Host factors in defense against bacterial and fungal pneumonia

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Appendix of Abbreviations

5-LO	5-Lipoxygenase
7AAD	7-Aminoactinomycin D
Akt	Protein Kinase B
ALT	Alanine transaminase
ANOVA	Analysis of variance
Akt	Protein Kinase B
AST	Aspartate transaminase
ATCC	American Type Culture Collection
BAL	Bronchoalveolar lavage
BrdU	5-Bromo-2'-deoxyuridine
BUN	Blood urea nitrogen
cAMP	Cyclic adenosine monophosphate
CC	Chemokine designation
CD	Cluster of differentiation
cDC	Conventional DC
CFU	Colony forming unit
cMoP	Committed monocyte progenitor
COPD	Chronic obstructive pulmonary disease
CPS	Capsular polysaccharide
CSF1R/CD115	Colony stimulating factor-1 receptor
CSF2R	Colony stimulating factor-2 receptor
CXC	Chemokine designation

DC	Dendritic Cell
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FSC	Forward scatter
FT	Fecal transplant
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-Macrophage colony stimulating factor
HBSS	Hanks buffered salt solution
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
I.P.	Intraperitoneal
I.T.	Intratracheal
LPS	Lipopolysaccharide
Mφ	Macrophage
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
MDP	Macrophage-DC progenitor
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MIP	Macrophage inflammatory protein
MPO	Myeloperoxidase

MMP	Matrix metalloproteinase
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa beta
NLRP	NLR family pyrin domain containing
P.I.	Post-infection
PBS	Phosphate buffered saline
PDCA-1	Plasmacytoid DC Ag-1
pDC	Plasmacytoid DC
PI3K	Phosphoinositide 3 kinase
PMN	Polymorphonuclear neutrophils
RFU	Relative fluorescence unit
ROS	Reactive oxygen species
SEM	Standard error of mean
SFB	Segmented Filamentous Bacteria
Src	Proto-oncogene tyrosine-protein kinase Src
SSC	Side scatter
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TREM-2	Triggering receptor expressed on myeloid cells-2
UVa	University of Virginia

Appendix of Figures and Tables

Chapter I.

Chapter II.

Chapter I Introduction

1.1 The Mononuclear Phagocyte System in bacterial and fungal pneumonia

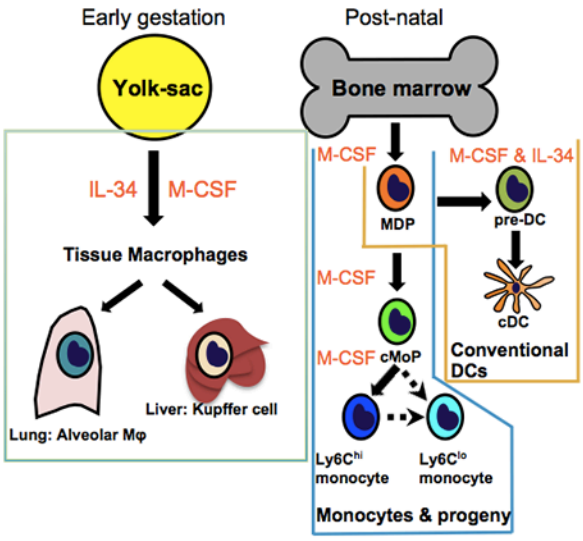
ABSTRACT

Pneumonia is a serious and life-threatening infection caused by a variety of microorganisms including bacteria and fungi. Antibiotic resistance among Gram-negative and Gram-positive bacteria is increasing, and even with treatment, mortality associated with infection is high. The mononuclear phagocyte system is a diverse group of immune cells consisting of three lineages: yolk-sac derived tissue-resident macrophages, conventionally derived dendritic cells, and monocytes and their progeny. The role of the mononuclear phagocyte system in host defense against bacterial and fungal pneumonia has been shown to be critical in microbial clearance, immune cell activation, and in initiating a productive adaptive immune response, but the contributions of each cell type to these functions is not well described. Herein, I will discuss the role of each mononuclear phagocyte in response to Gram-negative, Gram-positive, and fungal pneumonia.

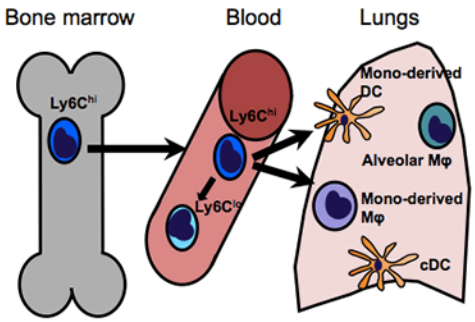
The mononuclear phagocyte system and M-CSF

The mononuclear phagocyte system is a diverse group of phagocytic cells consisting of tissue-resident macrophages, conventional dendritic cells (cDC), and monocytes and their progeny. Mononuclear phagocytes are not derived from a single lineage, but from progenitor cells from varying hematopoietic origins (1). Tissue-resident macrophages are derived early in gestation from yolk-sac progenitors, then home to specific tissues where they self-renew under homeostatic conditions (1). cDCs are derived from the pre-DC progenitor in the bone marrow postnatally and throughout adulthood (1,2). Finally, monocytes are derived in the bone marrow from the macrophage-DC progenitor (MDP) which then further differentiates into the committed-monocyte progenitor (cMoP) and finally into mature monocytes (3,4) (Fig. 1.1). In the mouse, the two phenotypically and functionally distinct monocyte subsets can be distinguished by their expression of the surface glycoprotein Ly6C; similar populations are identified in humans based on expression of surface antigens CD14 and CD16 (5). From the bone marrow, murine $Ly6C^{hi}$ monocytes extravasate into the blood and traffic to tissue where they will terminally differentiate into monocyte-derived macrophages and monocyte-derived dendritic cells (DCs), while $Ly6C^{lo}$ monocytes patrol the vascular endothelium and clear microparticles.

A: Sites of Mononuclear Phagocyte Hematopoiesis



B: Mononuclear Phagocytes in Tissue



C: Classification of Mononculear Phagocytes

Tissue	Lineage	Cell type	Phenotypic Marker
Bone marrow	HSC progenitor	MDP	CD45+, Lin-, CD115+, CD117+, CD135+, CD11b-, Ly6C-
Bone marrow	HSC progenitor	cMoP	CD45+, Lin-, CD115+, CD117+, CD135-, CD11b-, Ly6C+
Bone marrow and blood	Monocyte	Ly6C^{hi} monocyte	CD45+, CD11b+, Ly6G-, CD115+, Ly6C^{hi}
Bone marrow and blood	Monocyte	Ly6C^{lo} monocyte	CD45+, CD11b+, Ly6G-, CD115+, Ly6C^{lo}
Lungs	Tissue Macrophage	Alveolar macrophage	CD45+, CD11b+, Ly6G-, CD11c^{int} CD64+, CD103-
Lungs	Monocyte	Ly6C^{hi} monocyte	CD45+, CD11b+, Ly6G-, Ly6C^{hi}, MHCII-
Lungs	Monocyte	Ly6C^{lo} monocyte	CD45+, CD11b+, Ly6G-, Ly6C^{lo}, MHCII-
Lungs	Monocyte	Monocyte-derived macrophage	CD45+, CD11b+, Ly6G-, MHCII-, CD64+
Lungs	Monocyte	Monocyte-derived DC	CD45+, CD11b+, Ly6G-, CD11c+ CD64+, CD24-, MHCII+
Lungs	cDC	CD11b+ DC	CD45+, CD11b+, Ly6G-, CD11c+ CD64-, CD24+, MHCII+
Lungs	cDC	CD103+ airway DC	CD45+, Ly6G-, CD11c^{int} CD64-, CD103+, MHCII+
Lungs	cDC	Plasmacytoid DC	CD45+, Ly6G-, CD11c+, MHCII+, PDCA-1+

Figure 1.1. The mononuclear phagocyte system. (*A*) Sites of hematopoiesis and identification of the three lineages of the mononuclear phagocyte system. Macrophage colony stimulating factor (M-CSF) and IL-34 are required for the development of all three lineages at steady state. Mφ, macrophage. (*B*) Mononuclear phagocytes in the bone marrow, blood, and lungs. Ly6C^{hi} monocytes extravasate from the bone marrow and enter the blood where some differentiate into Ly6C^{lo} monocytes. Other Ly6C^{hi} monocytes will enter peripheral tissues like the lungs where they differentiate into monocyte-derived macrophages and monocyte-derived DCs. Tissue macrophages (alveolar macrophages) and cDCs (CD103+ Airway DC, CD11b+ DC, and plasmacytoid DC) are present in the airways and parenchyma of the lungs, respectively. (*C*) Identifying markers of cells in the mononuclear phagocyte system.

Mononuclear phagocytes require the cytokine macrophage-colony stimulating factor (M-CSF) for normal development and maintenance at steady state. M-CSF is a hematopoietic growth factor required for the proliferation, differentiation, survival, and function of mononuclear phagocytes during homeostasis (6-9). M-CSF is a 85 kDa glycoprotein that has 3 isoforms: a secreted glycoprotein, a secreted proteoglycan, and a membrane-spanning cell-surface glycoprotein that have overlapping functions (8,10-12). This growth factor is produced by hematopoietic stem cells (13), mononuclear phagocytes (13-15), endothelial cells, lung fibroblasts (14,16), and hepatocytes (17). The M-CSF receptor, CSF1R, is a homodimeric tyrosine kinase receptor constitutively expressed in monocyte lineage cells beginning with the macrophage-DC progenitor ((7,9)). M-CSF and CSF1R engagement results in proliferative and anti-apoptotic signaling cascades converging on the PI3K, Akt, mTOR, and Src downstream signaling pathways (8,9,18) and is therefore a common target in myeloid related cancer treatments. The alternative ligand for CSF1R is IL-34 (19) which has overlapping but distinct functions from M-CSF. IL-34 is primarily expressed in the central nervous system and is also involved in the derivation and maintenance of tissue macrophages and skin Langerhans cells at steady state (20-22).

Cells within the mononuclear phagocyte system have specific functions during homeostasis, but these functions change greatly during inflammation and infection (23-26). In the lungs, there are at least 5 populations of mononuclear phagocytes at steady state: long-lived and self-renewing tissue-resident

macrophages known as alveolar macrophages, short-lived monocyte-derived interstitial macrophages, conventional CD11b+ DCs, CD103+ airway DCs, and plasmacytoid DCs. Under inflammatory conditions, monocytes are recruited into the lungs and differentiate into monocyte-derived macrophages and DCs, while resident immune cells efflux or die, altogether comprising a shifting and dynamic cellular network. Here, I will provide an overview of the origins and dynamics of the mononuclear phagocyte system, as well as their roles and functions in the context of bacterial and fungal pneumonia.

