylated form of MAPKs as described in Materials and Methods. This is a representative of 3 separate experiments with similar results. B. Densitometric analysis of MAPK activation was performed from 3 separate blots. *, $p<0.05$ for difference between MCP-1$^{-/-}$ mice and their WT controls

B

Recombinant MCP-1 (rMCP-1) Induces Neutrophil Influx and Cytokine/chemokine Expression in the Lungs of MCP-1$^{-/-}$ Mice during *E. coli* Infection. Since MCP-1 deficiency causes attenuated neutrophil influx, we determined whether reconstituting MCP-1$^{-/-}$ mice with exogenous rMCP-1 prior to *E. coli* infection can cause neutrophil accumulation and cytokine/chemokine expression in the lungs. In this regard, MCP-1$^{-/-}$ and WT mice were i.t. infected with *E. coli* at 1 hour after i.t. rMCP-1(10 μg/mouse) administration. At 24 hours after infection, neutrophil count was increased in BALF of MCP-1$^{-/-}$ mice which received rMCP-1 (Figs.3.6A-B). Furthermore, MCP-1$^{-/-}$ mice reconstituted with rMCP-1 showed

increased expression of cytokines (TNF-α and IL-6) (Figs. 3.6C-D), and chemokines (KC, MIP-2) (Fig. 3.6E-F) in BALF after *E. coli* infection. Moreover expression of VCAM-1 and activation of NF-κB and MAPKs was increased in the lungs of MCP$^{-/-}$ mice reconstituted with rMCP-1 (Fig. 3.7A-C).
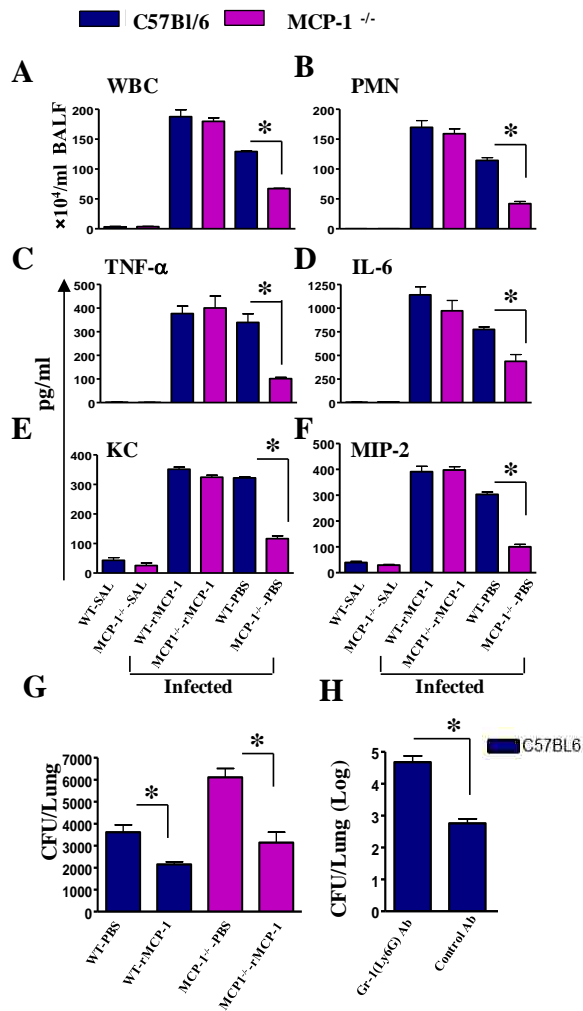

Figure 3.6. A-B. Cellular infiltration in BALF at 24 h after i.t. treatment with rMCP-1(50 μg) and *E. coli* infection. Fifty micrograms of rMCP-1 was i.t. administered 1h post *E.coli* infection and BALF was collected 24 h after infection. C-F. Cytokine (C and D) and chemokines (E and F) production in the lungs of infected animals post rMCP-1 treatment. For experiments indicated in A-F, n=4-6 mice/group and data are representative of 3 experimental repeats. G. Numbers of CFUs in the Lung of WT and MCP-1$^{-/-}$ mice after treatment with rMCP-1 following i.t. *E. coli* infection. The controls were treated with PBS. N=5-6 mice/group and the result shown is representative of 3 independent experiments. H. Lung CFUs Numbers of CFUs in the Lungs of neutrophil-depleted mice at 24 h after *E. coli* infection. Neutrophils were depleted by using anti Ly6G Ab intraperitoneally at 12 and 2 h prior to infection and control mice were treated with isotype Ab prior to infection. Lung samples were homogenised, diluted and plated to enumerate bacterial CFUs. (n=5-6 mice/group/time-point and are from 3 separate experiments). *, $P < 0.05$.

Since our findings show that rMCP-1 induced cytokine/chemokine secretion and neutrophil influx in the lungs, we wanted to determine whether rMCP-1 treatment affects bacterial clearance from the lungs. We reconstituted MCP-1$^{-/-}$ mice with rMCP-1 prior to infection and lung CFUs were enumerated at 24 h post infection. MCP-1$^{-/-}$ mice treated with rMCP-1 show reduced CFUs in the lung comparable to wild-type mice (Fig: 3.6G). These observations indicate that rMCP-1 improves *E. coli* clearance in the lungs. In order to

determine whether neutrophils are important for bacterial clearance, we depleted neutrophils in wild-type mice and challenged them with *E. coli*. We observed a substantial increase in the bacterial burden in the lungs of neutrophil-depleted mice as compared to control mice (Fig: 3.6H). These findings illustrate the importance of neutrophils in *E. coli* clearance from the lungs.

As we found that rMCP-1 causes neutrophil recruitment to the lungs of CP-1^{-/-} mice following infection, we determined whether administration of exogenous rMCP-1 in MCP-1^{-/-} mice can cause neutrophil recruitment in the absence of *E. coli* infection. In this regard, it has been shown that i.t. administration of 50 µg rMCP-1 in Balb/C mice did not cause any neutrophil influx (29). In our experiments, 2 doses of (10µg or 50µg/mouse) rMCP-1 were used in C57BL/6 mice via i.t. route and found that the neutrophil count was increased in BALF and lungs (Fig: 3.8A) of the rMCP-1 treated mice in a concentration-dependent manner.
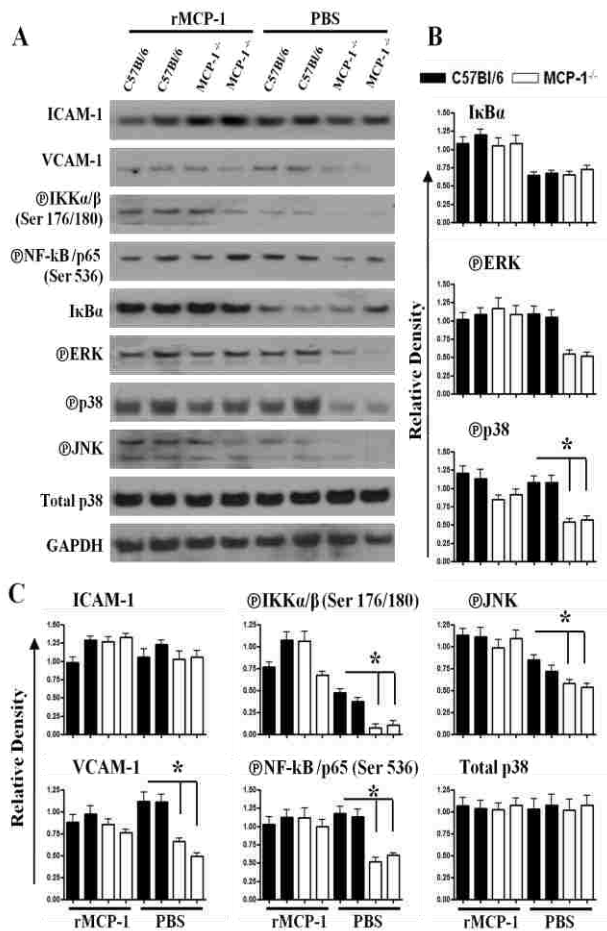
Figure. 3.7. (A) Activation of NF-κB and MAPKs, and expression of cellular adhesion molecules in lungs of mice administered rMCP-1 (10µg) at 24 h after *E. coli* infection. (B and C) Densitometric analysis of expression/phosphorylation levels of identified proteins normalized with GAPDH. The results are representative of 3 independent experiments with comparable results. *, *P* < 0.05; encircled P, phosphorylated form.

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Neutrophils Migrate Towards rMCP-1 I*n Vitro*. Since rMCP-1 can rescue neutrophil accumulation in MCP-1$^{-/-}$ mice in the presence or absence of *E. coli* infection, we examined whether this is due to the direct chemotactic effect of MCP-1. In this regard, we first used flow cytometry to determine whether neutrophils express CCR2. For these experiments, BALF and blood obtained from WT mice after *E. coli* infection were used since chemokines produced in the lungs after infection can induce neutrophil release from the bone marrow to blood. In this regard, we used dual staining of Gr-1/Ly6G-FITC/CCR2-APC to enumerate CCR2 expression on neutrophils. We first gated neutrophils based on their size and granularity and then determined the double positive cell population from the gated population of neutrophils. Notably, neutrophils in BALF and blood express CCR2 and this expression is enhanced during infection (Fig: 3.8B). In addition bone marrow neutrophils also express CCR2 and it is induced upon *E. coli* infection (Fig: 3.8C).

Since we observed CCR2 expression on neutrophils, we examined whether these neutrophils chemotax towards MCP-1. In this context, transwell transmigration assay was used in which we used 1×10^6 LPS activated neutrophils in DMEM 0.1% BSA. KC was used as a positive control at two different concentrations (500 ng, 1000 ng) whereas MCP-1 was used at 1000 ng. Neutrophils chemotax towards MCP-1 (Fig: 3.8D-E) although the number of cells that chemotax towards MCP-1 is much less than the number of cells that chemotax towards KC at the same concentration. Interestingly, neutrophils that are challenged with *E. coli* produce MCP-1 at 2 h post-infection (Fig: 3.8F). These findings demonstrate that neutrophils produce MCP-1 and respond to MCP-1. However this direct role of MCP-1 has a minimal effect on neutrophil recruitment.
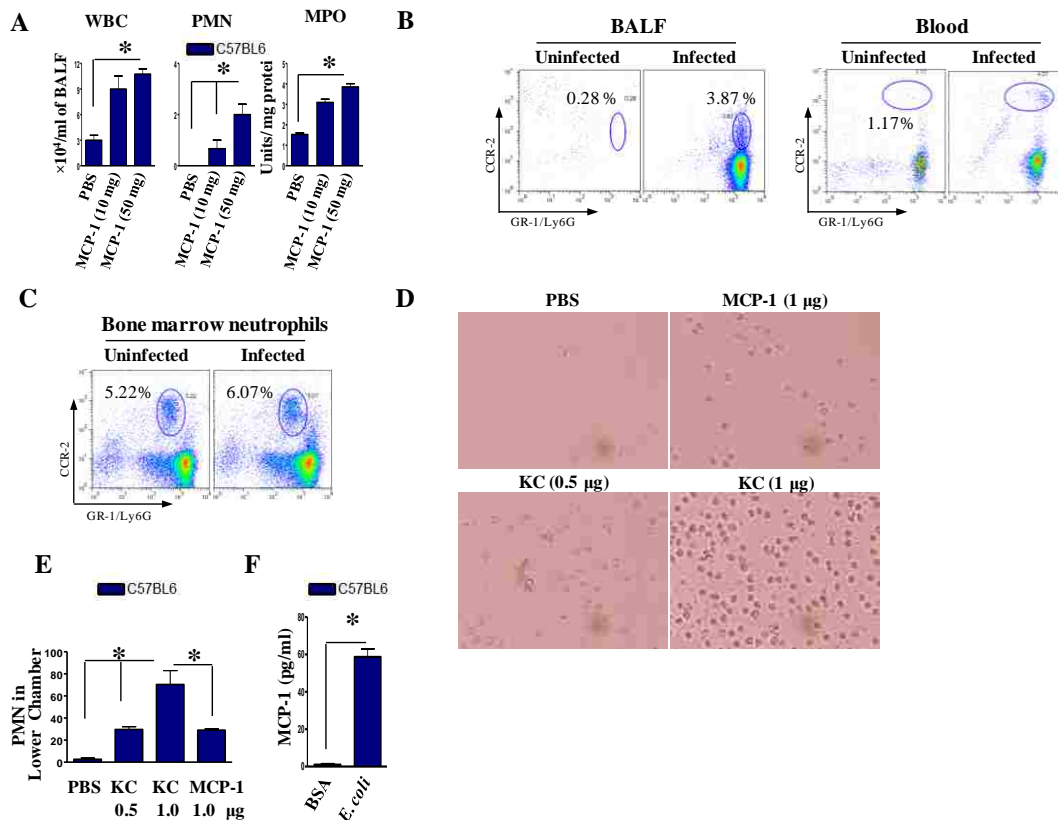
Figure 3.8. A. Cellular recruitment into the alveoli after i.t. treatment with rMCP-1(10μg and 50μg) alone. After 12 h, BALF was processed for cellular count. (n=5-6 mice/group; p<0.005, data are a representation of 3 individual experiments). MPO, myeloperoxidase. B. CCR2 expression in neutrophils. Flow cytometric analysis of BALF and blood from WT mice at 24h after i.t. *E. coli* ($1X10^6$ CFU/mouse) infection. This is a representative data of 3 independent experiments with similar results. D. Picture demonstrating the number of PMNs in the lower chamber of transwell plate after incubation with chemoattractants rMCP-1 and KC. The figure is representative of 20 random fields from 3 separate experiments. E. Chemotaxis of neutrophils towards MCP-1. PMN numbers in the lower chamber of transwell after 3 h of incubation with rMCP-1 and KC. Data shown here is a representation of 3 individual experiments where n=3-5 and p<0.05. F. Bone marrow neutrophils produce MCP-1 at 2 h after *E. coli* infection. (n=4-6 mice/group from 3 separate experiments).

Macrophages Obtained from MCP-1$^{-/-}$ Mice Show Reduced NF-κB and MAPK Activation. In order to confirm the activation of NF-κB and MAPKs in the lungs, we used alveolar macrophages obtained from WT and MCP-1$^{-/-}$ mice. Macrophages were infected with *E. coli* and samples were collected at 2 and 6 h for Western blotting. We found reduced NF-κB and MAPKs activation in MCP-1$^{-/-}$ mice following infection (Fig: 3.9A-B).
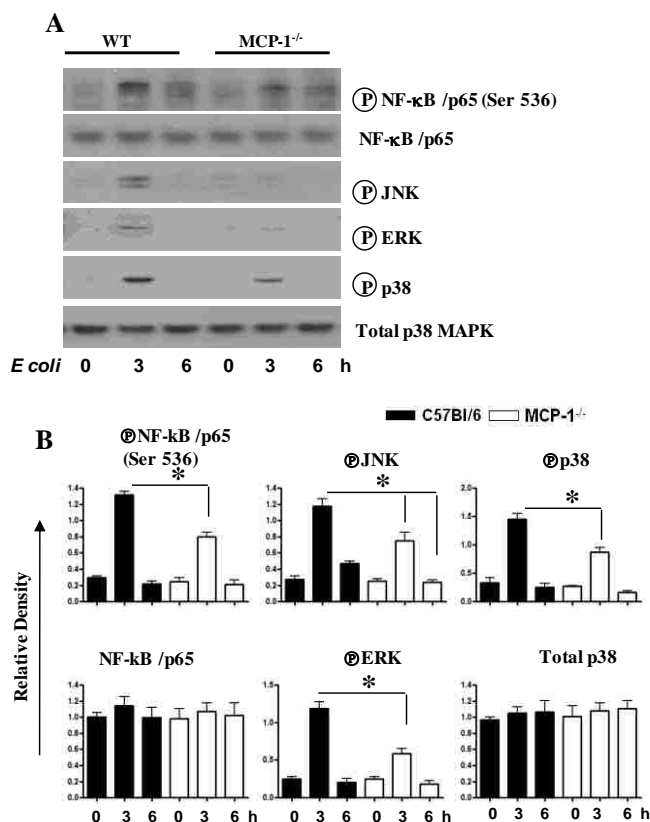
Figure 3.9. A-B. **Activation of NF-κB and MAPKs in alveolar macrophages obtained from WT, MCP-1^{-/-} mice following infection with _E. coli_.** Representative Western blots from 3 separate experiments are shown. B. Relative densities normalized against GAPDH are representatives of 3 independent experiments (n=4-5/mice/group; *, p<0.05). Encircled P, phosphorylated form

Discussion

In the present investigation, we sought to determine the importance of MCP-1 in an experimental model of bacterial pneumonia and in the human system. We found MCP-1 expression is substantially increased following intratracheal _E. coli_ infection and MCP-1 is important for bacterial clearance during _E. coli_ infection of the lungs. In addition we also show that MCP-1 is being produced by both myeloid and non-myeloid cells following _E. coli_ infection. Our study demonstrate that MCP-1 directly (via chemotaxis) and indirectly (via modulation of KC and MIP-2) contributes to neutrophil recruitment during _E. coli_ pneumonia (Figure 3.10).

In previous studies using mouse models, MCP-1 has been shown to play a protective role against pulmonary pathogens, including _Streptococcus pneumoniae_ (13, 45, 46), _Pseudomonas aeruginosa_ (2, 35) and _Cryptococcus neoformans_ (20). In studies of

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pneumococcal and *Pseudomonas* pneumonia, inhibition of MCP-1 by antibodies reduced monocyte but not neutrophil recruitment (2, 13). MCP-1 has also been shown to be associated with the influx of T lymphocytes to inflammatory sites (10). Our results demonstrate that rMCP-1 in the presence or absence of *E. coli* infection causes neutrophil and macrophage influx into the lungs. Our findings also demonstrate attenuated macrophage and neutrophil in the lungs of the gene deficient mice after *E. coli* challenge. These findings are consistent with an earlier report demonstrating that blocking MCP-1/CCR2 reduced neutrophil accumulation in the lung following i.t. treatment with MCP-1/LPS(30) or *Cryptococcus neoformans* infection (20). The Maus group (29) showed that administering MCP-1 alone didn't bring neutrophils to the lungs but MCP-1 plus LPS synergistically causes neutrophil trafficking to the lungs of BALB/c mice. However, we found rMCP-1 alone causes neutrophil influx into the lungs of the WT (C57BL/6) mice compared to the PBS controls. Further, we show that neutrophils from blood, BALF and bonemarrow express CCR2 and which is enhanced upon infection, bone marrow neutrophils migrate towards rMCP-1 suggesting that MCP-1 can act as a direct neutrophil chemoattractant. In addition, the Beck-schimmer group (4) has shown that LPS induced neutrophil recruitment depends on MCP-1 production. A study by Lida *et al*. has shown that murine bone marrow neutrophils express functional CCR2 and migrate towards MCP-1 (21). Additional studies have shown that normal neutrophils are not responsive to MCP-1, although neutrophils become responsive to MCP-1 when the animal become septic after cecal ligation and puncture (CLP) (40). A study by the Johnston group (23) shows that chronic inflammation induces neutrophils to express CCR2 in order to cause migration towards MCP-1. Furthermore, in other acute inflammatory models such as acute pancreatitis and colitis MCP-1 has been shown to be critical for neutrophil recruitment and blocking MCP-1 with bindarit reduced neutrophil numbers and inflammation (5, 6). These

observations strongly suggest that neutrophils are directly responsive to MCP-1 and our findings show the importance of MCP-1 in neutrophil recruitment to the lungs.
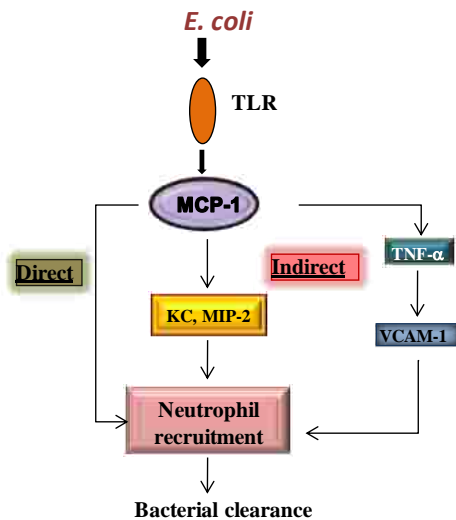

Figure 3.10. Proposed model of MCP-1 mediated neutrophil recruitment to the lung during *E. coli* infection. *E. coli* induces MCP-1 through MD-2 signaling pathway which involves Toll-like receptors. MCP-1 in turn regulate neutrophil recruitment directly via chemotaxis and indirectly though KC, MIP-2 as well as the cytokine TNF-α.

Our findings show that MCP-1 can indirectly regulate neutrophil influx by regulating the expression of CXC chemokines. We observed reduced expression of cytokines (TNF-α and IL-6) and chemokines (KC and MIP-2) in MCP-1$^{-/-}$ mice compared to the controls after *E. coli* infection and when MCP-1$^{-/-}$ mice were reconstituted with rMCP-1 the cytokine and chemokine levels returned to the level of WT mice during *E. coli* infection. In line with this finding a previous study in a rat gastric ulcer model suggests that MCP-1 can regulate the expression of CXC chemokines and cause neutrophil recruitment(44). Moreover, Gouwy *et al*. showed that extracellular signal by CCL2 significantly enhances CXCL-8 expression on monocyte (15) suggesting that CCL2/CCR2 signaling can regulate the expression of CXC chemokines. Further, the Herbold group (17) demonstrated that CXCR-2 signaling can lead to macrophage recruitment during *S. pneumoniae* infection. These observations suggest that there is an interplay between CC and CXC chemokines.

LTB$_4$ is a known neutrophil chemotactic lipid which can be generated by leukocytes from arachidonic acid (19) and act via G-protein coupled receptors to cause leukocyte accumulation, microbial killing and generation of pro-inflammatory mediators (26, 36). In an earlier study using a cecal ligation and puncture (CLP) model, it has been shown that MCP-1 inhibition attenuated neutrophil influx via the down-regulation of LTB$_4$ production (37). From our results, it is also possible that the attenuated level of LTB$_4$ was the result of the reduced number of migrating neutrophils in MCP-1$^{-/-}$ mice. However, the attenuation was significant at 6 h and not significant at 24 h post- *E. coli* infection. Since LTB$_4$ itself can lead to generation of other proinflammatory mediators and neutrophil chamoattractants (36), reduction of the neutrophil chemoattractant lipid, LTB$_4$, at 6 h would have lead to reduced neutrophil influx at 24 h.