Tissue-resident macrophages

Tissue-resident macrophages are derived early on in development from yolk-sac progenitors (2,27,28). These early progenitors require CSF1R stimulation by M-CSF and its second ligand IL-34, as well as CSF2R stimulation via granulocyte-macrophage colony stimulating factor (GM-CSF) to proliferate, differentiate, and survive (1,21,29). Tissue macrophages are given unique names based on the organ in which they reside: brain microglia, liver Kupffer cells, and lung alveolar macrophages are examples of this nomenclature. Tissue-resident macrophages are critical in host defense during infection as first responders that phagocytose and kill invading pathogens.

Alveolar macrophages are long-lived and self-renewing during homeostasis (1,28,30). Some early studies suggest that murine Ly6C^{lo} monocytes contribute to the pool of alveolar macrophages (31-33), but more recent studies have revealed caveats to these findings. The major criticisms of these earlier studies are methodological: many of these experiments relied on bone marrow chimeras following myeloablation. Due to the tissue damage that is generated during irradiation, myeloablation may result in inhibition of proliferative capacity of tissue macrophages and their precursors (2,30,34), and therefore, data resulting from these experiments may not represent homeostatic conditions. More recent studies rely on parabiosis and fate-mapping approaches, and show that alveolar macrophages proliferate and self-renew locally during homeostasis with little to no repopulation from monocytes (28,30,34). The ability of alveolar macrophages to self-renew during inflammation or infection is not well described, and it is less

clear if the recovery of alveolar macrophages results only from proliferation of surviving alveolar macrophages, or if Ly6C^{hi} and Ly6C^{lo} monocytes are able to repopulate this lineage.

Conventional dendritic cells

Conventional DCs develop in the bone marrow postnatally and throughout adulthood. During hematopoiesis, the common DC progenitor (CDP) differentiates into the pre-DC progenitor (1,35) and in a FLT3-L dependent manner, terminally differentiates into the cDC (36,37). Interestingly, some studies implicate a role of CSF1R in the development of cDCs (21,36), perhaps suggesting that monocyte precursors could have some involvement in the derivation of this lineage (23), but this requires further investigation. In the lungs, CD11b+ DCs were long thought to be phenotypically indistinguishable from their monocyte-derived counterparts, but recent studies have shown that these lineages can be distinguished by CD24 and CD64 surface expression: conventionally derived CD11b+ DCs express CD24 but not CD64, while monocyte-derived DCs express CD64 but not CD24 (37,38). Apart from these phenotypic differences, CD11b+ DCs and monocyte-derived DCs have distinct but overlapping functions. In the lungs, CD11b+ DCs acquire antigen, upregulate surface expression of CCR7, and traffic to the draining lymph node to present antigen to their cognate T cell (23). During inflammation and infection, CD11b+ DCs egress from peripheral tissue and undergo rapid death associated with homeostasis (39,40).

In the airway, CD103+ CD11b- DCs known as CD103+ airway DCs, reside beneath the bronchial epithelium. While it is generally accepted that these cDCs are derived from the pre-DC progenitor under the control of FLT3L, Id2, and IRF8, (29,36), others have shown that CD103+ DCs can also arise from the

Ly6C^{hi} monocytes in the lungs at steady state (23). CD103+ DCs acquire and transport apoptotic cells to the lung-draining lymph node (41) where they become CD8+ DCs (24) and are implicated in antigen cross-presentation (25). The ability of monocytes to repopulate this lineage in the context of inflammation or infection has not been well defined.

Like the CD11b+ DC and CD103+ airway DC, plasmacytoid DCs (pDCs) are also derived from the pre-DC progenitor. pDCs reside in the lung interstitium (37) and are major contributors in viral immunity due in large part to their production of type I interferons. While pDCs, like other conventionally-derived DCs, are derived in the bone marrow, the dynamics and repopulation of these cells have not been fully defined. In some cases, Ly6C^{hi} monocytes can contribute to generating pDCs (42), but more evidence is required to support this conclusion.

Monocytes and their progeny:

Bone marrow monocytes develop from the MDP progenitor cell. Monocyte development requires M-CSF to develop from the MDP into the cMoP at steady state (4,43). These differentiated monocytes are then recruited into the blood via CCR2 (44,45), where Ly6C^{hi} monocytes differentiate into Ly6C^{lo} monocytes. The role of Ly6C^{lo} monocytes is incompletely described, but evidence suggests these cells patrol the vascular endothelium to scavenge microparticles and clear cellular debris, and more recently, Ly6C^{lo} monocytes have been implicated in neutrophil recruitment culminating in necrosis of vascular endothelial cells (46). Conversely, Ly6C^{hi} monocytes leave the blood and enter peripheral tissue where they differentiate into monocyte-derived macrophages and monocyte-derived DCs and carry out effector functions (44,47-49).

Monocyte-derived macrophages and DCs are present in small numbers at steady state but their numbers increase dramatically during infection and inflammation. Monocyte-derived macrophages can be distinguished from differentiating monocytes by the down regulation of CD115 (CSF1R) and Ly6C surface expression (50), while monocyte-derived DCs can be distinguished from CD11b+ DCs based on their surface expression of CD24 and CD64. These cells are professional phagocytes that clear invading microorganisms by phagocytosis and oxidative and non-oxidative killing (44,47,48) while monocyte-derived DCs are also involved in initiating adaptive immunity.

Mononuclear phagocytes are a diverse group of phagocytic cells that originate from disparate hematopoietic precursors and carry out a variety of

effector functions. The role and function of mononuclear phagocytes, as well as their degree of plasticity in repopulating different lineages of mononuclear phagocytes is largely dependent on the context of the perturbation (e.g. infection) as well as the specific nature of this perturbation (e.g. bacterial or fungal). Henceforth, I will discuss the pathogenic mechanisms utilized by microorganisms during pneumonia, as well as the roles and functions of mononuclear phagocytes in the context of infection.

Pneumonia and the pathogenic mechanisms of its causative organisms: Gram-negative and Gram-positive bacteria and *Aspergillus fumigatus*

Pneumonia is an acute lower respiratory infection typically caused by infectious agents including bacteria and fungi. Acute lower respiratory infections cause greater burden of disease worldwide than many infectious and non-infectious diseases including HIV, malaria, cancer, or heart attacks (51), and in the United States, more disease and death than any other infection (52). The outcome of infection depends on the virulence of the causative microorganism as well as the resulting host immune response. Herein, I will identify the most common causes of bacterial and fungal pneumonia and provide an overview of their pathogenic mechanisms during pneumonia. Microbes can utilize various virulence factors to evade the host immune system or to kill host cells. Virulence factors vary between organisms, but can include endotoxin, secreted toxins, effector proteins, fimbriae, and capsular polysaccharide. I will focus on the exotoxins of the specific Gram-negative and Gram-positive organisms I will address later in this chapter, with special attention given to virulence factors in the microbes I will address in chapter 2 and 3: *Klebsiella pneumoniae* and *Aspergillus fumigatus*.

Gram-negative bacteria including *Pseudomonas aeruginosa*, *Bordetella pertussis*, and *Legionella pneumophila* and Gram-positive bacteria including *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Staphylococcus aureus* all produce toxins that aid in immune evasion or damage and kill host cells. For example, the Gram-negative bacterium *P. aureginosa* utilizes exotoxin A and exoenzyme S to evade phagocytosis and induce host cell death (53,54).

While distinct from each other, both toxins are ADP-ribosyltransferases that inhibit protein synthesis in eukaryotic cells and are cytotoxic. Furthermore, multiple studies have shown that expression of exoenzyme S correlates to increased lung injury during *Pseudomonas* infection (55,56). Similarly, the Gram-negative bacterium *L. pneumophila* utilizes the glucosyltransferases Lgt1 Lgt2, and Lgt3 to inhibit host cell protein synthesis resulting in target cell death (57-59). Likewise, *B. pertussis* utilizes pertussis toxin and adenylate cyclase toxin to combat host immune defenses during infection. Once inhaled, *B. pertussis* produces pertussis toxin and adenylate cyclase toxin to colonize the lungs and subvert immune function in mononuclear phagocytes by increasing cAMP levels resulting in reduced oxidative burst (60-64). Toxins produced by Gram-positive bacteria have similar effects during pneumonia. For example, *S. pneumoniae* produces pneumolysin, a pore-forming toxin that induces host cell apoptosis (65). The absence of this toxin results in improved outcomes during *S. pneumoniae* infection (66,67). In addition, *S. aureus* produces the pore-forming toxin α-hemolysin to induce host cell death during pneumonia (68-70) while the M protein of *S. pyogenes* has anti-phagocytic properties that subvert the host immune response during infection (71,72). Thus, toxins produced by Gram-negative and Gram-positive bacteria promote pathogenicity and are important in subverting host immune responses and inducing host cell death during pneumonia. In contrast to these Gram-negative and Gram-positive bacteria, *Klebsiella pneumoniae* and *Aspergillus fumigatus* can be considered true opportunistic pathogens in that *Klebsiella* is present in healthy individuals and

does not produce exotoxin, while *Aspergillus* is ubiquitous in the environment and has only one known exotoxin. Below, I will briefly summarize the pathogenicity of *Klebsiella* and *Aspergillus* during pneumonia.

K. pneumoniae is a Gram-negative bacillus in the family *Enterobacteriaceae*. It is found in the gut microbiota of healthy individuals, but can colonize the nasopharynx upon bodily injury or prolonged hospital or health-care associated stay. In the nasopharynx, *K. pneumoniae* can enter the alveoli of the lungs by microaspiration where it can replicate and cause severe pneumonia resulting in host death. While various *Klebsiella* species contain type III fimbriae and nutrient acquisition molecules like siderophores, the two major components of *K. pneumoniae* pathogenicity are capsular polysaccharide (CPS) and lipopolysaccharide (endotoxin). The *Klebsiella* CPS allows the bacterium to subvert the immune system by evading mononuclear phagocyte-mediated phagocytosis (73,74). Likewise, endotoxin has been associated with lung injury during infection and has been implicated in *Klebsiella* pathogenicity (75,76). Thus, while *Klebsiella pneumoniae* lacks exotoxins, the capsular polysaccharide and endotoxin allow the bacterium to evade the immune system and also cause lung injury during infection.

Aspergillus fumigatus is a ubiquitous saprophytic fungus and one of the most prevalent molds in the world (77-79). *A. fumigatus* has only one known toxin known as gliotoxin, which has been shown to kill murine plasmacytoid DCs (80) but is not a major component of disease pathogenesis in humans (81). *Aspergillus fumigatus* causes invasive disease known as invasive pulmonary

aspergillosis only in the immunosuppressed host. As I will discuss in chapter 3, immune cell defects are required for establishment invasive pulmonary aspergillos. In light of this, the most important factor resulting in *Aspergillus* infection is its small, asexual spores commonly known as conidia. The conidia of *A. fumigatus* are particularly small compared to other species in the *Aspergillus* genus (77). The small diameter of *A. fumigatus* conidia allows the fungus to bypass the nasal turbinates and branched airways of the respiratory tract. This is an important component to *A. fumigatus* pathogenicity: the branched airways are a turbulent environment that normally deposit larger conidia on airway surface fluid, allowing for their removal by airway ciliary action. In bypassing this anatomical barrier, *A. fumigatus* arrives in the airways and alveoli of the lungs where they germinate and lose their proteinaceous, hydrophobic layer. Loss of this layer exposes inner cell wall polysaccharides consisting of β-glucan, mannan, chitin, and galactomannan (82,83) and ultimately results in the growth of branching multicellular hyphae, which then invade lung tissue and cause severe pneumonia and death (84). While inherently different, these data suggest that virulence factors across various microorganisms contribute to pathogenicity during pneumonia.