We observed reduced NF-κB activation and activation of MAPKs in MCP-1 gene deficient mice during *E .coli* infection. These observations suggest that MCP-1/CCR2 axis regulates NF-κB and MAPKs activation. Consistent with this finding, Viedt *et al*. showed that MCP-1 can activate NF-κB and MAPKs during renal inflammation(43). It is also possible that other transcription factors, such as AP-1 and STAT-1 can be activated via MAPKs by MCP-1 (14). Therefore, our results suggest that MCP-1/CCR2 signaling regulates the expression of CXC chemokines (MIP-2 and KC) through NF-κB and MAPKs. Regulation of CXC chemokines and cytokines through NF-κB and MAPKs by MCP-1 can be attributed to macrophage recruitment as MCP-1 is primarily a monocyte chemoattractant therefore we used alveolar macrophages from wild-type and MCP-1$^{-/-}$ mice to determine whether reduced MCP-1 levels have any effect on activation of NF-κB and MAPKs. We observed reduced activation of transcription factor NF-κB and MAPKs in the MCP-1$^{-/-}$ macrophages following

E. coli infection. These findings confirm our *in vivo* data that in addition to macrophage recruitment, MCP-1 could regulate NF-κB and MAPK mediated macrophage activation.

Recruitment of leukocytes to the tissues involves up regulation of cell adhesion molecules(3). In this regard, we found that VCAM-1 up regulation was dependent on MCP-1. This was most pronounced at 24 h following *E. coli* infection when neutrophil influx in the lungs was at peak. These observations suggest that VCAM-1 plays an important role in the transmigration of both neutrophils and monocytes to the lungs during *E. coli* infection in a MCP-1 dependent manner.

Previous studies have shown that MCP-1 is important for bacterial killing by macrophages (35, 38). Furthermore, studies have shown that neutrophils play an important role in the bacterial clearance during gram-negative bacterial infections (27, 47). Since we found reduced neutrophil numbers and enhanced bacterial burden in MCP-1$^{-/-}$ mice following *E. coli* infection, we depleted neutrophils in wild-type mice to determine the role of neutrophils in augmenting bacterial clearance. Our results show that neutrophils are indeed important for the clearance of *E. coli* in the lungs. Further experiments involving MCP-1 restoration in MCP-1 $^{-/-}$ mice demonstrate the importance of MCP-1 in bacterial clearance from the lungs. Although there is a higher bacterial burden in the lungs of MCP-1$^{-/-}$ mice, both WT and MCP-1$^{-/-}$ mice cleared infection after 24 h, and no mortality was observed after infection.

Overall, our study reveals a novel role for MCP-1, that MCP-1 directly as well as indirectly is involved in neutrophil recruitment during *E. coli* pneumonia. Herein we show evidence of MCP-1 being a direct neutrophil chemoattractant and indirectly it regulates the expression of other chemokines (KC and MIP-2) and cytokines (TNF-α and IL-6) to cause neutrophil trafficking (Fig. 2.10). Identification of these novel molecular MCP-1 mediated

mechanisms triggered by *E. coli* could be useful to design better therapeutic strategies during

infections by gram-negative bacterial pathogens.

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Chapter 4

Intrapulmonary G-CSF Reverses Neutrophil Recruitment to the Lung and   Neutrophil Release to Blood in Klebsiella pneumoniae Infection in MCP-1$^{-/-}$ Mice

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are caused by multiple factors, including aspiration, sepsis, hyperoxia and bacterial pneumonia (1). *Klebsiella pneumoniae* (Kp) is one of the most frequently isolated Gram-negative pathogens associated with nosocomial pneumonia (2, 3). In light of the emergence of carbapenem resistant Kp along with increasing numbers of immunocompromised individuals, an understanding of innate immune mechanisms of the lung and how they may be augmented is critical (4).

Recruitment of neutrophils to the lungs is a multistep process involving granulopoiesis, neutrophil release, expression of neutrophil chemoattractants, cellular adhesion molecules and other proinflammatory mediators and eventual transmigration of neutrophils into the alveolar spaces (5-7). In this regard, neutrophils play a key role in mucosal defense against intrapulmonary Kp infection since neutrophil depletion by anti-Gr-1 Ab makes the host more susceptible to infection (8, 9). Production of chemokines in the lung is the first critical step in neutrophil recruitment (5-7) .

Monocyte chemoattractant protein 1 (MCP-1/chemokine [C-C motif] ligand 2 [CCL2]) is a potent monocyte/macrophage and T cell chemoattractant against bacteria infecting the lung (10-13). MCP-1 is produced by a variety of cells, including endothelial cells (14), epithelial cells , fibroblasts(15), and myeloid cells (monocytes/macrophages) (16). It binds to the G protein-coupled transmembrane receptor chemokine receptor 2 (CCR2) to induce downstream signaling events (17). The important role of MCP-1 in monocyte/macrophage-mediated host defense against bacterial infection has been documented by previous studies. MCP-1 over expression augments bacterial clearance during *Streptococcus pneumoniae* infection (18). Using gene-deficient (MCP-1$^{-/-}$) mice, Winter et

al. (19) have reported that MCP-1-induced macrophage migration is required to prevent bacterial dissemination following intrapulmonary *Streptococcus pneumoniae* infection. In a mouse model of *Pseudomonas aeruginosa* infection, MCP-1 promoted resolution of inflammation and repair of the lung by augmenting uptake of neutrophils by alveolar macrophages (20). In addition, $CCR2^{-/-}$ mice exhibit reduced macrophage influx-mediated bacterial clearance from the lungs and distal organs in response to intravenous challenge with *Listeria monocytogenes* (21). MCP-1 is also shown to be essential for the survival of mice and macrophage-mediated bacterial killing during *P. aeruginosa* and *Salmonella* enterica serotype *Typhimurium* infections (10). We recently demonstrated that neutrophils expressing CCR-2 and MCP-1 can recruit neutrophils directly as well as indirectly by regulating the expression of CXC neutrophil chemokines during *E. coli* infection (5). Our study was limited in that *E. coli* neither disseminates nor induces death in mice following intrapulmonary infection and the role of bone marrow and/or resident cells in host immunity is unclear; and the mechanisms underlying neutrophil accumulation in the lungs against *E. coli* have not been explored.

In this study, we demonstrate the role of MCP-1 in Kp lung infection because Kp is an important Gram-negative pulmonary pathogen that also extensively disseminates to extrapulmonary organs with a small intrapulmonary inoculum. Using $MCP-1^{-/-}$ mice, we found that MCP-1 is vital for bacterial clearance from the lungs following i.t. Kp infection and ultimately survival of the mice. In addition, MCP-1 regulates neutrophil recruitment to the lungs directly through CCR2 upregulation and indirectly via regulating CXC neutrophil chemokines in the lungs. To test the hypothesis that G-CSF protein is necessary and sufficient to restore MCP-1 deficiency, we administered granulocyte colony stimulating factor (G-CSF) by intratracheal instillation into the lungs of Kp-infected mice. Impairment in

survival, bacterial clearance and neutrophil mobilization to the circulation in MCP-1^{-/-} mice were reversed by administration of G-CSF.

Materials and Methods

Mice: Eight to 10-wk-old female mice genetically deficient in MCP-1 (22) were used while age- and gender-matched C57Bl/6 mice were used as controls. All animal studies were approved by the Louisiana State University Animal Care and Use Committee. The mice ranged from 19 to 25 g in weight.

Murine Model of Infection: Bacteria were prepared for mouse inoculation, as described in previous studies (9, 23). 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 20×10^6 CFU/ml. Mouse strains were anesthetized with i.p. ketamine/xylazine (250 mg/kg), followed by intratracheal (i.t.) inoculation of 50 µl of bacteria (10^3 CFU/mouse) where as control mice were i.t. inoculated with 50 µl of saline. The initial mouse inoculums 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 for 30 s, 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 15 s for bacterial culture. Bacterial colonies were counted after incubation overnight at 37°C.

Bronchoalveolar Lavage Fluid (BALF) Collection: BALF was collected and total and differential cell counts and cytokine/chemokine concentrations 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 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°C (5, 24-26).

Lung Pathology: The lungs were perfused from the right ventricle of heart with 10 ml isotonic saline at 24 h post infections described earlier (23). Lungs were then removed and fixed in 4% phosphate-buffered formalin. Fixed tissue samples were processed and stained with hematoxylin and eosin (H&E). Semiquantative histopathology was 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.

Measurement of Cytokines and Chemokines: We used BALF and lungs that were obtained from animals after *Kp* infection or saline instillation. ELISA kits for TNF-α, IL-6 and MCP-1 were obtained from eBiosciences, PA whereas kits for KC and MIP-2 were obtained from R&D systems, MN. The minimum detection limit is 8 pg/ml cytokine protein (5, 26).

Immunoblotting: The lungs were collected at the designated time points and used for Immunoblotting as described in our previous publications (23, 24). The primary Abs to phospho-NF-κB/p65(ser 536), NF-κB/p65,, phospho-IκBα (ser 32), 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, and GAPDH (Santa Cruz Biotechnology, CA) were added at 1:5,000 dilution. Immunostaining was performed using 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.

MPO Activity in Neutrophils. MPO release by the neutrophils was measured as previously described in our publications (27).

Bone Marrow Chimeras. Donor and recipient mice (6-8 wk old) were used to generate chimeras, as described in our previous publications (5, 28). Recipient mice were γ irradiated from a cesium source in two 525-rad doses 3 hours apart. Bone marrow cells (8×10^6/mouse) were injected into the irradiated recipients via the tail vein. Chimeric mice were maintained on 0.2% neomycin sulfate for the first 3 weeks. The reconstituted mice were used 8 weeks post-transplantation. We found that greater than 86% of blood leukocytes were derived from donor mice at the time the mice were used for experiments. Irradiated mice that were not transplanted with donor cells died between days 20 and 23 post-transplantation.

Administration of either rMCP-1 or rG-CSF: MCP-1 $^{-/-}$ mice were treated i.t. with rMCP-1 (5 μg/animal) or rG-CSF (1 μg/mouse) 30 mins after *Kp* infection (10^3 CFUs/50μl/animal) and the control mice were treated with an equal volume of PBS with BSA. At 24 h post-infection, BALF was collected and processed for cellular enumeration and the determination of CFUs. For survival experiments mice were observed for 14 days post treatment.

Absolute Blood Neutrophil Count: Total leukocyte count was determined in 100 μl of blood. Differential leukocyte count was determined in 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 of WT and MCP-1$^{-/-}$ mice treated with saline or Kp was aliquoted into flow cytometry tubes and Fc blocked. A total of 10 µl of mouse conjugated anti-mouse Gr-1, CCR2, 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 dark. Cells were washed by adding 2 ml of 1X PBS and centrifuged at 1000 rpm (200 x g)/8 min. RBCs were lysed using 2 ml of NH$_4$Cl lysing buffer to each sample tube, mixed well and incubated at room temperature for 10 min. Samples were centrifuged immediately at 1000 rpm (200 x g)/8 min and the supernatant was removed. Cells washed twice with PBS. Cells were fixed by adding 200 µl of cold 1% formaldehyde-PBS and stored at 2-6 $^{\circ}$C for FACS analysis (5, 29).

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

Results

Effect of MCP-1 Deletion on Survival and Bacterial Clearance. First we assessed whether MCP-1 is protective in mice against i.t. Kp infection of the lungs. We infected MCP-1$^{-/-}$ mice and WT (C57Bl/6) controls with 10^3 Kp CFUs and monitored for up to 10 days. We observed MCP-1$^{-/-}$ mice were more susceptible to Kp infection compared to the wild-type controls. All knockout mice died within 6 days post infection while 85% of WT mice survived (Fig. 4.1A).

In order to determine whether this impaired survival was due to a defect in bacterial clearance from the lung and/or bacterial dissemination, bacterial loads in the lung and extrapulmonary organs, including blood, spleen and liver were determined. Mice were infected with i.t. Kp and sacrificed at 24 and 36 h post-infection and the lungs, blood, spleens and livers were collected to quantitate bacterial CFUs. In MCP-1$^{-/-}$ mice, higher bacterial loads were found in the lungs accompanied by enhanced dissemination to spleen and liver and blood (Figs. 4.1B-E). Thus, MCP-1 clearly plays a role in the survival and bacterial clearance of *K. pneumoniae* infection.

Effect of MCP-1 Deletion on Neutrophil Recruitment. MCP-1 is important for neutrophil and macrophage recruitment to the lungs in response to i.t. *E. coli* infection (5). The antibacterial defenses of the lung to Kp include the recruitment of the PMN from the systemic vasculature (5, 6, 30). Having determined that MCP-1 mediates survival and bacterial clearance to Kp infection, we next examined the total leukocyte and PMN numbers in BALF at 24 and 36 h post-infection. We found reduced neutrophil numbers, but macrophage numbers were not significantly reduced at 24 and 36 h post infection in the knockout mice (Figs. 4.1F-H). Lung myeloperoxidase (MPO) activity showed significant differences in neutrophil accumulation in lung parenchyma between WT and MCP-1$^{-/-}$ mice at 24 and 48 h post-Kp infection (Fig. 4.1I). Histopathology of MCP-1$^{-/-}$ lungs showed a reduction in neutrophil numbers in lung parenchyma (Fig. 4.1J) whereas higher numbers of bacteria were found in these lungs 36 h after Kp infection (Fig. 4.1J). In marked contrast, photomicrographs of lung sections in MCP-1$^{-/-}$ and WT mice challenged with saline showed no significant changes (Fig. 4.1J).
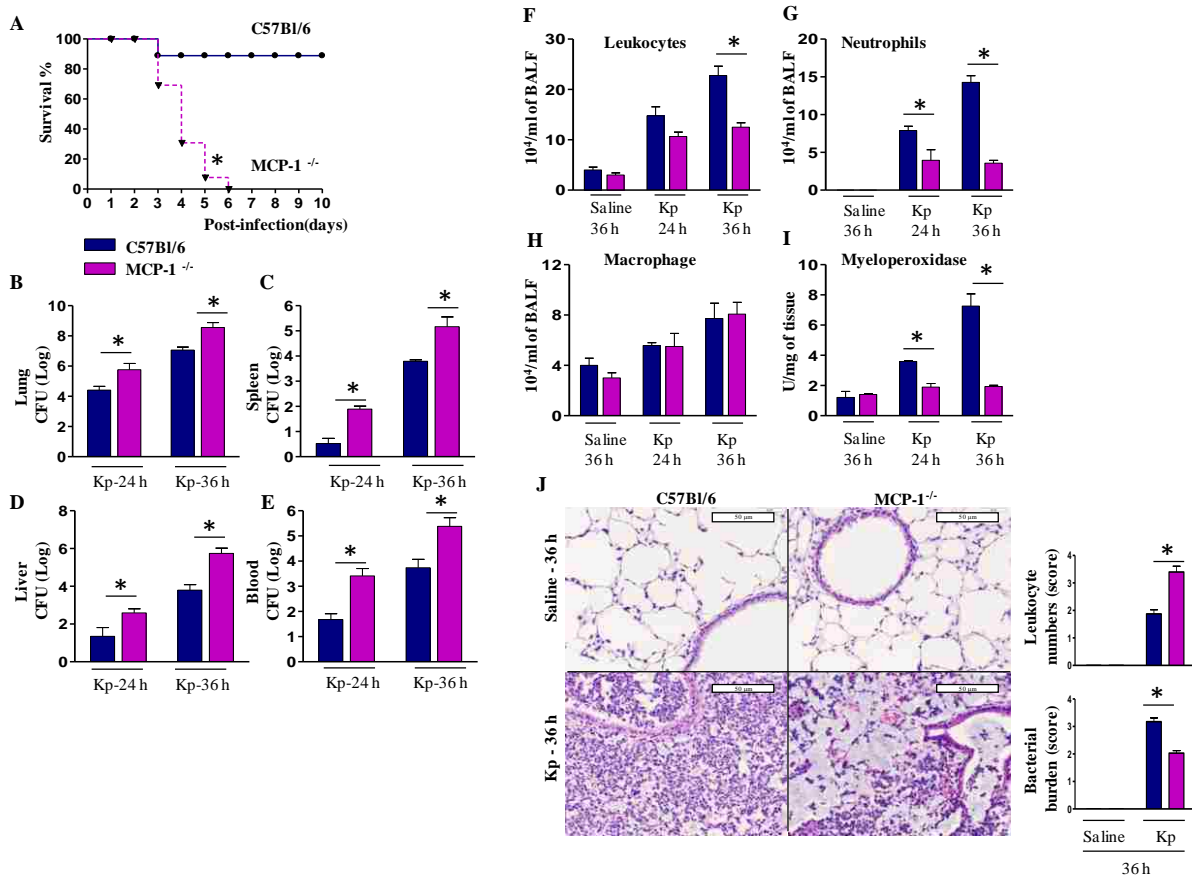
Figure 4.1: Importance of MCP-1 in host defense against pulmonary Kp infection: A. Reduced survival in MCP-1^{-/-} mice following i.t. Kp infection. MCP-1^{-/-} and WT (C57Bl/6) mice were i.t. inoculated with 10^3/mouse of Kp, and survival was monitored up to 10 days. n=14 mice in each group. *, P<0.05 determined by Wilcoxon Rank Sign Test. B-E. Impaired bacterial clearance in the in MCP-1^{-/-} mice after i.t. Kp infection (10^3/mouse). Bacterial CFUs in mice were determined in lungs, spleens, livers and blood of MCP-1^{-/-} and WT mice after Kp infection. Data represent mean $\pm$ SE of 4-6 mice at each time point. *, Significant differences between MCP-1^{-/-} and WT mice (P<0.05). F-H. Total leukocyte, neutrophil and macrophage numbers in the lungs of MCP-1^{-/-} mice after Kp inoculation. Both MCP-1^{-/-} and WT animals underwent BAL fluid and lung collection after challenge with Kp. I. MPO activity in homogenized (unlavaged) whole lungs of WT and MCP-1^{-/-} mice infected with *K. pneumoniae* (10^3 CFU/mouse) for 24 and 36 h. Data are presented as means $\pm$ SEM. n=6-8 mice/group. (* indicates p<0.05 compared with MCP-1^{-/-} mice). J. Lung histology in MCP-1^{-/-} mice following Kp inoculation. Lung sections were made 36 h after bacterial or saline challenge and stained with H&E. Score for leukocyte numbers in the lesion and bacterial numbers were calculated as described in Materials and Methods. These are representative sections of 4 mice in each condition with comparable results (Magnification x200).

Determination of Proinflammatory Cytokines/Chemokines Following i.t Kp Challange.

Neutrophil recruitment is a sequentially regulated process in which cytokine/chemokine

production plays a critical role. Since neutrophil recruitment to the lungs was reduced in MCP-1$^{-/-}$ mice following Kp infection, we then examined the MCP-1 dependence of cytokine/chemokine concentrations in the BALF induced by Kp infection. We found a universal reduction in cytokines (TNF-α and IL-6) and neutrophil chemoattractants (KC and MIP-2) in MCP-1$^{-/-}$ mice at 36 h after i.t. Kp inoculation (Figs.4.2A-D). These data provide evidence impaired expression of cytokines and neutrophil chemoattractants in the absence of MCP-1 during Kp infection.

Assessment of Activation of NF-κB and MAPKs Following Kp *Administration*. Expression of inflammatory genes is regulated by NF-κB and MAPKs (6, 31, 32). Having found reduced expression of cytokines and chemokines in BALF following Kp infection, we then investigated the activation of NF-κB and MAPKs in the lungs following Kp infection. As demonstrated in Figs. 4.2E-F, the data show reduced NF-κB activation in MCP-1$^{-/-}$ mice after 36 h of Kp challenge. In addition, MCP-1$^{-/-}$ mice infected with Kp exhibit reduced activation of p38, p42/44 MAPK and JNK at 24 h and 36 h of infection (Figs. 4.2G-H). These data demonstrate that MCP-1$^{-/-}$ mice have a defect in NF-κB and MAPK activation.

Administration of rMCP-1 to MCP-1$^{-/-}$ Mice after Kp Infection Restores Survival, Bacterial Clearance and Neutrophil Accumulation Following i.t. Challenge with Kp. To validate the possibility that impaired survival, bacterial clearance and neutrophil accumulation in the lungs of MCP-1$^{-/-}$ mice is indeed due to a genetic defect, we treated the MCP-1$^{-/-}$ mice i.t. with recombinant MCP-1 (rMCP-1) 1 h after i.t. Kp infection and examined survival, CFUs in lungs and distal organs as well as neutrophil influx. The effect of MCP-1 post-treatment on survival after i.t. challenge with Kp in MCP-1$^{-/-}$ is shown in figure 4.3A. MCP-1 markedly enhanced survival in MCP-1$^{-/-}$ mice treated with rMCP-1 (Fig. 4.3A). Moreover, post-treatment with MCP-1 significantly attenuated bacterial CFUs in lungs and spleen in MCP-1$^{-/-}$ mice (Fig.

4.3B-C). Furthermore, rMCP-1 post-Kp treatment augmented neutrophil accumulation in MCP-1$^{-/-}$ mice (Figures 4.3D-E).
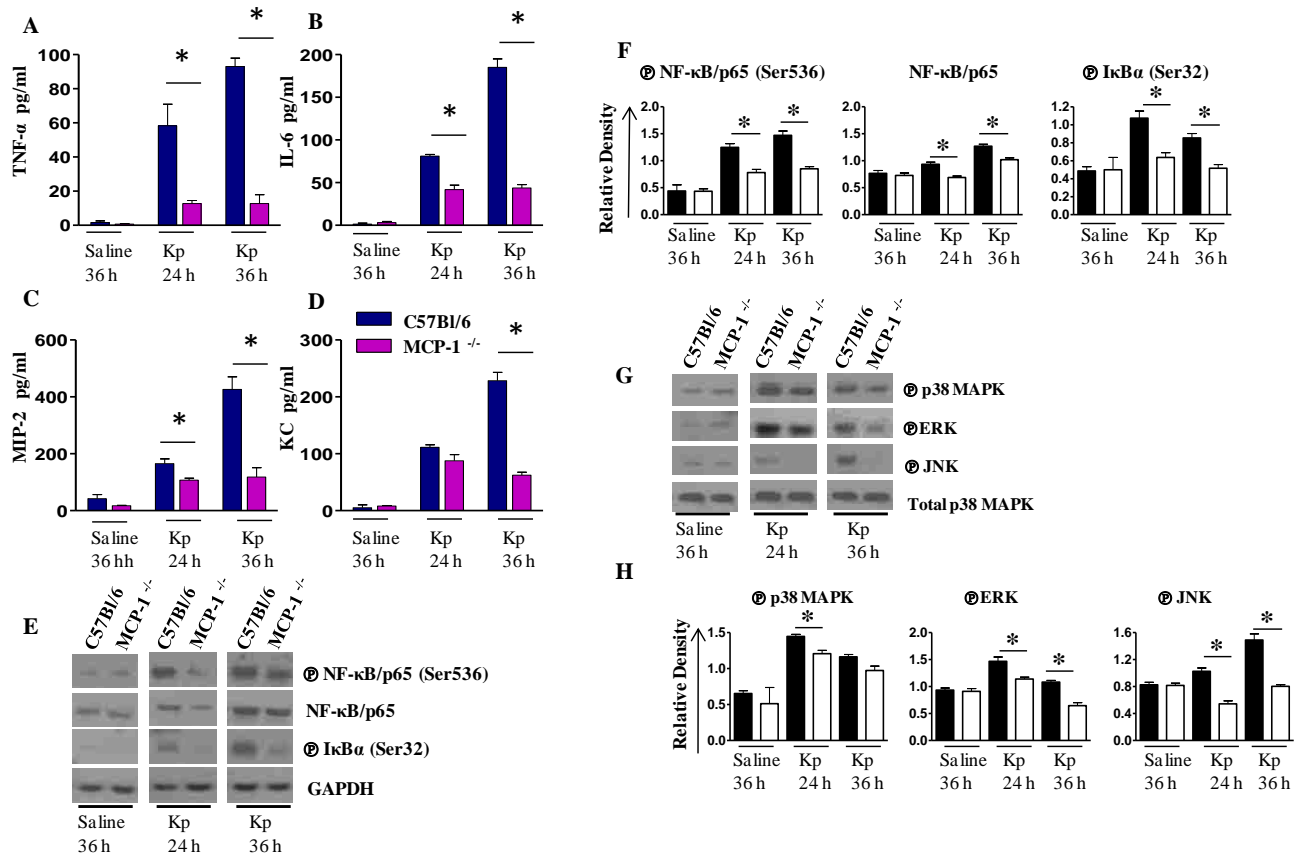
Figure 4.2: Impairment of airspace cytokine responses in MCP-1$^{-/-}$ mice after infection with Kp. A-D. Cytokine (TNF−α, IL-6) and chemokine (MIP-2 and KC) concentrations in BAL fluid were measured by sandwich ELISA after infection with Kp. Protein concentrations (pg/ml) are expressed as mean $\pm$ SE and 4-6 animals were used at each time point. Significant differences between MCP-1$^{-/-}$ and WT mice are indicated by asterisks (p<0.05). E-F. Activation of NF-κB in the lung following infection with Kp. Lung homogenates from MCP-1$^{-/-}$ mice and their controls were prepared at 24 and 36 h after infection with Kp. NF-κB expression and phosphorylation of NF-κB and IκBα were determined using western blots of lung homogenates. The blots are representative of 3 independent experiments with similar results. F. Relative densities normalized against GAPDH are representatives of 3 independent experiments. G. Activation of MAPKs in the lung following Kp infection. Total proteins in the lung were isolated from MCP-1$^{-/-}$ and control mice at 24 and 36 h after infection with Kp, 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 of 3 separate experiments with similar results. H. Densitometric analysis of MAPK activation was performed from 3 separate blots. *denotes the difference between MCP-1$^{-/-}$ mice and their WT controls (p<0.05).