The mononuclear phagocyte system in response to Gram-negative pneumonia

Gram-negative bacterial pneumonia is a severe and life-threatening infection commonly caused by *Pseudomonas aeruginosa*, *Bordetella pertussis*, *Legionella pneumophila*, and *Klebsiella pneumoniae*. The involvement of mononuclear phagocytes is critical to host defense during Gram-negative pneumonia, albeit to varying degrees. For instance, numbers of mononuclear phagocytes increase during infection with *Klebsiella pneumoniae*, and cytokine expression is critical in promoting microenvironments conducive to bacterial clearance and resolution of infection (50,85-87). In contrast, *Legionella pneumophila* subverts the host immune response by infecting monocytes and macrophages (88). Herein, I will discuss the role of each lineage of mononuclear phagocytes in bacterial clearance, immune cell activation and function, and in the initiation of the adaptive immune response as part of host defense mechanisms during Gram-negative bacterial pneumonia.

Alveolar Macrophages in Gram-negative pneumonia:

Alveolar macrophages are arguably the most important mononuclear phagocyte in host defense against bacterial pneumonia, as depletion or reduced function of alveolar macrophages in the context of infection results in increased host mortality. Alveolar macrophages are the first leukocytes to come in contact with inhaled pathogens and are therefore critical in phagocytosis and bacterial killing during Gram-negative pneumonia. Below, I will focus on the role of alveolar macrophages in host defense against *Klebsiella*, *Pseudomonas*, and *Bordetella* pneumonia.

Depletion of alveolar macrophages by clodronate-loaded liposomes results in increased mortality and increased bacterial burden in the lungs and blood during *Klebsiella* pneumonia and in *Bordetella pertussis*, even with rapid recruitment of neutrophils (89,90). The absence of mononuclear phagocytes results in reduced neutrophil infiltration and poor host outcome during infection. During *Klebsiella* pneumonia, Broug-Holub et al. reported increased transcript levels of tumor necrosis factor alpha (TNF-α) and macrophage inflammatory protein-2 (MIP-2) and found that neutralization of these cytokines resulted in reduced neutrophil infiltration and reduced host defense during infection (89).

Alveolar macrophages require specific cytokines and TLR agonism to function properly in the context of infection. Macrophage inflammatory protein-1α (MIP-1α) has been implicated in host defense against *Klebsiella* pneumonia by promoting alveolar macrophage-mediated phagocytosis (91). MIP-1α is required for host defense against *K. pneumoniae* as evidenced by increased mortality and

reduced bacterial phagocytosis in mice deficient for MIP-1α. Along these same lines, alveolar macrophages and monocyte-derived macrophages both require the monocyte chemoattractant protein-1 (MCP-1) for the phagocytosis of apoptotic neutrophils in the context of *Pseudomonas* infection (92). *Pseudomonas aeruginosa* infection resulted in the rapid recruitment of neutrophils that died by apoptosis and caused deleterious lung inflammation. Amano et al. showed that alveolar macrophages efferocytosed apoptotic neutrophils in an MCP-1 dependent manner, ultimately resulting in resolution of lung inflammation and improved host outcome (92). In the context of *Bordetella pertussis*, the TLR adapter protein Mal is required for alveolar macrophage survival and antimicrobial ability, and its absence is associated with poor host outcome (93). Taken together, these data show that the absence or reduced function of alveolar macrophages during Gram-negative bacterial pneumonia results in poor host outcomes. Altogether, these data suggest that alveolar macrophages are critical mediators of bacterial phagocytosis and are critical in the clearance of apoptotic bodies associated with prolonged lung inflammation during Gram-negative pneumonia.

Additional studies implicate specific lipid mediators and proteins in alveolar macrophage function during Gram-negative bacterial pneumonia. In a model of *Klebsiella* pneumonia, multiple studies implicated a role for leukotrienes and surfactant proteins on alveolar macrophage phagocytosis and oxidative killing during infection (94-96). Leukotrienes are inflammatory lipid mediators produced by the 5-lipoxygenase pathway (5-LO) of arachidonic acid metabolism.

Early studies showed that 5-LO was required in host defense during *Klebsiella* pneumonia and that 5-LO knock-out mice exhibited marked increase in bacterial dissemination during pneumonia. Mechanistically, 5-LO was required for alveolar macrophage phagocytosis of *K. pneumonia* (94), and additional studies showed that administration of particular leukotrienes, specifically LTB4, LTC4, and 5-HETE, resulted in enhanced alveolar macrophage phagocytosis of *K. pneumoniae* with therapeutic implications (95). This idea was later supported by evidence showing that leukotrienes, specifically LTB4, not only enhanced alveolar macrophage mediated bacterial phagocytosis, but also alveolar macrophage oxidative killing of *Klebsiella* (96). Additional studies implicated surfactant proteins in alveolar macrophage function during Gram-negative pneumonia. Co-incubation of human alveolar macrophages with surfactant protein A (SP-A) attenuated *Klebsiella pneumoniae* growth compared to controls (97) and SP-A increased human, rat, and guinea pig alveolar macrophage killing of *Klebsiella* by stimulating phagocytosis and the production of reactive oxygen species (97,98). Taken together, these data suggest that alveolar macrophages are critical in host defense against Gram-negative bacterial pneumonia by enhancing phagocytosis and antimicrobial function.

Conventional DCs in Gram-negative pneumonia:

Compared to alveolar macrophages, the role of conventionally derived DCs in the context of Gram-negative pneumonia is less studied. However, their role in bacterial phagocytosis, recruitment of additional immune cell mediators, and in the initiation of the adaptive immune response has been assessed in the context of *Bordetella pertussis*, *Legionella pneumophila,* and to a lesser extent, in *Klebsiella pneumoniae*. CD103+ DCs and CD11b+ DCs, but not monocyte-derived DCs, were critical in mediating defense against *B. pertussis* by recruiting additional immune cells to control bacterial dissemination (99). Independent depletion of CD103+ and CD11b+ DCs resulted in increased bacterial burden and decreased cellular infiltration into the lungs during infection, suggesting that some conventional DCs are critical in host defense against *Bordetella*. The role of CD11b+ DCs has also been studied in the context of infection with *Legionella pneumophila*. These cDCs are critical in providing protective immune responses by priming naive T cells; cDCs restricted intracellular growth of *Legionella in vitro* and initiated protective T cell-mediated responses against this pathogen (100). Less is known about the role of conventionally-derived DCs in the context of *Klebsiella* pneumonia, but the dynamics of conventional dendritic cells during infection has been somewhat assessed (50,101). Multiple studies have shown that plasmacytoid DCs increased in the lungs during *Klebsiella* pneumonia compared to mock-infected controls, and this was associated with production of DC-subset-specific pro-inflammatory cytokines and interferons: pDCs produced type I interferon, CD103+ airway DCs producde type II interferons, IL-4, and IL-

13, and CD11b+ DCs produced IL-19 and IL-12p35. Similar to their roles in *Bordetella* and *Legionella*, CD103+ airway DCs and CD11b+ DCs were the most potent activators of naive CD4+ T cells (101).

Although the role of pDCs has not been fully examined in the context of *Klebsiella* pneumonia, their role has been described in the context of *B. pertussis* and *L. pneumophila* pneumonia. pDCs were rapidly recruited to the lungs of *Legionella*-infected mice, and depletion of pDCs resulted in increased bacterial burden during infection (102). Interestingly, the ability of pDCs to combat infection did not require type I interferon, perhaps suggesting an interferon-independent role of pDCs in *Legionella*, however, this remains unknown. In direct contrast to the protective role of pDCs during *Legionella* infection, recent work implicates a detrimental role of pDCs in *Bordetella pertussis.* T-helper 17 cells (Th17) and IL-17 family cytokines are critical in host defense against *B. pertussis* (103). Interestingly, the role of the pDC might be detrimental during infection due to the production of type I interferon, which has been shown to restrict Th17 cell differentiation (104). pDC-derived type I interferon inhibited Th17 cells resulting in poor host outcomes, and neutralizing type I interferon early in infection resulted in increased Th17 cell frequency associated with beneficial inflammation and decreased lung bacterial burden (104). These data suggest that pDCs have distinct roles in the context of infection and that this is dependent on the identity of the causative microorganism. Altogether, these findings suggest distinct functional roles for each cDC subset during Gram-negative pneumonia, but future studies should further assess the contribution of each conventional DC

during *Klebsiella* pneumonia, as well as the specific roles of CD103+ airway DCs during *Bordetella pertussis* and *Legionella pneumophila* pneumonia.

Monocytes in Gram-negative pneumonia:

Although it is accepted that the role of monocytes in Gram-negative pneumonia is to differentiate into monocyte-derived macrophage and DC effector cells, there is some evidence suggesting that monocytes themselves are critical mediators against Gram-negative pneumonia, independent of their role in producing macrophages and DCs. Depletion of CCR2+ monocytes resulted in delayed recovery from infection across 5 different strains of *K. pnuemoniae* (105). Interestingly, neutrophils were dispensable for host defense against all but one of the strains tested, suggesting that neutrophils and monocytes might have unique functions during infection. Notably, this study did not address the mechanisms by which monocytes conferred protection during infection. A reasonable explanation is that in the absence of CCR2+ monocytes, the host is more susceptible to infection due to a reduction in monocyte numbers resulting in inadequate bacterial clearance, or due to reduced numbers of differentiated effector macrophages and DCs. More recently, the same researchers suggested a potential mechanism by which monocytes conferred protection: enhanced immune function by monocyte-mediated production of TNF-α, which acted on innate lymphoid cells to enhance IL-17 production (106). IL-17 mediated protection against *Klebsiella* pneumonia by enhanced bacterial phagocytosis and killing, and monocyte depletion or TNF-α deficiency attenuated this IL-17-mediated resolution of pneumonia. The authors concluded that inflammatory monocytes and innate lymphoid cells engage in a positive feedback loop during *Klebsiella* pneumonia that promotes host defense during infection.

The role of monocytes in host defense against *Bordetella pertussis* and *Legionella pneumophila* has also been shown to involve bacterial clearance. Monocytes are critical mediators of bacterial clearance during *B. pertussis* (107). Pertussis toxin and LPS inhibited bacterial phagocytosis, but interestingly, did not impact bacterial killing. Similarly, IFN-γ activated human monocytes exerted antimicrobial effects and enhanced bacterial phagocytosis of *L. pneumophila* (108), altogether suggesting that monocytes have specific roles in host defense against Gram-negative pneumonias independent of their ability to propagate monocyte-derived macrophages and DCs.