A
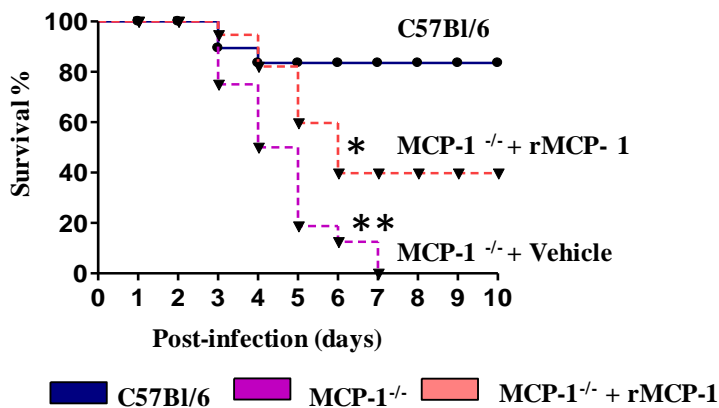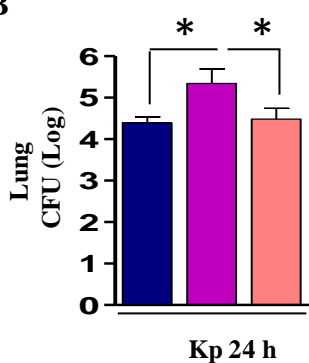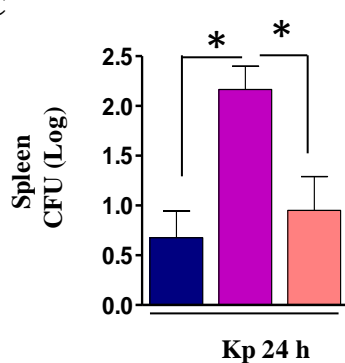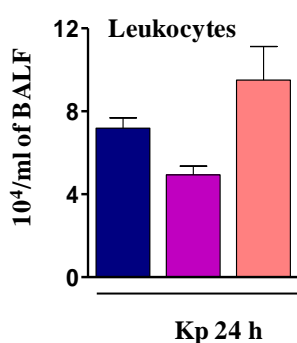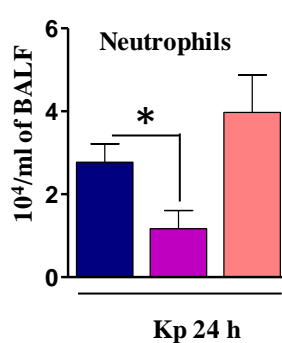
Figure 4.3: Impaired survival, bacterial clearance and neutrophil influx in the lungs of MCP-1$^{-/-}$ mice are restored by rMCP-1. Mortality (A) in WT and MCP-1-/- mice infected with 10^{3}CFUs of *K.pneumoniae* and administered with rMCP-1 (5 µg/mouse) or vehicle (BSA) 1 h later, and survival was assessed up to 10 days. Data are presented as % survival (n=20 mice/group) and analyzed using Wilcoxon signed-rank test. * indicates the difference between rMCP-1 or vehicle (BSA) control treated MCP-1$^{-/-}$ mice (p<0.05) and ** indicates the difference between WT and MCP-1$^{-/-}$ mice.) Bacterial clearance (B, C) in the lungs and spleen were examined following exogenous MCP-1 administration in MCP-1$^{-/-}$ mice at 24 h post-Kp challenge (10^{3}CFUs/mouse). Data are presented as Mean $\pm$ SE (n=5-6 mice/group). (*

indicates p<0.05 compared with BSA (vehicle) administered mice. Cellular infiltration (D,E) in airspaces at 24 h after i.t. treatment with rMCP-1 (5 µg/mouse) or vehicle (BSA) control.

Neutrophil Recruitment in Response to Kp Iinfection Requires MCP-1 by BM-Derived and Resident Lung Cells. Since host response to bacteria in the lung is derived from both resident alveolar cells and/or recruited bone marrow cells, we examined which cell-derived MCP-1 was essential in promoting neutrophil recruitment, thus host innate immunity. To

address this question, we used irradiation (500 rad 3 hrs apart) to eliminate all bone marrow cells in the recipient mice. These lethally irradiated recipient WT and MCP-1$^{-/-}$ mice were reconstituted with bone marrow from donor WT or MCP-1$^{-/-}$ mice to generate four groups of chimers: 1) WT mice reconstituted with WT marrow (WT→WT); 2) WT mice reconstituted with MCP-1-/-marrow (MCP-1$^{-/-}$→WT); 3) MCP-1$^{-/-}$ mice reconstituted with WT marrow (WT→MCP1$^{-/-}$); and 4) MCP-1$^{-/-}$ mice reconstituted with MCP1$^{-/-}$ marrow (MCP-1$^{-/-}$→ MCP1$^{-/-}$). At 8 wk post-transplantation, these bone marrow chimeric mice were i.t. inoculated with Kp and inflammatory parameters in the lungs were examined. At 48 h post-Kp infection, neutrophil recruitment to the lungs in response to *K. pneumoniae* infection was increased in irradiated WT$^{-/-}$ reconstituted with WT bone marrow (Fig. 4.4B) whereas Kp-induced neutrophil influx was attenuated in irradiated MCP-1$^{-/-}$ reconstituted with MCP-1$^{-/-}$ bone marrow (Fig. 4.4B). Irradiated MCP-1$^{-/-}$ mice reconstituted with MCP-1$^{+/+}$ bone marrow and irradiated MCP-1$^{+/+}$ mice reconstituted with MCP-1$^{-/-}$ bone marrow did show a similar degree of neutrophil influx in response to *K. pneumoniae* infection (Fig. 4.4B) Not surprisingly, neutrophil recruitment to the lung was not observed in reconstituted mice after saline challenge in all 4 chimera groups (data not shown). These observations suggest that MCP-1 by both resident and bone marrow cells is required for neutrophil-mediated host immunity against Kp.

A Leukocytes

C57Bl/6 → C57Bl/6
MCP-1$^{-/-}$ → MCP-1$^{-/-}$
C57Bl/6 → MCP-1$^{-/-}$
MCP-1$^{-/-}$ → C57Bl/6

B Neutrophils

36 h Kp
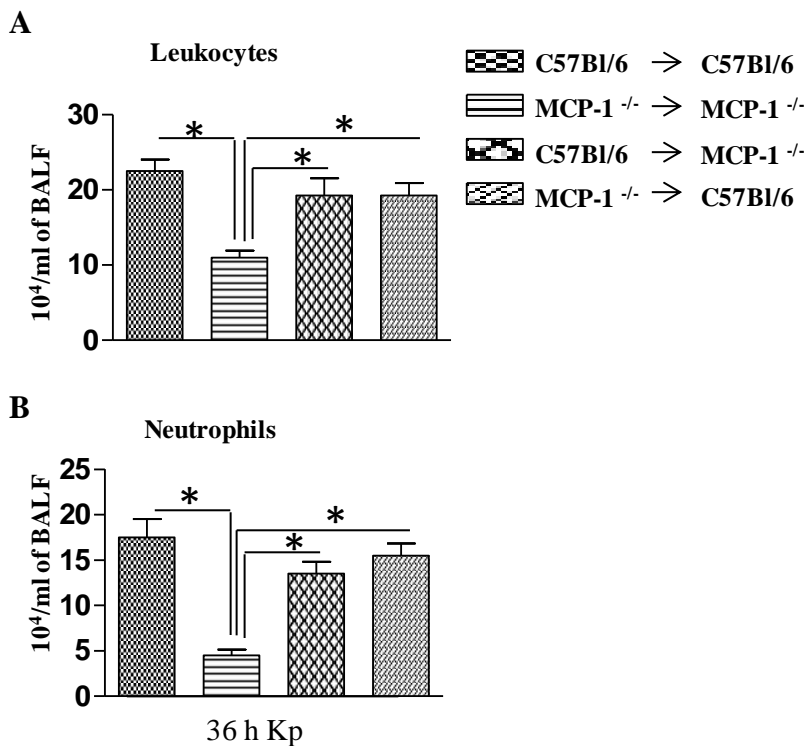
Figure 4.4: Total leukocyte numbers and neutrophil numbers in bone marrow chimeras following Kp infection. Bone marrow transplantation was performed between WT and MCP-1$^{-/-}$ mice in four combinations as is described in the materials and methods section and infected with Kp i.t. and at 36 h post infection BALF was collected to determine the cellular counts A-B. *indicates significant difference between different chimeras; $p < 0.05$; n=4-6/group.