Apart from their ability to phagocytose and kill bacteria, monocytes have been implicated in the initiation of the adaptive immune response against *B. pertussis*. Boschwitz et al. showed that infecting human monocytes with *B. pertussis* abrogated T cell proliferation caused by adenylate cyclase toxin and filamentous hemagglutinin (109), however, a mechanism by which monocytes affected T cell proliferation was not assessed. It is important to note that ablation or reduction of monocyte numbers directly impacts numbers of monocyte-derived DCs. Based on other studies implicating adenylate cyclase toxin in the death of mononuclear phagocytes (60-62) one possible explanation for this finding is that monocyte infection with *B. pertussis* resulted in abrogated T cell responses due to reduced numbers of antigen-presenting monocyte-derived DCs, the hypothesis being that adenylate cyclase toxin induces the death of monocytes which results in fewer cells able to differentiate into monocyte-derived DCs. While this hypothesis remains to be tested, the results suggest that monocytes

have distinct roles and functions in host defense against bacterial pneumonia independent of their role in propagating macrophages and DCs.

Monocyte-derived macrophages and DCs in Gram-negative pneumonia:

Apart from alveolar macrophages, monocyte-derived macrophages and monocyte-derived DCs should be considered the next most important mononuclear phagocytes against Gram-negative bacterial pneumonia due to their rapid recruitment and effector function during infection. For instance, monocyte-derived macrophages mediate defense against *B. pertussis* by phagocytosis and antimicrobial properties (110), which occurs intracellularly in the phagolysosome, and by oxidative killing by nitric oxide species (110-112). Interestingly, monocyte-derived macrophages control infection not only by enhancing bacterial clearance, but also by undergoing programmed cell death. In fact, the ability to undergo programmed cell death is critical in host defense against intracellular pathogens. For example, in *Legionella* pneumonia, monocyte-derived macrophages sensed cytosolic flagellin in a TLR5-independent pathway that leads to rapid caspase-1-dependent cell death, which was critical in restricting bacterial growth (88). Similarly, flagellin was required for NLRC4-dependent responses to *Legionella*, which triggered caspase-1-dependent death associated with restriction of bacterial growth in monocyte-derived macrophages (113), suggesting that immune cell death can be critical to host defense in certain infections. Overall, monocyte-derived macrophages and DCs impart host defense during Gram-negative pneumonia in a variety of ways. Below, I will discuss the contribution of bacterial clearance as well as immune cell recruitment, activation, and initiation of the adaptive response in monocyte-derived macrophage and DC-mediated host defense against Gram-negative

pneumonia.

Monocyte-derived macrophages and DCs are critical in bacterial clearance during Gram-negative pneumonia. In the context of *Pseudomonas aeruginosa*, pre-treating monocyte-derived macrophages with exogenous neutrophil-derived myeloperoxidase (MPO) enhanced the microbicidal activity of the macrophages, suggesting a potentially useful therapeutic application (114). Similarly, treatment of monocyte-derived macrophages with GM-CSF and IFN-γ resulted in enhanced antimicrobial function against *Pseudomonas* in the context of Cystic Fibrosis (115). Additionally, triggering receptor expressed on myeloid cells-2 (TREM-2), a cell surface receptor on myeloid lineage cells including macrophages and DCs, was required for monocyte-derived macrophage mediated killing of *Pseudomonas*, but is dispensable for bacterial phagocytosis (116). Enhanced killing of bacteria by TREM-2 agonism was associated with enhanced production of reactive oxygen species (ROS), altogether suggesting that monocyte-derived macrophages and their effectors are critical mediators of bacterial clearance during *Pseudomonas* pneumonia.

Others have shown that monocyte-derived macrophages require TNF-α and type I interferon signaling to control bacterial growth (117). Monocyte-derived macrophages lacking the cytosolic pathogen-recognition receptor Naip5 were unable to control bacterial growth, suggesting that certain cytokines and interferons are required for monocyte-derived macrophage mediated host defense against *Legionella*. Along these same lines, toll-like receptor 9 (TLR9) knockout mice infected with *Legionella pneumophila* exhibited increased

mortality and bacterial burden during infection compared to controls (118), which is additionally supported by others (119), altogether suggesting that monocyte-derived macrophages are critical in bacterial clearance in Gram-negative pneumonia.

Macrophage migration inhibitory factor (MIF) is required for host defense during infection by mediating bacterial killing during *K. pneumoniae* (120). MIF was required for monocyte-derived macrophage bacterial killing, but was dispensable for bacterial phagocytosis, similar to the finding of TREM-2 in *Pseudomonas*. Killing deficiency could be due to decreased monocyte-derived macrophage activation, as evidenced by decreased NFκB activity and TNF-α production in stimulated MIF-deficient cells. Additional studies suggest similar mechanisms for monocyte-derived DCs in the context of *Klebsiella* pneumonia. Using bacterial mutants for *Klebsiella* capsular polysaccharide (CPS) and LPS O antigen, Evrard et al. showed that CPS allows bacteria to evade phagocytosis, which was associated with reduced cytokine production and monocyte-derived DC maturation (121). *In vitro*, LPS-stimulated DCs were more activated as measured by increased production of IL-12, TNF-α, and IL-10 in response to *K. pneumoniae*. These data suggest that activated monocyte-derived DCs are critical in phagocytosis and cytokine production against *Klebsiella pneumoniae*, however, the authors do not address the implications of this in host defense against infection. While these studies might suggest that monocyte-derived cells require cytokine activation to mount a protective response during infection, this is not always the case; decreased cytokine production does not always correlate

with worse outcomes during Gram-negative pneumonia.

In some cases, cytokine production can be associated with detrimental or prolonged inflammation associated with worse host outcome during Gram-negative pneumonia. Studies using *Pseudomonas* showed that infection resulted in assembly of the NLRP3 inflammasome in human monocyte-derived macrophages, resulting in the secretion of caspase-1 and IL-1β (122). Importantly, activation of this inflammasome led to a reduction in monocyte-derived macrophage mediated bacterial killing without affecting the production of antimicrobial peptides, reactive oxygen species, or nitric oxide. Most importantly, IL-1β decreased the macrophage-mediated killing of *Pseudomonas*, suggesting that cytokine production did not necessarily result in protective host responses during Gram-negative pneumonia. Additionally, it has recently been suggested that monocyte-derived macrophages are required to attenuate IL-1β production associated with detrimental inflammation and lung injury during *Klebsiella* pneumonia (123). Monocyte-derived macrophages reduced IL-1β associated lung injury by producing the IL-1r antagonist IL-1ra. The administration of exogenous IL-1ra and adoptive transfer of IL-1ra$^{+/+}$ mononuclear cells, but not IL-1r$^{-/-}$ cells, attenuated alveolar inflammation, epithelial cell apoptosis, and loss of barrier function associated with lung injury during infection (123). Altogether, these results suggest that there is a balance between protective and deleterious inflammation, and monocyte-derived macrophages are critical cellular mediators in their capacity to control both types of inflammation during *Klebsiella* pneumonia.

Immune cell recruitment and activity have also been implicated in host defense against Gram-negative bacteria, albeit by disparate means. In the context of *Pseudomonas* pneumonia, specific members of the matrix metalloproteinase (MMP) family have implications in the immune response to infection. MMP-28 was required for the dynamics of monocyte-derived macrophage-recruitment during *Pseudomonas* pneumonia (124). MMP-28 knock-out mice had enhanced monocyte-derived macrophage influx to the lungs during infection with *Pseudomonas* which was associated with decreased bacterial burden compared to controls. Along the same lines, the transcription factor C/EBTδ was required for myeloid lineage-mediated host defense during *Klebsiella* pneumonia, but did not affect the ability of these cells to produce cytokines or oxidative burst *in vitro*, suggesting that a mechanism independent of immune cell activation is critical in host defense during infection (125). F4/80+ macrophages were reduced in the lungs of C/EBTδ knockout mice during infection compared to controls, suggesting a role for C/EBTδ in maintaining macrophage populations associated with host protection during *Klebsiella* pneumonia. Altogether, these data suggest that the maintenance, recruitment, and activity of monocyte-derived macrophages and DCs are critical in host defense against Gram-negative pneumonia.

Monocyte-derived DCs have also been implicated in host defense against Gram-negative pneumonia by initiating the adaptive immune response. Monocyte-derived DCs were involved in the effective induction of adaptive T-cell-mediated immunity during *Legionella* pneumonia (126). CX_3CL1-expressing

monocyte-derived DCs mediated host defense against *L. pneumophila* by promoting protective Th1 responses. Likewise, in the context of *Bordetella pertussis*, monocyte-derived DCs are critical in the initiation of protective Th1 responses during infection (127). Upon bacterial phagocytosis, monocyte-derived DCs matured and exhibited antigen-presenting cell functions, suggesting implications for *B. pertussis* vaccine studies (103,127). Taken together, these results suggest that monocyte-derived macrophages and DCs are critical in host defenses against Gram-negative pneumonia. A summary of mononuclear phagocytes in Gram-negative pneumonia can be found in Table 1.1.

Organism	Bacterial clearance	Immune cell recruitment & activation	Programmed cell death
Klebsiella pneumoniae	Alveolar Mφ, Monocyte-derived Mφ	pDC, Monocytes, Monocyte-derived DC	
Pseudomonas aeruginosa	Monocyte-derived Mφ	Monocyte-derived Mφ	
Bordetella pertussis	Alveolar Mφ, Monocytes, Monocyte-derived Mφ	CD103+ airway DC, CD11b+ DC, pDC, Monocyte	
Legionella pneumophila	Monocyte-derived Mφ	CD11b+ DC, Monocyte-derived DC	Monocyte-derived Mφ pyroptosis

Table 1.1. Mononuclear phagocytes in host defense against Gram-negative pneumonia. Monocyte lineage cells and alveolar macrophages are primarily involved in bacterial clearance by phagocytosis and killing. Conventional DCs and to a lesser degree, monocyte lineage cells, are involved with immune activation and initiation of the adaptive response. In the context of *Legionella* pneumonia, monocyte lineage cell pyroptosis mediates protection during infection.

The Mononuclear phagocyte system in response to Gram-positive pneumonia

Gram-positive bacterial pneumonia is predominantly caused by *Streptococcus pneumoniae* (128), but *Staphylococcus aureus* and *Streptococcus pyogenes* (Group A Strep) have also been implicated in causing disease (129-133) In contrast to the majority of the Gram-negative bacteria previously discussed, these Gram-positive bacteria normally colonize the host. For example, *S. pneumoniae* and *S. aureus* are normally resident in the nasopharynx, but can be aspirated into the lungs and cause disease in a manner similar to *Klebsiella*.

The involvement of the mononuclear phagocyte system in host defenses against Gram-positive pneumonia has been somewhat assessed. The critical mediators in infection have been shown to predominantly involve the tissue-resident macrophage and monocyte lineages within the mononuclear phagocyte system, while substantially less is known about the conventional DC lineage (134). In stark contrast to Gram-negative pneumonia, infection by *S. pneumoniae*, *S. pyogenes*, and *S. aureus* usually occurs by subversion of host immune responses associated with phagolysosome evasion and initiation of programmed cell death of mononuclear phagocytes and neutrophils (135,136). To combat infection by Gram-positive organisms, mononuclear phagocytes impart defenses against Gram-positive pneumonia by bacterial clearance, immune cell activation and function, and initiation of the adaptive immune response in a manner similar to but distinct from Gram-negative pneumonia.

Herein, I will summarize what is known about the role of each lineage of mononuclear phagocytes in host defense against Gram-positive pneumonia.

Alveolar Macrophages in Gram-positive pneumonia:

The role of alveolar macrophages in host defense against Gram-positive pneumonia is similar to the role of alveolar macrophages in Gram-negative pneumonia in that the absence of these cells results in significantly worse outcomes during infection (137-140). Studies on alveolar macrophages in Gram-positive pneumonia have focused on the impact of leukocyte activation by pattern recognition receptors and cytokine production on phagocytic capacity, antimicrobial function, and enhanced leukocyte recruitment culminating in bacterial clearance during infection.

Recognition and opsonization of bacteria by alveolar macrophages is a critical aspect of host defense during Gram-positive pneumonia. Agonism of pattern recognition receptors, specifically TLR2, has been shown to be critical in alveolar macrophage-mediated defense during infection. In a model of *S. pneumoniae* infection, TLR2 stimulation with synthetic macrophage-activating lipopeptide-2 (MALP-2) increased levels of the chemokine CCL5 which resulted in enhanced leukocyte recruitment and reduced levels of the anti-inflammatory cytokine IL-10 (141), ultimately resulting in increased host survival during infection. Likewise, TLR2 recognition of *S. pneumoniae* resulted in production of TNFα, IL-1β, and IL-6 during pneumonia, which was associated with NFκB activation and host defense during infection (142). Along these same lines, the NFκB protein RelA has been implicated in alveolar macrophage-mediated host defenses against Gram-positive pneumonia by influencing TLR2 induction and cytokine production associated with enhanced host defense, but its effect is

evident only at early time points (143). Surfactant proteins have also been implicated in alveolar macrophage-mediated bacterial clearance. In a model of *S. aureus* pneumonia, opsonization of the bacteria by lung surfactant protein A mediated recognition and enhanced antimicrobial function by alveolar macrophages (144). These results suggest that recognition of bacteria by alveolar macrophages results in host defense against Gram-positive pneumonia. In some cases, this protection is mediated by pro-inflammatory cytokine production (142), though the specific mechanisms by which protection is conferred is not addressed.

Cytokine production during Gram-positive pneumonia can impact alveolar macrophage activation and function in a variety of ways. In contrast to its effect on monocyte-derived macrophages in Gram-negative pneumonia, the role of IFN-γ on alveolar macrophages during Gram-positive pneumonia has been shown to be both beneficial and detrimental in host defense during infection. Researchers have shown that exposure of alveolar macrophages to IFN-γ during infection with *S. pneumoniae* resulted in decreased phagocytic capacity and reduced antimicrobial function of alveolar macrophages (145), while others showed that the detrimental role of IFN-γ on alveolar macrophages occurred only at early time points post-infection (146). These authors showed that early exposure of alveolar macrophages to IFN-γ resulted in reduced bacterial internalization, but that this effect diminished over time. These data suggest that IFN-γ has distinct, time-dependent effects on alveolar macrophage-mediated host defense against Gram-positive pneumonia. Altogether, these data suggest

that bacterial recognition and cytokine expression is critical to alveolar macrophage-mediated host defenses during Gram-positive pneumonia, but that the initiation and duration of cytokine expression greatly contribute to outcome during infection.

Along these lines, it is important to note that pro-inflammatory responses to Gram-positive pneumonia are not always beneficial to host defenses. In fact, apoptosis of alveolar macrophages has been shown to be critical in promoting host defense during infection. One group showed that macrophage migration inhibitory factor (MIF) was detrimental during *Streptococcus pneumoniae* infection due in large part to its role in inhibiting the efflux of macrophages during infection (147,148). Although MIF expression in the nasopharynx is responsible for retaining *S. pneumoniae* and preventing its dissemination into the lungs, MIF expression was detrimental in the lungs by promoting prolonged inflammation and promoting the extended survival of alveolar macrophages. These data suggest that the anti-inflammatory environment created by apoptotic alveolar macrophage is beneficial in host defenses during infection. Similarly, decreased alveolar macrophage apoptosis resulted in increased pulmonary inflammation and worse outcome during infection in a murine model of *S. pneumoniae* infection (149). It is important to note, however, that increased apoptosis of alveolar macrophages does not always result in protective host responses during pulmonary infection with *S. pneumoniae*. Should leukocyte apoptosis increase while phagocytic capacity decreases, this could result in prolonged inflammation due to failure to clear dead cells, as suggested in other studies (139).

Importantly, the impact of alveolar macrophage death during infection should be considered cell-specific and not a general mechanism of mononuclear phagocyte-mediated defense during infection, as it does not always result in protective host responses.

Conventional DCs in Gram-positive pneumonia:

The role of cDCs in the context of Gram-positive pneumonia is unknown, but the role of pDCs during infection has been somewhat examined in the context of *S. aureus* pneumonia, albeit indirectly (150). For example, it is well known that pDCs are the major producers of type I interferon and that type I interferons are detrimental to host defense against *S. aureus* pneumonia (151). Additional studies have shown that *S. aureus* infection activates TLR9 signaling resulting in type I interferon production associated with increased bacterial burden during infection (152). These data implicate detrimental impacts of pDCs during Gram-positive pneumonia. Future studies should assess the contribution of CD11b+ DCs and CD103+ airways DCs in host defense against Gram-positive pneumonia.

Monocytes in Gram-positive pneumonia:

Similar to their role in Gram-negative pneumonia, monocytes in Gram-positive pneumonia are not only important in propagating monocyte-derived macrophages and DCs, but might also be involved in bacterial clearance. In a murine model of *S. pneumoniae* pneumonia, researchers showed that monocytes rapidly egressed from the bone marrow and into the lungs (153), which was associated with host defense during infection (154). This rapid recruitment was associated with nitric oxide release and enhanced bacterial clearance (155), though it is unclear if this bacterial clearance resulted from monocyte phagocytosis or from the generation of monocyte-derived macrophages and DCs and their resulting effector function. In a model of *S. pneumoniae* pneumonia, researchers showed that CCL2 was required for controlling bacterial dissemination and resulting sepsis during infection, which was associated with reduced recruitment of monocyte-derived macrophages during infection (156). Altogether, these data suggest that monocytes are critical in host defense against Gram-positive pneumonia by producing monocyte-derived macrophages, and to a lesser extent, in their ability to phagocytose bacteria. Because the differentiation of monocytes into macrophages and DCs is critical to host defenses during infection, additional studies should further assess the direct contribution of monocytes in bacterial clearance during Gram-positive pneumonia.

Monocyte-derived macrophages and DCs in Gram-positive pneumonia:

The role of monocyte-derived macrophages and monocyte-derived DCs in host defense against Gram-positive pneumonia has been assessed in the contexts of antimicrobial function and bacterial clearance, immune cell function, as well as in the initiation of the adaptive immune response. IL-1β has been implicated in host defense against *S. pneumoniae* by enhancing antimicrobial function (157). Mice deficient in IL-1r had reduced numbers of neutrophils and macrophages associated with increased bacterial colonization, and intranasal instillation of IL-1β promoted monocyte-derived macrophage mediated bacterial clearance. These results suggest that monocyte-derived macrophages are critical mediators in host defense against Gram-positive pneumonia, which is further supported by additional studies in the context of *S. pyogenes* (158) and *S. aureus* (159). Altogether, these results suggest that monocyte-derived macrophages are critical in bacterial clearance by cytokine activation, culminating in host defense against Gram-positive pneumonia.

Interestingly, and in stark contrast to the role of immune cell function in Gram-negative pneumonia, programmed cell death of monocyte-derived macrophages in Gram-positive pneumonia is associated with increased severity of infection in mouse models of *S. aureus* pneumonia (69,70,160,161). Infection with *S. aureus* resulted in NLRP3 inflammasome activation and subsequent pyroptosis of monocyte-derived macrophages, while caspase-3 activation and subsequent apoptosis of monocyte-derived macrophages resulted in bacterial escape from the phagosome and subsequent dissemination during *S. aureus*

pneumonia (130). Likewise, monocyte-derived macrophage and alveolar macrophage death by necroptosis resulted in increased lung damage durin *S. aureus* pneumonia (70). Taken together, these data suggest that programmed cell death, and specifically pyroptosis, apoptosis, and necroptosis, is detrimental during Gram-positive pneumonia. This is particularly intriguing given the beneficial impact of apoptosis on alveolar macrophages during Gram-positive pneumonia, and in comparison to the beneficial impact of apoptosis in Gram-negative pneumonia. This is especially interesting given that apoptosis is associated with reduced inflammation, and researchers have shown that reduced lung inflammation during *S. aureus* pneumonia resulted in improved outcome during infection (162,163). Future studies should further assess not only the additional contributions of monocyte-derived macrophages in host defense against Gram-positive pneumonia, but also the mechanisms and dynamics of programmed cell death during specific Gram-positive pneumonias.

Monocyte-derived DCs have been shown to be critical in host defense against Gram-positive pneumonia by mounting effective adaptive immune responses during infection. In the context of *S. pneumoniae*, monocyte-derived DCs matured after interaction with bacteria and initiated productive Th-1 responses associated with host protection (164). Additionally, monocyte-derived DCs engulfed *S. aureus* and subsequently upregulated the expression of costimulatory molecules which was associated with proliferation and IFN-γ production in CD4+ and CD8+ T cells (165). These data suggest that monocyte-derived DCs can respond to *S. aureus* and subsequently mature and induce

protective Th1 adaptive immune responses during Gram-positive pneumonia, which is further supported by additional studies (40,166). Taken together, these results suggest that monocyte-derived DCs are critical in host defense against Gram-positive pneumonia by initiating protective adaptive immune responses during infection. Altogether, these results suggest that monocyte-derived macrophages and DCs are required for host defenses in Gram-positive pneumonia by promoting the clearance of bacteria and in initiating protective adaptive responses. A summary of mononuclear phagocytes in Gram-positive pneumonia can be found in Table 1.2.