Kp Infection Augments CCR2 Expression on Blood Neutrophils. As lung macrophage recruitment was not altered during Kp-induced inflammation, we hypothesized that the accumulation of neutrophils in lung inflammation may reflect increased chemotaxis, in addition to the reduced chemokine concentrations. Specifically, we studied whether the increased accumulation of neutrophils in the lungs required the expression of CCR2 selectively on this cell type. To perform this, we infected MCP-1$^{-/-}$ and WT mice i.t. with 10^3 CFUs of Kp and collected blood at 24 and 36 h post-infection and then stained with CCR2 antibody and Ly6G antibody for flow cytometry. We found that the level of CCR2 expression on neutrophil populations was increased with time following Kp infection (Fig. 4.5A-B).
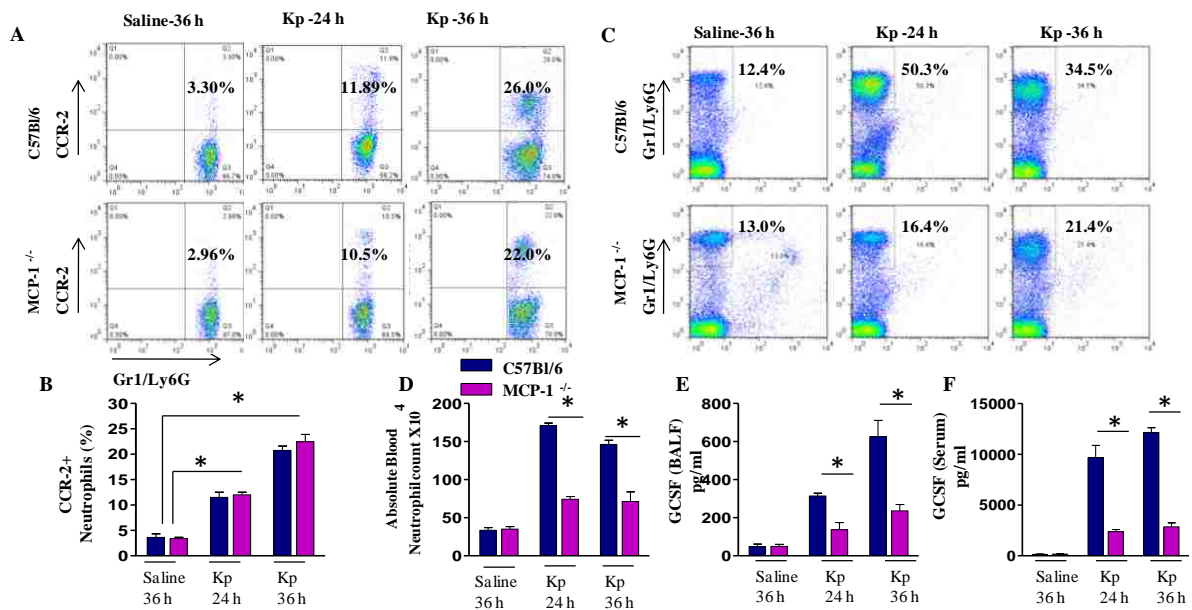
Figure 4.5: CCR2 expression in blood neutrophils of WT and MCP-1^{-/-} mice after Kp infection. Flow cytometric analysis of blood from WT mice at 24 and 36 h after i.t. Kp ($1X10^3$ CFU/mouse) infection using tagged antibodies against CCR2 and Gr1(Ly6G)(A-B). This is representative of 3 independent experiments with comparable results. C. Neutrophil numbers in blood of both WT and MCP-1^{-/-} mice following Kp infection. Neutrophils were gated and the percentage of Gr-1/Ly6G positive cells were determined. G-CSF concentrations in BALF and in blood of WT and MCP-1^{-/-} mice following i.t. Kp infection (E-F) determined by using sandwich ELISA. n=4-8 and $*p<0.05$.

Kp-Induced MCP-1 Modulates Blood Neutrophil Numbers. Neutrophil recruitment to the site of infection is a multistep event which involves neutrophil release from bone marrow (33). We found more bacterial dissemination (Fig. 4.1A-D) in MCP-1^{-/-} mice upon i.t. Kp infection along with reduced neutrophil influx to the site of infection (Figs. 4.1F-I). We therefore asked whether it is the defect in recruitment to the lung or release of neutrophils to the blood from marrow. In order to explore these possibilities, we infected both WT and MCP-1^{-/-} mice i.t. with Kp and collected blood at 24 and 36 h post infection and determined the blood neutrophil numbers. We found neutrophil numbers in both WT and MCP-1^{-/-} mice were significantly increased at 24 and 36 h post Kp infection but the magnitude was less in MCP-1^{-/-} mice (Fig. 4.5C-D).

MCP-1 Regulates the Expression of G-CSF. G-CSF is a known cytokine implicated in granulopoiesis and neutrophil mobilization (34, 35). Since we found reduced neutrophil numbers in the circulation of MCP-1$^{-/-}$ mice, we studied whether MCP-1 affects the expression of G-CSF in blood following Kp exposure using ELISA. We found reduced G-CSF concentrations in MCP-1$^{-/-}$ mice in serum and as well as BALF as compared to their littermates (Fig. 4.5E-F).

Instillation of MCP-1 in MCP-1$^{-/-}$ Mice Restores Blood Neutrophil Numbers and G-CSF Concentrations. After identifying a pronounced requirement of MCP-1 for G-CSF production and neutrophil release to the blood, we evaluated whether MCP-1 is necessary and sufficient to induce neutrophil release to the blood. Next we reconstituted MCP-1$^{-/-}$ mice with rMCP-1 and found increased blood neutrophil numbers in blood and BALF, even in the absence of infection in a concentration-dependent manner (Figs. 4.6A-C). We also observed a substantial increase in G-CSF expression in blood and BALF following rMCP-1 administration in a concentration-dependent manner (Fig. 4.6D). In addition, we performed similar experiments in the presence of Kp infection. Kp infected mice were administered with rMCP-1 at 1 h post-infection. We found that rMCP-1 rescues blood neutrophil counts (Fig. 4.7A-B), G-CSF concentrations in blood (Fig. 4.7C) and G-CSF concentrations in blood/serum (Fig. 4.7C). These observations suggest that MCP-1 regulates neutrophil numbers in the blood through G-CSF production in the absence or presence of Kp infection.
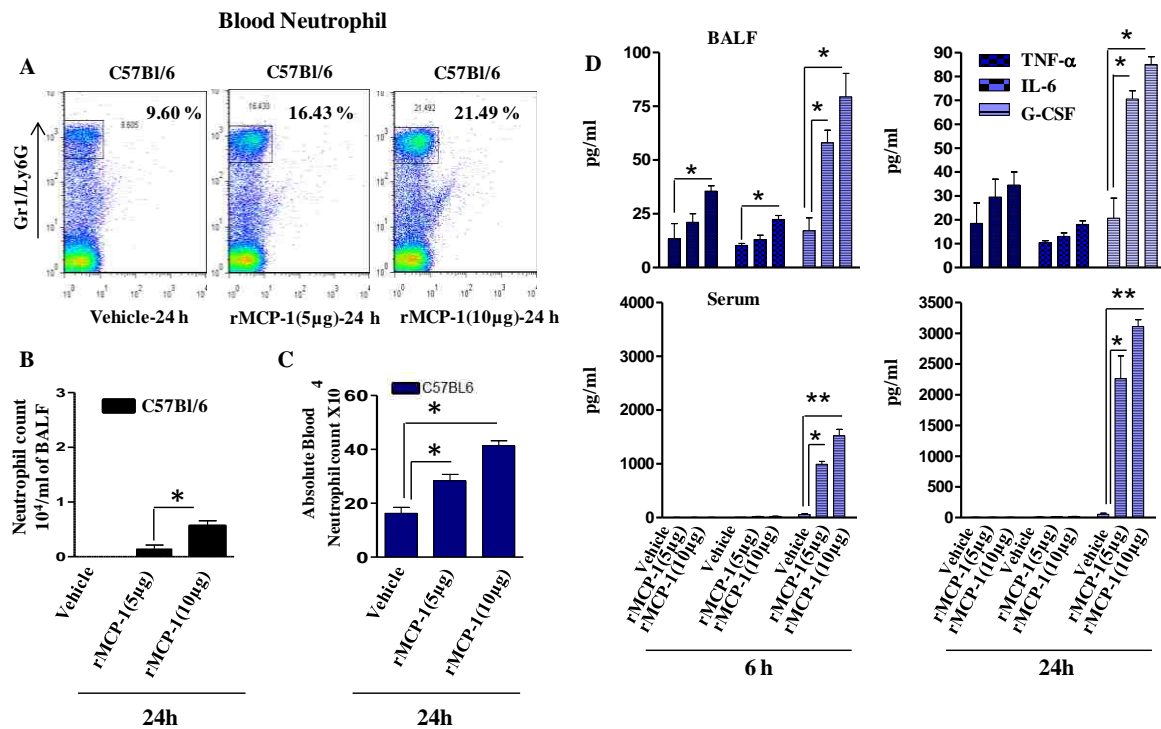
Figure 4.6: Increased neutrophil numbers in BALF and blood in a dose dependent manner after i.t. treatment with rMCP-1 (A-C). A-C. Blood neutrophil numbers were determined 24 h after i.t. treatment with rMCP-1 in two different doses (5 and 10μg/mouse) using Gr-1/Ly6G antibodies. Absolute blood neutrophil numbers were determined in total blood volume of mice as described in *Materials and Methods*. D. Protein concentrations of TNF-α, IL-6 and G-CSF in BALF and in serum were determined after treatment with 5 and 10 μg rMCP-1/mouse using sandwich ELISA. n=4-6 and *p<0.05., ** significance between two doses where p<0.05.

rG-CSF Treatment Reverses the Host Defense Defect in the MCP-1^{-/-} Mice in Response to Kp Challenge. Since we observed reduced neutrophil numbers in the blood associated with reduced G-CSF concentrations, we wanted to determine whether G-CSF treatment can improve host defense in MCP-1^{-/-} mice following Kp infection. We treated MCP-1^{-/-} mice i.t. with 1 μg G-CSF 1 h post Kp infection and observed that the survival of the knockout mice was significantly improved (Fig. 4.8A). In a similar manner, we found reduced bacterial burden in the lungs and dissemination to the extrapulmonary organs, including spleen in mice i.t. treated with 1 μg G-CSF 1 h post-Kp infection (Figs. 4.8B-C). Furthermore, neutrophil