Organism	Bacterial clearance	Immune cell recruitment & activation	Programmed cell death
Streptococcus pneumoniae	Alveolar Mφ, Monocytes, Monocyte-derived Mφ, Monocyte-derived DC	Alveolar Mφ, Monocyte-derived DC	Alveolar Mφ
Streptococcus pyogenes	Monocyte-derived Mφ	Monocyte-derived Mφ	Monocyte-derived Mφ pyroptosis
Staphylococcus aureus	Alveolar Mφ	pDC	Monocyte-derived Mφ Pyroptosis, Apoptosis, Necroptosis,

Table 1.2. Mononuclear phagocytes in host defense against Gram-positive pneumonia. Monocyte lineage cells and alveolar macrophages are primarily involved in bacterial clearance by phagocytosis and killing. All three lineages are involved in immune activation or the initiation of the adaptive response. In stark contrast to Gram-negative pneumonia, programmed cell death in the context of Gram-positive pneumonia is beneficial or deleterious depending on the causative organism. Alveolar macrophage apoptosis and monocyte-derived macrophage pyroptosis against *Streptococcus* is protective to the host during infection. Alternatively, alveolar macrophage necroptosis and monocyte-derived macrophage necroptosis, apoptosis, and pyroptosis are all associated with poor host outcomes during *Staphylococcus aureus* pneumonia.

Mononuclear phagocytes in response to *Aspergillus fumigatus*

Invasive pulmonary aspergillosis is a severe and life-threatening pneumonia most commonly caused by the fungus *Aspergillus fumigatus*. Invasive pulmonary aspergillosis develops in immunocompromised individuals and most commonly in individuals with qualitative or quantitative defects in neutrophils resulting from chemotherapy and corticosteroid treatments. Invasive aspergillosis is the second most common cause of health-care associated fungal infections and carries the greatest incremental hospitalization cost of other systemic fungal infections (167,168). Even with treatment, mortality rates for those infected are between 30-50% (169-171), underscoring the need to better understand the host response to infection and determine additional risk factors associated with the onset of disease. As I discussed above, *Aspergillus* is an opportunistic pathogen with few typical virulence factors, and thus, the host response to the organism is the most important factor in whether the host will clear the fungus or develop disease. The importance of neutrophils in clearing *Aspergillus* conidia and preventing disease is well documented (172-174), but in the context of neutropenia and in defense against invasive disease, cells in the mononuclear phagocyte system are critical in combating infection and protecting the host. The role of mononuclear phagocytes during invasive pulmonary aspergillosis has been extensively reviewed previously (83). Thus, I will summarize the role of mononuclear phagocytes in host defense against invasive pulmonary aspergillosis.

Alveolar Macrophages against *A. fumigatus*

As in bacterial pneumonia, alveolar macrophages are the first leukocytes that come in contact with inhaled *A*. *fumigatus* and mediate fungal killing by phagocytosis and NADPH oxidase (175,176). Phagocytosis required actin rearrangement and PI3K activation, while conidial killing required acidification of the phagolysosome (177). Furthermore, reactive oxygen species are additionally required for fungal killing. In the absence of components of the NADPH oxidase complex, alveolar macrophages were unable to kill conidia as efficiently as their wild-type counterparts (175,178). Interestingly, reactive nitrogen intermediates were not required for alveolar macrophage-mediated defense against *A. fumigatus* (179,180), suggesting a specific role of reactive oxygen species in alveolar macrophage mediated defense against *A. fumigatus*. Altogether, these data suggest that alveolar macrophages are important mediators in host defense against *A. fumigatus*.

Conventional DCs against *A. fumigatus*

Conventional DCs mediate fungal clearance, produce anti-inflammatory cytokines following hyphal clearance, and initiate adaptive immunity in response to *A. fumigatus*. Lung CD11b+ DCs produced the inflammatory cytokines TNF-α and IL-12p70 following conidial phagocytosis, but produced anti-inflammatory cytokines IL-4 and IL-10 upon engulfment of hyphae (181,182). These data suggest that CD11b+ DCs are critical in fungal clearance and produce inflammatory cytokines following engulfment of hyphae, perhaps to initiate tissue repair mechanisms. In addition, pDCs phagocytosed swollen conidia and had direct antifungal activity against *A. fumigatus* hyphae (80). Upon interaction with *A. fumigatus* hyphae, plasmacytoid DCs released TNF-α and IFN-α, and although the downstream effect of these cytokines were not assessed, previous studies reported TNF-α as a critical cytokine in defense against invasive aspergillosis (183,184). Furthermore, CD11b+ DCs initiate the adaptive immune response following interaction with *A. fumigatus*. Within three days following challenge with conidia or hyphae, CD11b+ DCs and monocyte-derived DCs matured and primed CD4+ T cells (181,185). Taken together, these data suggest that conventional DCs are critical mediators in host defense against *A. fumigatus*.

Monocytes and their progeny against *A. fumigatus*

Monocytes and monocytic progeny mediate phagocytosis of resting conidia and produce pro-inflammatory cytokines in response to *A. fumigatus*. While the role of monocyte-derived macrophages has not been directly assessed in the context of *A. fumigatus* pneumonia, the roles of monocytes and monocyte-derived DCs have been well described. Human monocytes phagocytosed resting conidia and produced TNF-α in response to germinating conidia, but only CD14+ CD16- human monocytes were able to inhibit and restrict conidial germination (186). Similar to findings in bacterial pneumonia, the absence of CCR2+ monocytes during infection resulted in abrogated adaptive responses associated with reduced conidial transport from the lungs to the draining lymph nodes during infection and resulted in reduced CD4+ T cell responses (187). It is unclear if this occurred due to monocytes themselves or was artifact of reduced numbers of monocyte-derived dendritic cells. Given the role of monocyte-derived DCs in initiating the adaptive immune response during infection, it is likely that these findings resulted from reduced numbers of monocyte-derived DCs, and while this remains to be directly tested, additional works suggests this could be a possible mechanism. Depletion of monocyte-derived DCs during invasive pulmonary aspergillosis resulted in increased lung fungal burden (183), suggesting that monocyte-derived DCs are critical mediators in host defense during invasive aspergillosis. A summary of mononuclear phagocytes against *Aspergillus fumigatus* can be found in Table 1.3.

Organism	Fungal clearance	Immune cell recruitment & activation
Aspergillus fumigatus	Alveolar Mφ, Monocytes, Monocyte-derived Mφ Monocyte-derived DC	CD11b+ DC Monocytes Monocyte-derived DC

Table 1.3. Mononuclear phagocytes in host defense against *Aspergillus fumigatus*. All three lineages of mononuclear phagocytes are implicated in fungal clearance. Conventional DCs and monocyte lineage cells are involved in immune cell recruitment and activation.

Conclusions

Bacterial pneumonia and invasive pulmonary aspergillosis are serious and life-threatening infections commonly caused by Gram-negative and Gram-positive organisms including *Bordetella, Legionella, Pseudomonas, Klebsiella, Streptococcus,* and *Staphylococcus* and the fungus *Aspergillus fumigatus*. In light of increasing antibiotic resistance among the causative organisms and ineffective treatment options for invasive aspergillosis, increased knowledge and understanding of the host response during pneumonia is critical to inform future drug discoveries and potential host-focused therapies to combat infection. Cells in the mononuclear phagocyte system are critical mediators in host defense against pneumonia. Mononuclear phagocytes mediate host defense in a variety of ways, most commonly resulting in bacterial or fungal clearance by phagocytosis and oxidative and non-oxidative killing, immune cell maintenance, recruitment, and activation, as well as in programmed cell death in the context of specific bacterial infections. Henceforth, the focus on these two topics will diverge: in chapter 2, I will focus on mononuclear phagocytes in bacterial pneumonia, and in chapter 3, I will discuss the role of the gut microbiota in host defense against invasive pulmonary aspergillosis. As I have previously discussed, mononuclear phagocyte effector function has been well described in the context of bacterial pneumonia, but the factors involved in the generation, development, and survival of mononuclear phagocytes in the context of bacterial pneumonia are unknown. In chapter 2, I will assess the role of M-CSF in the survival and function of mononuclear phagocytes during *Klebsiella* pneumonia.

1.2 The role of the microbiota in lung immunity

Abstract

The gut microbiota consists of bacteria, fungi, and viruses that reside in the intestine in a symbiotic relationship with the host. Over the last 50 years, the composition of the gut microbiota has been shown to influence the host response to local infection or diseases such as irritable bowel disease. This has in part inspired the study of other mucosal sites such as the lungs, and the impact of the resident microbiota on local immunity. Interestingly, the composition of the gut microbiota has recently been implicated in influencing immunity in peripheral tissue including the lungs, suggesting more far-reaching effects of the gut microbiota on immunity than previously appreciated. Below, I will discuss the contributions of the resident lung microbiota on lung immunity, as well as the impact of the gut microbiota on host immune responses to disease state and lung infections.

Microbiota and the host

It is well known that host mucosal surfaces harbor millions of microorganisms and viruses. These microbes and viruses are commonly referred to as the microbiota when referring to the composition of microorganisms, and as the microbiome when referring to the genomes of these microorganisms. The most well-known, well-studied, and most diverse microbiota reside in the gastrointestinal tract and are often referred to as the gut microbiota (188,189). The relationship between the gut microbiota and the host is symbiotic: the host provides environmental and nutritional requirements for the microbiota, and in turn, the microbiota aid in digestion and provide essential dietary metabolites to the host. In addition, research over the last 50 years has implicated the gut microbiota in shaping the local host immune response: examples of this include autoimmune diseases such as irritable bowel diseases like Crohn's Disease, as well as immunity to gastrointestinal infections like pathogenic *E. coli* and *C. difficile*. Data published in the last 10 years have implicated gut microbiota imbalance termed "dysbiosis," induced by broad-spectrum antibiotic treatment, as the major contributor to disease state and susceptibility to intestinal infections (190). With the recent advent of genomic deep-sequencing and sophisticated computing tools, the ability to identify one specific genus or species of the microbiota and its influence on the intestinal immune response has been possible. While there is rich literature on the various ways in which the gut microbiota can influence the local immune response, current gaps in our knowledge are how extra-intestinal microbiota can influence local immunity, as

well as how the gut microbiota itself can influence immunity in peripheral tissue.

Segmented Filamentous Bacteria

Segmented Filamentous Bacteria are found in the gut microbiota of certain populations of vertebrates and invertebrates and greatly shape the host immune response during steady state and disease. SFB are Gram-positive spore-forming obligate anaerobes in the family *Clostridiaceae* (191). These bacteria were originally named for their morphology and not defined taxonomically due to the inability to culture these bacteria *in vitro* (192). However, the creation of SFB mono-associated mice has been a useful tool in further characterizing SFB (193,194). Using 16S rRNA, Snel et al. found that SFB from different host species were not identical and suggested the name *Candidatus arthromitus* for rodent SFB (191), however, these bacteria are still commonly referred to only as SFB, regardless of their origin. In recent years, it has become apparent that SFB are present in the gut microbiota of mice from certain commercial vendors and notably absent from others (195-197), and that there are greater than 400 different microbial taxa between genetically identical C57bl/6 mice purchased from different commercial vendors (195). These results have prompted researchers to take special care in comparing and interpreting results from experiments that use different cohorts of mice, and likewise, in interpreting results from studies using knock-out mice created in academic institutions compared to control mice purchased from commercial vendors.

SFB are found in the intestines of some populations of rodents, chickens, domestic pets and humans (191,192). SFB predominantly colonize the small intestine of mice, but are found in smaller numbers in the cecum (198). In the

murine intestine, SFB tightly adhere to small intestine epithelial cells in the ileum (199,200) where they shape host immunity by specifically inducing Th17 cells (195,198,201-203), and are critical in host defense against certain intestinal pathogens (195,204).

SFB colonization in mice peaks at day 28 after birth and steadily declines until at least day 70 (205). Colonization is a multi-step process that occurs in the small intestine and has been reviewed in detail (206). SFB spores germinate and form single-celled bacteria that use flagella to reach the epithelial surface of the terminal ileum. These bacteria induce actin rearrangement to imbed themselves in the epithelial cells overlaying peyer's patches and M-cells. After attachment, SFB grow to 5 μM in length before dividing and forming primary filaments (200). Primary filaments continue to grow from their free end before beginning a second round of segmentation. At this stage, SFB divides each primary segment to form a larger mother cell that engulfs a smaller daughter cell. This daughter cell further divides within the mother cell to produce additional daughter cells. This process results in two fates: under favorable growth conditions, the mother cell falls away and releases the inner daughter cells, allowing for the colonization process to begin again. Under less favorable conditions, the daughter cells within the mother cell are surrounded by a spore coat and will develop into a mature spore within the mother cell. These spores can then be released from the filament and transmitted to a new host (206).

Until recently, SFB could not be cultured, but in 2015, Schnupf et al. cultured SFB *in vitro* for the first time. The resulting *in vitro*-derived SFB

colonized murine cecum rather than small intestine, resulting in reduced cytokine expression compared to mice colonized with fecal-derived SFB from SFB-colonized mice (206). Thus, the use of fecal-derived SFB rather than *in vitro* derived SFB should be used to assess the role of SFB in host immunity. Furthermore, the use of SFB mono-associated stool from SFB gnotobiotic mice is a powerful tool to directly assess the contribution of SFB to the host immune response.

The lung microbiota in lung immunity

Aside from the gastrointestinal tract, there are several other mucosal surfaces known to have site-specific microbiota, including the skin, vaginal tract, and the oral and nasal cavities. The lungs are another notable mucosal tissue, but until recently, they were thought to be devoid of microbiota and deemed sterile (207) In the last 5-10 years, using culture-independent methods, researchers have shown that the lungs, like other mucosal sites, also harbor resident microbiota (208,209). The emerging field of the lung microbiota is currently narrow, but the three major aspects of this field include: 1) identifying microorganisms that comprise the resident microbiota by culture-independent methods (210), 2) establishing methods to delineate between lung-resident microbiota and oral microbiota (211,212) and 3) identifying the role of the lung microbiota in susceptibility to pulmonary diseases from COPD to asthma (213,214)

Unlike the trillions of microorganisms that comprise the gut microbiota (215), there are far fewer microorganisms in the lung microbiota: the number of lung microbiota is estimated to be in the order of 10^3 per cm^2 (209). Thus, the lung microbiota is comparatively smaller than the gut microbiota. This might mean that, while the gut microbiota can influence immunity locally as well as peripherally, and influences autoimmunity as well as outcome during infection, the lung microbiota might only influence local immunity, and might influence local immunity only in specific diseases like asthma. However, this remains to be studied. While the role of the lung microbiota in lung disease continues to be

defined, yet another field is emerging involving the ability of tissue-specific microbiota to influence the immune response in peripheral tissue. This hypothesis is currently being assessed in two fields: 1) the ability of the gut microbiota to influence brain immunity and neurological behavior (216) and 2) the ability of the gut microbiota to influence lung immunity. Herein, I will provide an overview of the latter.

The gut microbiota and its influence in lung immunity

The hypothesis that the composition of the gut microbiota influences lung immunity has emerged only in the last few years. Within this field, most of the literature has focused on how the composition of the gut microbiota influences host susceptibility to allergic asthma, the main focus of which has been on two topics: 1) how the absence or ablation of the gut microbiota influences susceptibility to allergic asthma, and 2) identifying which specific microorganisms influence susceptibility to disease state.

To assess the role of the gut microbiota in susceptibility to allergic asthma, Noverr et al. showed that oral antibiotic treatment resulted in overgrowth of the fungus *Candida albicans* in a mouse model, and that upon subsequent challenge with *A. fumigatus* conidia, the host developed allergic asthma (217). This effect was independent of differences in host genetics, altogether suggesting that the gut microbiota influence immunity to allergic asthma. It was later suggested that the mechanism underlying this phenomenon was due to M1 versus M2 polarization in macrophages (218), which was supported by later work utilizing germ-free mouse models.

Germ-free mouse models and antibiotic treatment have allowed researchers to assess the contribution of the gut microbiota on the development of allergic asthma. Herbst et al. tested this hypothesis by inducing allergic asthma in germ free, specific pathogen free, or recolonized mice. The absence of gut microbiota resulted in aberrant leukocyte homing and increased production of local Th2 family cytokines, which were highly associated with the allergic

response (219). Additionally, antibiotic treatment resulted in similar outcomes in that treatment of neonatal mice with vancomycin, but not streptomycin, resulted in changes in gut microbiota and subsequent susceptibility to allergic asthma (220,221). These studies show that the absence of the gut microbiota, or depletion by treatment with antibiotics, is sufficient to promote allergic asthma. Important work stemming from these earlier reports has defined the roles of specific microorganisms in mediating protection or susceptibility to allergic asthma.

The addition of *Lactobacillus johnsonii* to the gut microbiota protected against dust-allergy induced airway pathology in a mouse model and this was associated with significantly reduced Th2 family cytokines in the lung, suggesting that specific additions to the gut microbiota can influence lung immunity (222). Altogether, these data show that the gut microbiota can influence immunity in peripheral tissue with specific implications for lung immunity. Interestingly, there are fewer than 10 papers that have assessed the ability of the gut microbiota to influence host defense against lung infections, and all but two of these papers have focused on the host response only to bacterial infections.

The gut microbiota and its influence on immunity in lung infections

Using germ-free mice or microbiota depletion, multiple groups showed that inoculation with *Klebsiella pneumoniae, Streptococcus pneumoniae* or influenza A results in increased mortality, bacterial burden or viral load during infection (223-225), implicating a role of the gut microbiota in host defense against lung infections. Others have shown that the addition of specific microorganisms to the gut microbiota resulted in disparate host responses depending on the microorganisms involved. In a model of experimental tuberculosis, early intestinal colonization with *Helicobacter hepaticus* resulted in increased susceptibility to *Mycobacterium tuberculosis* which was rescued by treatment with anti-IL-10 receptor antibody (226). In contrast, in a model of experimental *Klebsiella* pneumonia, the addition of *Bifidobacterium longum* 5^{1A} to the gut microbiota of mice resulted in protection associated with ROS production and modulation of lung inflammation (227). Finally, two groups have implicated SFB as the microorganism responsible for inducing the cytokines IL-17 and IL-22 in host defense in a model of *Staphylococcus* pneumonia and *Aspergillus* infection (196,197). Using SFB-negative mice from The Jackson Laboratory and SFB-positive mice from Taconic Farms, Gauguet et al. showed that microbiota-induced production of IL-17 and IL-22 resulted in a protective host response by promoting neutrophilia, while McAleer et al. used Jackson and Taconic mice to implicate SFB in modulating host responses to *Aspergillus*, Altogether, these data suggest that the gut microbiota can influence host defenses against lung infections, but overall very little is known about the components of the gut

microbiota that can influence lung immunity during infection. Future studies should further assess the contribution of the gut microbiota and identidy specific microorganisms and the mechanisms by which they influence host defenses during lung infection. In chapter 3, I will assess the contribution of the gut microbiota in defense against invasive pulmonary aspergillosis as well as implicate a role of SFB in host defense during infection.

AUTHOR CONTRIBUTIONS

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A.B., M.D.B., A.K., and B.M. conceived and designed experiments; A.B., Z.Z., K.M., R.E.C., I.S.V., M.D.B., A.K., and B.M. performed experiments; A.B., I.S.V., and A.K. analyzed data; I.S.V. and A.K. contributed reagents and analysis tools; A.B. and B.M. wrote the paper; and all authors revised and approved the text.

Chapter II

M-CSF mediates host defense during bacterial pneumonia by promoting the survival of lung and liver mononuclear phagocytes

ABSTRACT

Gram-negative bacterial pneumonia is a common and dangerous infection with diminishing treatment options due to increasing antibiotic resistance among causal pathogens. The mononuclear phagocyte system is a heterogeneous group of leukocytes composed of tissue-resident macrophages, dendritic cells, and monocyte-derived cells that are critical in defense against pneumonia, but mechanisms that regulate their maintenance and function during infection are poorly defined. M-CSF has myriad effects on mononuclear phagocytes but its role in pneumonia is unknown. We therefore tested the hypothesis that M-CSF is required for mononuclear phagocyte-mediated host defenses during bacterial pneumonia in a murine model of infection. Genetic deletion or immunoneutralization of M-CSF resulted in reduced survival, increased bacterial burden, and greater lung injury. M-CSF was necessary for the expansion of lung mononuclear phagocytes during infection but did not affect the number of bone marrow or blood monocytes, proliferation of precursors, or recruitment of leukocytes to the lungs. In contrast, M-CSF was essential to survival and antimicrobial functions of both lung and liver mononuclear phagocytes during pneumonia, and its absence resulted in bacterial dissemination to the liver and hepatic necrosis. We conclude that M-CSF is critical to host defenses against

bacterial pneumonia by mediating survival and antimicrobial functions of mononuclear phagocytes in the lungs and liver.

Introduction

Pneumonia caused by aerobic Gram-negative bacilli is among the most common and dangerous nosocomial infections and an important cause of death, prolonged hospital stay, and increased healthcare costs. Gram-negative bacilli colonize the upper aerodigestive tract as part of the normal response to acute illness and cause pneumonia when introduced into the lower respiratory tract by microaspiration (51,228). The progressive rise in antibiotic resistance among the causative organisms in recent decades has markedly diminished the available treatment options for these infections, lending new impetus to mechanistic studies that may inform the development of better therapeutic strategies.