accumulation in the lungs was enhanced in mice following G-CSF administration (Figs. 4.8D-E).
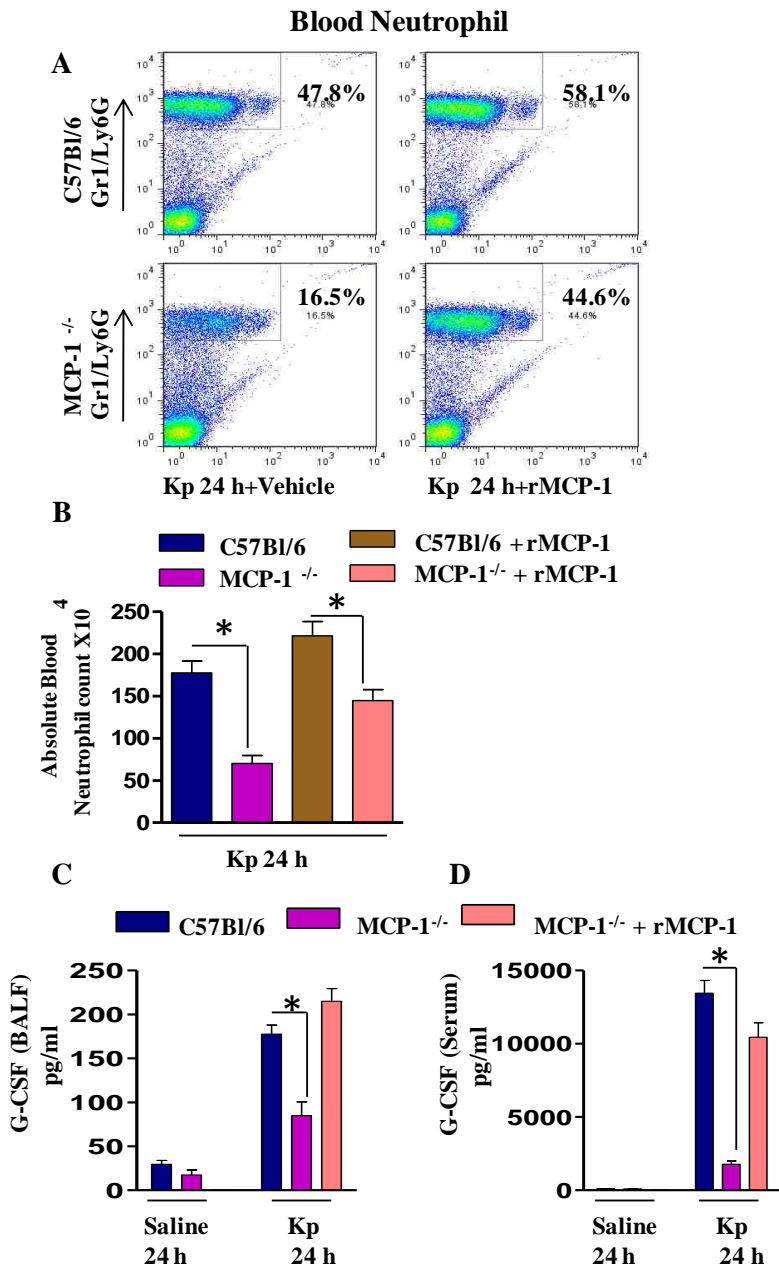
Figure 4.7: Higher blood neutrophil numbers and G-CSF concentrations in BALF and in serum after Kp infection and rMCP-1 treatment of the lung. Blood neutrophil percentage and absolute blood neutrophil numbers were determined following i.t. Kp infection and rMCP-1 (5 μg/mouse) (A-B). **C.** G-CSF protein concentrations in BALF and in serum were determined by using sandwich ELISA. Data shown here is a representation of 3 individual experiments where n=4-6 and *p<0.05.

Discussion

We recently reported that MCP-1 can recruit neutrophils to the lung during *E. coli* infection directly through chemotaxis and indirectly through the production of neutrophil

chemoattractants, including KC and MIP-2. Nevertheless, several limitations noted in this model: 1) *E. coli* is rapidly cleared from the lungs; 2) *E. coli* does not induce bacterial dissemination. Because of these limitations, we used Kp in the current investigation to examine host immunity in the lungs and extrapulmonary organs since this bacterium disseminates following intrapulmonary inoculation and thus, induces both local and systemic immune responses.
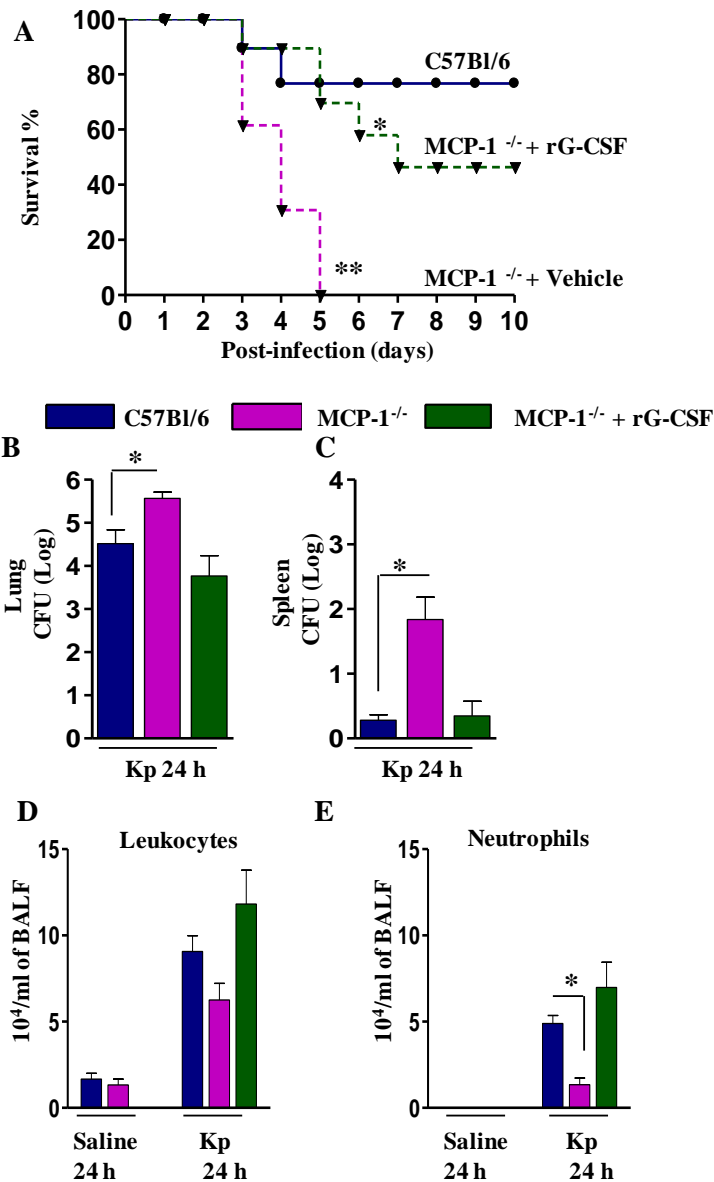
Figure 4.8: Attenuated survival, bacterial clearance, neutrophil influx, and cytokine/chemokine production in the lungs of MCP-1$^{-/-}$ mice are restored by exogenous rG-CSF. A. Survival of WT and MCP-1$^{-/-}$ mice after infection with 10^3 CFUs of Kp and administered with rG-CSF (1 µg/mouse) or vehicle (BSA) 1 h later, and survival was assessed up to 10 days. Data are presented as % survival (n=20 mice/group) and analyzed using Wilcoxon signed-rank test. * indicates the difference between rG-CSF or vehicle (BSA) control treated MCP-1$^{-/-}$ mice (p<0.05) and ** indicates the difference between WT and MCP-1$^{-/-}$ + rG-CSF mice.) Bacterial load (B) in the lungs and bacterial dissemination (C) were examined in lung homogenates of rG-CSF or BSA administered WT and MCP-1$^{-/-}$ mice at 24 h post-*K. pneumoniae* challenge (10^3 CFUs/mouse). Data are presented as Mean + SE (n=5-6 mice/group). * indicates p<0.05 rG-CSF compared with BSA (vehicle) administered mice.

Cellular infiltration (D-E) in airspaces at 24 h after i.t. treatment with rG-CSF (1µg/mouse) or vehicle (BSA) control. n=6-8 mice/group. (* indicates p<0.05 as compared to BSA administered mice).

Antimicrobial function of phagocytes is contingent on the ability of these phagocytes to leave the bloodstream and enter the site of infection (5, 6). Numerous studies show that MCP-1 is a potent monocyte/macrophage chemoattractant: In mouse of pneumococcal and *Pseudomonas* pneumonia, blocking MCP-1 reduced monocyte but not neutrophil recruitment (20, 36). In subsequent studies, MCP-1 has been shown to be associated with T lymphocyte recruitment to the site of inflammation (11). We previously demonstrated that rMCP-1 in the presence or absence of *E. coli* infection causes neutrophil and macrophage influx into the lungs. In the current investigation, our findings show that MCP-1 induces neutrophil, but not monocyte recruitment, to the lungs in response to i.t. Kp infection. These results are consistent with earlier reports showing that blocking MCP-1/CCR2 attenuated neutrophil influx in the lung following i.t treatment with MCP-1/LPS (37) or *Cryptococcus neoformans* infection (38). In addition, the other study (39) implied that administering MCP-1 alone did not cause neutrophil influx in the lungs but MCP-1 along with LPS synergistically induced neutrophil migration to the lungs.

Recruitment of neutrophils by chemoattractants during the inflammatory process involves chemotaxis (direct). Our results showed that neutrophils from blood and BALF express CCR2, which was increased upon infection. In this context, we showed in our earlier report that bone marrow neutrophils migrate towards rMCP-1 (5). Several other reports support this. For instance, the Beck-schimmer group has indicated that LPS induced neutrophil influx depends on MCP-1 production (40). Murine neutrophils express functional CCR2 and migrate towards MCP-1 as demonstrated earlier (41). Further studies have shown that unstimulated neutrophils are not responsive to MCP-1 despite the fact that neutrophils become responsive to MCP-1 during sepsis in mice (42). In other acute inflammatory models in mice, such as acute pancreatitis and colitis, MCP-1 has shown to be critical for neutrophil

migration and blocking MCP-1 attenuated neutrophil counts and inflammation (43, 44). In chronic inflammation, neutrophils upregulate CCR2 (45).

The other mechanism of neutrophil recruitment is that cytokines modulate the expression of other neutrophil chemoattractants via indirect cascades. The present study shows that MCP-1 regulates the expression of key neutrophil chemoattractants, including KC and MIP-2. These findings are similar to our previous report which shows a) reduced expression of chemokines (KC and MIP-2) in MCP-1$^{-/-}$ mice in response to *E. coli* infection (5); and b) when MCP-1$^{-/-}$ mice were reconstituted with rMCP-1 the chemokines concentrations returned to the level of WT mice during *E. coli* infection (5). These observations also suggest that there is a complex interplay between CC and CXC chemokines. This has been documented in earlier studies: 1) extracellular signal by CCL2 augments CXCL-8 expression on monocytes (46); and 2) CXCR-2 signaling can lead to macrophage recruitment during *S. pneumoniae* infection (47).

NF-κB is one of the well-studied transcription factors that mediate the induction of numerous inflammatory cytokines and chemokines (5, 32). Studies have demonstrated that NF-κB activation is a critical event in the pathogenesis of bacterial pneumonia (5, 6, 48). We observed decreased NF-κB and MAPK activation in MCP-1 gene deficient mice during *E. coli* infection. These observations suggest that the MCP-1/CCR2 axis regulates the activation of NF-κB and MAPKs. In this regard, it has been shown that MCP-1 can activate both NF-κB and MAPKs during renal inflammation (49). Our findings suggest that transcription factors other than NF-κB, such as AP-1 and STAT-1 can be activated via MAPKs in a MCP-1-dependent manner. Together, our findings suggest that the MCP-1/CCR2 signaling axis regulates the expression of CXC chemokines (MIP-2 and KC) through NF-κB and MAPKs.

The antibacterial defenses of the lung include the resident alveolar macrophages and epithelium as well as the neutrophils and/or macrophages that migrate into the lung parenchyma from the bloodstream (7, 33). Any defect in these functions will ultimately result in infectious complications in the host. The relative contribution of hematopoietic/bone marrow cells versus resident tissue cells in neutrophil recruitment to the lung from the bloodstream has not been clear. However, the contribution of specific cell type is dependent on the specific stimulus, because both of these cell types are exposed to PAMPs and danger associated molecular patterns (DAMPs) during the insult. Bone marrow-derived cells in the lung produce several neutrophil chemokines such as KC (50, 51) and MIP-2 (52, 53) whereas resident cells, including alveolar epithelial type II cells, produce other neutrophil chemoattractants, such as LIX (54). An interesting finding of this investigation was the dependence of MCP-1 from both bone marrow cell and resident cells for neutrophil accumulation in the lung in response to *Kp* infection. These findings are consistent with previous reports demonstrating the role of bone marrow versus resident cells in lung inflammation: a) MyD88 derived from bone marrow cells is more important for LPS-induced expression of TNF-α and IL-12p40 (55), although both bone marrow and resident cell-derived MyD88 are essential for LPS-induced neutrophil recruitment (56-58); b) MD-2 signaling cascades in both cell types is essential for neutrophilic inflammation, and the expression of MIP-2, TNF-α, and IL-6 is mediated by both cell types in the lungs after LPS administration (59); and c) the neutrophil chemokine KC produced by both bone marrow and resident cells is important for bacterial clearance and neutrophil accumulation in the lung upon Kp infection (60).

G-CSF is a cytokine produced by numerous cell types in the lungs, including macrophages, fibroblasts, epithelial and endothelial cells (61-63). G-CSF has been shown to be important for granulocyte production/maturation and neutrophil functions, including

phagocytosis, chemotaxis and the production of reactive oxygen intermediates (64). We have demonstrated that MCP-1 modulates the production of G-CSF in the blood and BALF in response to Kp infection (Fig. 4C-F). Thus, rG-CSF reverses host defense defects in the blood and BALF of MCP-1$^{-/-}$ mice (Fig. 6). These findings are consistent with earlier observations that i.t. G-CSF instillation induced neutrophil infiltration to the lung (65, 66). G-CSF in the lung could result in PMN migration through augmenting PMN adhesion to endothelium (67, 68), and through enhancing PMN chemotaxis (68, 69). The movement of PMNs from capillaries to the lung parenchyma requires transmigration of PMN across both endothelial and epithelial cells. Our data suggest that intrapulmonary administration of G-CSF following Kp infection can induce both transendothelial and transepithelial migration of neutrophils. Based on our findings, it appears that the augmented neutrophil influx in the lungs could have contributed to bacterial clearance from the lungs and blood (Fig. 6).

In conclusion, these studies suggest a role for MCP-1 in antibacterial defenses of the lung via the production of G-CSF. To our knowledge, this is the first time that G-CSF was used to overcome the MCP-1-dependent impairment in antibacterial defenses of the lung. In humans, serum G-CSF concentrations increased during the acute phase of respiratory infections, suggesting an essential physiologic role of G-CSF in determining the neutrophil-mediated host defenses within the lung during pneumonia (70). The availability of recombinant G-CSF affords the clinical use of G-CSF to augment innate host defense in patients who either lack or have malfunctional MCP-1. However, the concentration of G-CSF needs to be optimized to make sure that the dose of G-CSF does not induce PMN-induced acute lung injury and possibly, ARDS. It has also been demonstrated that MCP-1 polymorphism is associated with increased susceptibility to *Mycobacterium tuberculosis* infection (71, 72) and bacterial peritonitis (73).

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Chapter 5

Conclusions

Conclusions

The respiratory system is the second largest system of the body that is directly exposed to the environment. Therefore, it constitutively encounters numerous infectious agents and thus bacterial lung infections are a leading burden of disease in the world and the most common cause of acute lung injury. It is also a major cause of mortality in the US [1-3]. Tremendous advances have been made in the treatment of pneumonia using broad spectrum antibiotics. However, these strategies have resulted in the emergence of multi-drug resistant bacterial strains[4, 5]. In addition to this, increasing populations of immunocompromised individuals who are susceptible to pneumonia make the treatment of bacterial pneumonia increasingly difficult. This necessitates novel therapeutic approaches to treat lower respiratory tract bacterial infections. Immunotherapy to augment the host immune response can serve as an adjunct to antibiotic treatment [3, 6]. Although both innate and adaptive immune responses play important roles in the host defense, the innate immune response is the primary defense against most acute infections. Innate defenses consist of structural defenses, antimicrobial molecules, and phagocytosis by resident alveolar macrophages (AMs) and recruited polymorphonuclear cells (PMNs)[7, 8]. According to studies with bacterial infections in different models, neutrophils play a predominant role in containing the infection [9, 10]. Our results also indicate that neutrophil depletion significantly impairs bacterial clearance during *E. coli* infection in mice [11, 12].

Neutrophil recruitment is a very well orchestrated multi-step process. This involves a complex network of cytokine/chemokine signaling. Therefore, cytokines/chemokines can be used to boost neutrophil mediated host immune response. Many cytokines have pleiotropic effects and can be involved in multiple facets of host defense[7, 8, 13]. MCP-1 has been extensively studied for its role in monocyte recruitment and activation[14-16]. Different studies indirectly indicated a role of MCP-1 with regard to neutrophil recruitment [17-19]. In

chapter 2 we have shown that MCP-1 can act as a neutrophil chemoattractant directly and indirectly through other cytokines (TNF-α and IL-6) and chemokines (KC and MIP-2) during *E. coli* infection of the lungs. We observed CCR2 expression on neutrophils which is increased with infection both *in-vitro* and *in-vivo*. In an *in-vitro* transmigration assay we observed neutrophils migrated towards rMCP-1. When we administered rMCP-1 to WT as well as MCP-1$^{-/-}$ mice it caused neutrophil influx with or without infection. However, only 4-6% of neutrophils expressed CCR2. Therefore, the remarkable reduction in neutrophil recruitment observed in the gene deficient mice could not be solely attributed to the direct role of MCP-1 in neutrophil recruitment. With regard to the indirect role of MCP-1 by controlling TNF-α, IL-6, KC and MIP-2, it was possible that reduced cytokines/chemokines levels in the knock-out mice could be a result of lower numbers of recruited neutrophils in the lungs because previous reports indicate that neutrophils also produce these cytokines and chemokines during infection [20-22]. In addition, in this study, although we found higher bacterial burden in the lungs of MCP-1 gene deficient mice, *E. coli* gets cleared off and doesn't disseminate to distal organs to cause mortality in bothe groups of mice after infection.

Because of these limitations, to delineate the mechanism of neutrophil recruitment by MCP-1 in acute bacterial infections we have chosen the pathogen *Klebsiella pneumoniae* in

Chapter 3. Interestingly we found not only neutrophil numbers in BALF but also blood neutrophil numbers were low in MCP-1$^{-/-}$ mice after *Klebsiella* infection. We also found that MCP-1 regulates G-CSF production with or without infection in mice. A single G-CSF dose i.t. improved the neutrophil mediated host defense in MCP-1$^{-/-}$ mice after *Klebsiella* infection. Our data indicate a direct role of MCP-1 in G-CSF regulation. However, the mechanism of how MCP-1 regulates G-CSF is yet to be determined. In order to determine this, it is important to determine the source of G-CSF in lungs upon *Klebsiella* infection. In previous

studies, it has been shown that in addition to monocytes/macrophages[23], fibroblasts[23, 24], alveolar epithelial cells[24, 25] and endothelial cells[26] can also produce G-CSF upon infection. Since we saw a remarkable increase in G-CSF levels in serum upon rMCP-1 treatment alone, it is possible that endothelial cells are the predominant source of G-CSF production because they constitutively express the CCR2 receptor. However, in our model this needs to be determined.

G-CSF is a member of the glycoprotein family that is essential for survival, proliferation differentiation and release of hematopoietic cells and activation of neutrophils and monocytes[23, 27]. Local administration of rG-CSF intratracheally has resulted in systemic effects such as increasing blood neutrophil numbers, their microbicidal activity, and extravasation. In animal experiments, including ours, it has been found that recombinant G-CSF (filgrastim, pegfilgrastim or lenograstim) treatment has improved bacterial clearance either by increasing neutrophil numbers or enhancing their function[23]. In a rabbit model, rG-CSF improved survival during bacterial pneumonia/sepsis when combined with antibiotics[3]. These findings are consistent with ours. In contrast, it has been shown that prophylactic treatment with G-CSF worsened the outcome during *Klebsiella pneumoniae* infection of the lungs in mice[27, 28]. Similar to this finding, during *E. coli* pneumonia, administration of G-CSF impaired bacterial clearance and increased pulmonary injury and mortality[27]. However, G-CSF has been FDA approved as a therapy in several neutropenic conditions, such as patients with cancer receiving myelosuppressive chemotherapy, after induction or consolidation chemotherapy for acute myelogenous chemotherapy, for myeloid reconstitution after hematopoietic stem cell transplantation, for mobilization and collection of peripheral blood stem cell for transplantation, and for severe chronic neutropenia[27, 29]. The most common side effect with such treatment is mild-to-moderate bone and/or musculoskeletal pain that occurs in 20-30% patients and resolves with discontinuation of the

treatment[27]. Therapeutic investigations in patients with community acquired pneumonia receiving intravenous antibiotics and G-CSF for ten days have shown an increase in circulating neutrophils, however, it did not affect time to resolution of morbidity, mortality or length of hospitalization. The development of sepsis related organ failure or acute respiratory distress syndrome was very much reduced[3, 27].

With regard to *Klebsiella* infection, studies have been done with other cytokines such as TNF-α, KC, MIP-2, IFN-γ and IL-12. Inhibition of endogenous TNF-α has resulted in increased mortality associated with higher bacterial burden in mice upon i.t. Kp infection[30]. When mice were treated either with TNF-α agonists or TNF-α expressing adenoviral vectors prior or concomitant with infection, bacterial clearance and survival was much improved[6]. Although TNF-α blocking decreased neutrophil numbers and administering TNF-α increased neutrophils following Kp infection, the beneficial effects of TNF-α has not been defined because TNF-α itself is not chemotactic to neutrophils[30]. In addition, during Kp infection, our lab and other labs have shown that neutrophil chemokines such as KC and MIP-2 are essential for survival and bacterial clearance [6, 9, 31-33]. In our recent report, we have shown that neutrophil defects in KC knock-out mice can be compensated by another neutrophil chemokines, LtB4[34]. Another cytokine that has been shown to be critical for the host defense against Kp infection is IFN-γ. During Kp infection, IFN-γ knock-out mice showed significant mortality and when mice were administered with rIFN-γ host defense was improved against Kp in alcohol intoxicated mice[30]. Similar to these findings, IL-12 has also been shown to be essential for Kp clearance and survival upon infection in mice[30, 35]. However, less work has been done with regard to clinical investigations of these cytokines/chemokines. Our study is the first to show the critical role of MCP-1 in neutrophil recruitment and host defense during Kp infection. The mechanism of how MCP-1 controls

the expression of G-CSF needs to be investigated in detail. Since cytokines have a pleiotropic effect and several cytokines can have a common function, it is important to clearly dissect out the signaling mechanisms during acute bacterial infections in order to utilize them as therapies. However, when compared to the above mentioned cytokines G-CSF has potential as a therapeutic agent in acute bacterial pneumonia with low side effects[29].

From a therapeutic stand point, it is important to select a cytokine that has a broad function in the host defense. In this regard, MCP-1 has been shown to have multiple roles in the host defense. MCP-1 is important for the recruitment of monocytes[15], neutrophils[11] and T lymphocytes[36]. MCP-1 plays an important role in clearing apoptotic neutrophils[37] and aids in the healing process by recruiting endothelial cells and promoting angiogenesis[38]. In our study, MCP-1 regulates G-CSF and blood neutrophil numbers. Therefore, MCP-1 can serve as a potential therapeutic agent to treat gram-negative bacterial pneumonia.

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Appendix I

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Appendix II

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Vita

Gayathriy (Gaya) Balamayooran was born in Jaffna, Sri Lanka, to Vallipuram Mahadevan and Virasadevy Mahadevan. She has a sister, loving and supportive husband and a beautiful baby Kaitlyn. Gayathriy 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, she was a lecturer at the same institution. During her veterinary studies she became interested in immunology of infectious diseases. In 2008 fall, she joined the Lung Biology Laboratory at the Department of Pathobiological Sciences, Louisiana State University to pursue her doctoral degree under the guidance of Dr. Samithamby Jeyaseelan. In lung biology laboratory, she has learned pulmonary innate immune mechanisms involved with bacterial infections. Gayathriy will graduate in May 2012 with her Doctor of Philosophy degree. After graduation, Gaya is interested in pursuing her research interests in the field of immunology of infectious diseases.