The mononuclear phagocyte system encompasses heterogeneous populations of cells from three distinct lineages, namely yolk sac–derived and self-renewing tissue-resident macrophages, Flt3 ligand–dependent pre–dendritic cell (DC)-derived cells, and monocytes and their progeny (229). The latter lineage, derived from circulating monocytes that express high levels of the surface glycoprotein Ly6C in mice, can differentiate into diverse populations of mononuclear phagocytes depending on environmental cues: under homeostatic conditions, mouse $Ly6C^{hi}$ monocytes terminally differentiate into endothelial-patrolling $Ly6C^{lo}$ counterparts in the blood and also give rise to some short-lived populations of tissue macrophages. In response to tissue injury, $Ly6C^{hi}$ monocytes extravasate into the damaged tissue and differentiate into inflammatory macrophages, repopulate some tissue-resident macrophage populations, and differentiate into monocyte-derived inflammatory DC that are

phenotypically similar to, but transcriptionally distinct from, CD11b+ pre-DC–derived conventional DC (37). The mouse lung, in particular, contains at least five populations of mononuclear phagocytes under homeostatic conditions, namely the tissue-resident and long-lived alveolar macrophages, short-lived monocyte-derived interstitial macrophages, CD11b+ conventional DC, CD103+ airway DC, and plasmacytoid DC (230). In the context of injury, $Ly6C^{hi}$ and $Ly6C^{lo}$ monocytes, monocyte-derived DC, and macrophages enter the lungs and resident cells display accelerated efflux and death, comprising a complex and dynamic network.

M-CSF was the first cytokine described for its induction of macrophage colonies from bone marrow progenitors in soft agar (15,231) and is a key regulator of the development and homeostasis of the mononuclear phagocyte system (42,232,233) In different experimental conditions, M-CSF has been shown to mediate diverse functions in the differentiation, proliferation, survival, and effector functions of multiple cell types from different mononuclear phagocyte lineages (8) . In addition, the sole receptor for M-CSF, CSF1R, or CD115 is shared with a structurally unrelated ligand, IL-34 (19). M-CSF and IL-34 have distinct tissue expression patterns and, at least in the development of osteoclasts, microglia, and skin Langerhans cells, have nonredundant roles (234) Most recently, protein tyrosine phosphatase-z was described as a second receptor for IL-34 (20,235).

Evidence from multiple experimental systems support a critical role for mononuclear phagocytes in host defenses but the mechanisms that control the

development, homing, and survival of these cells during specific infections are incompletely defined. In the context of bacterial pneumonia, monocytes are recruited to the lungs via a CCR2-dependent mechanism, and both recruited and resident mononuclear phagocytes mediate host defenses by direct killing of bacteria and NF-kB–mediated recruitment of other leukocytes (50,94,105,143,236). In this context, we previously defined the dynamic changes in the various mononuclear phagocyte populations during experimental Gram-negative pneumonia and reported that CCR2 was necessary not only to the recruitment of monocyte-derived lineage cells but, unexpectedly, also controlled the number and phenotype of alveolar macrophages and all DC populations in this infection (50). Little is known about the role of the M-CSF/IL-34 biologic axis in the context of infection, and many of the published studies in the field predate current understanding of the lineages of mononuclear phagocytes (237-241). To our knowledge, this mechanism has not been investigated in pulmonary infections to date. We therefore sought to define the role of this biological axis in pneumonia by testing the hypothesis that M-CSF is required for mononuclear phagocyte-mediated host defense during *Klebsiella* pneumonia.

Materials and Methods

Animals and in vivo procedures

We used a previously characterized model of experimental bacterial pneumonia (242,243). C57Bl/6 and *op*/+ mice, purchased (The Jackson Laboratory, Bar Harbor, ME), were bred and maintained under pathogen-free conditions and in compliance with institutional animal care regulations. Age- and sex-matched 6- to 8-wk-old mice were used in all experiments. *Klebsiella pneumoniae* strain 43816 (American Type Culture Collection, Manassas, VA) was grown overnight at 37˚C to midlog phase in tryptic soy broth. Bacteria were administered intratracheally as previously described (50). Unless otherwise noted, all mice received i.p. injections of 100 mg/100 mL anti–M-CSF–neutralizing Ab (clone 5A1), anti-CSF1R–neutralizing Ab (clone AFS98), IgG1 isotype control (clone HRPN), or IgG2A isotype control (clone 2A3) beginning 2 h before intratracheal inoculation and then every 24 h through day 2 postinfection (all from BioXCell, West Lebanon, NH). In some experiments, mice received three i.p. injections of 2 mg BrdU (BD Biosciences, San Jose, CA) in 0.2 ml PBS, every 3 h until 2 h before bacterial inoculation, as described (28). At designated time points, mice were euthanized with an injection of ketamine and xylazine.

Tissue harvest

Blood was collected in heparinized syringes from the right ventricle. Lung and liver vasculature were perfused with 3 mL PBS with 2 mM EDTA before excision. Blood and homogenized lungs and right liver lobes were serially diluted and cultured on blood agar plates for quantification of bacterial content.

Bronchoalveolar lavage was performed as previously described (242). Histology was performed on lungs and left liver lobes as previously described (242,244). In other experiments, whole lungs, left liver lobes, and plasma were processed for ELISAs as described (243).

Flow cytometry

Flow cytometry was performed as previously described (183,242,245). Cell suspensions of whole lungs, median liver lobes, bone marrow, and peripheral blood were prepared as previously described (243,244,246). The following reagents were used to label cells for flow cytometry (from BD Biosciences, San Jose, CA; eBioscience, San Diego, CA; or BioLegend, San Diego, CA): 7-aminoactinomycin D (7-AAD), anti-CD3–PE-Cy7 (clone 17A2), anti-CD11b–allophycocyanin-Cy7 (clone M1/70), anti-CD11c–PE-Cy7 (clone HL3), anti-CD19–PE-Cy7 (clone 1D3), anti-CD24–allophycocyanin (clone M1/69), anti-CD45–PerCP or AmCyan (clone 30-F11), anti-CD64–BV421 (clone X54-5/7.1), anti-CD103–allophycocyanin (clone 2E7), anti-CD115 (clone AFS98), anti-CD117-FITC (clone 2B8), anti-CD135–BV421 (clone A2F10.1), anti-F4/80–Pacific Blue (clone BM8), anti-F4/80-PE (clone T45- 2342), Fc block (anti-CD16/CD32), anti–I-A/I-E–FITC or AmCyan (clone M5/114.15.12), Ki-67–PE and isotype control (clones B56 and MOPC-21), anti-Ly6C–PE or Pacific Blue (clone HK1.4), anti-Ly6G–PE-Cy7 (clone 1A8), anti-NK1.1–PE-Cy7 (clone PK136), and anti–plasmacytoid DC Ag-1–allophycocyanin (clone 129c1). In some experiments, cells were labeled, fixed, and permeabilized using a commercial kit (Cytofix/Cytoperm; BD Biosciences) before staining for intracellular Ags. Data

were acquired on an FACS Canto II instrument using BD FACSDiva software (version 8.0; BD Biosciences) and analyzed using FlowJo software (version 8.8.6; Tree Star, Ashland, OR). Leukocyte subsets were identified as previously described (37,38,50) with minor modifications, as depicted in the figures. The absolute number of each cell type was determined as the product of the percentage of the cell type and the total number of cells in the sample, as determined on an automated cell counter (Countess; Invitrogen, Carlsbad, CA).

ELISA and assays for alanine transaminase, aspartate aminotransferase, urea nitrogen, and lactate

ELISAs for M-CSF and IL-34 were performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Albumin ELISA was performed using 5 mg/ml goat anti-mouse albumin (A90-134A), 0.025 mg/ml goat-HRP detection (A90-134P), mouse reference serum (rs10-101) (Bethyl Laboratories; Montgomery, TX), and 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific). Plasma alanine transaminase, aspartate aminotransferase, and urea nitrogen were measured using commercial kits (Liquid ALT Reagent Set and Liquid AST Reagent Set; Pointe Scientific, Canton, MI; and DetectX Urea Nitrogen Detection Kit; Arbor Assays, Ann Arbor, MI). Plasma lactate was measured using a commercial device (Lactate Plus Meter; Nova Biomedical, Waltham, MA).

In vitro studies

Bone marrow cells were cultured as described to generate macrophages (247). In viability assays, macrophages were washed and resuspended in DMEM with 10% FCS without antibiotics, and 1 x 10^5 cells were cultured overnight in opaque flat-bottom 96-well tissue-culture plates (BD Falcon, Franklin Lakes, NJ). Cells were then incubated with or without 5 ng M-CSF for 2 h before the addition of *K. pneumoniae*. After 150 min, plates were washed with sterile HBSS, and viability was measured using a commercial kit (LIVE/DEAD viability/cytotoxicity kit for mammalian cells; Invitrogen Molecular Probes, Eugene, OR). For phagocytosis assays, bacteria were labeled with a pH-sensitive cyanine dye that acquires fluorescence in acidic environments (248,249): 1 x 10^5 macrophages were seeded into round-bottom tissue culture–treated plates (Costar, Corning, NY) and incubated with or without M-CSF as above. Midlog phase *K. pneumoniae* cells were suspended at 1 x 10^9 /mL in sterile saline, fixed in 5 ml 70% ethanol for 5 min, washed, and resuspended in sterile HBSS; CypHer5E dye (GE Healthcare Life Sciences, Pittsburgh, PA) was added at a final concentration of 5 µmol before incubation at room temperature for 30 min. Bacteria were then washed, and 1 x 10^8 bacteria were added to wells containing macrophages. Plates were centrifuged for 30 s to achieve cell contact and incubated at 37˚C and 5% CO_2 for 30 min, then placed on ice, washed in cold HBSS, and analyzed immediately by flow cytometry. Positive gating was set using an unstained control.

Statistical analysis

Data were analyzed using Prism statistical software (version 5.0d; GraphPad Software, San Diego, CA). Data from survival experiments were analyzed using the log-rank test. Values between two groups over multiple time points were compared with two-way ANOVA. Comparisons between two groups at a single time were performed using the nonparametric Mann–Whitney U test. Comparisons between multiple groups at a single time point were performed using the Kruskal–Wallis nonparametric test with Dunn comparison posttest. Comparison of paired samples receiving different treatments was made using Wilcoxon matched-pairs signed-rank test or two-way repeated-measures ANOVA. The p values < 0.05 were considered statistically significant.

Results

Role of M-CSF during pulmonary infection with K. pneumoniae

We began by measuring the concentration of M-CSF and IL-34 protein in whole-lung homogenates during experimental pneumonia induced by *K. pneumoniae*. Both ligands were detectable in uninfected lungs. As compared with animals challenged with intratracheal saline vehicle, the concentration of M-CSF increased ~2-fold as the infection progressed but the concentration of IL-34 did not change significantly (Fig. 2.1A, 2.1B). In contrast, the concentration of plasma M-CSF protein remained undetectable in plasma throughout the time course, suggesting that M-CSF was produced locally in the lungs during infection. Given these findings, we focused our studies on the role of M-CSF during *Klebsiella* pneumonia.

To assess the contribution of M-CSF during bacterial pneumonia, we next examined the outcome of animals deficient in M-CSF during infection. Mice homozygous for an inactivating mutation in the M-CSF locus (*op*/*op*) had notably increased mortality during experimental pneumonia as compared with littermate controls, with no animals surviving beyond 2 d; this was associated with increased incidence and severity of bacteremia on the first day of the infection (Fig. 2.1C, 2.1D).

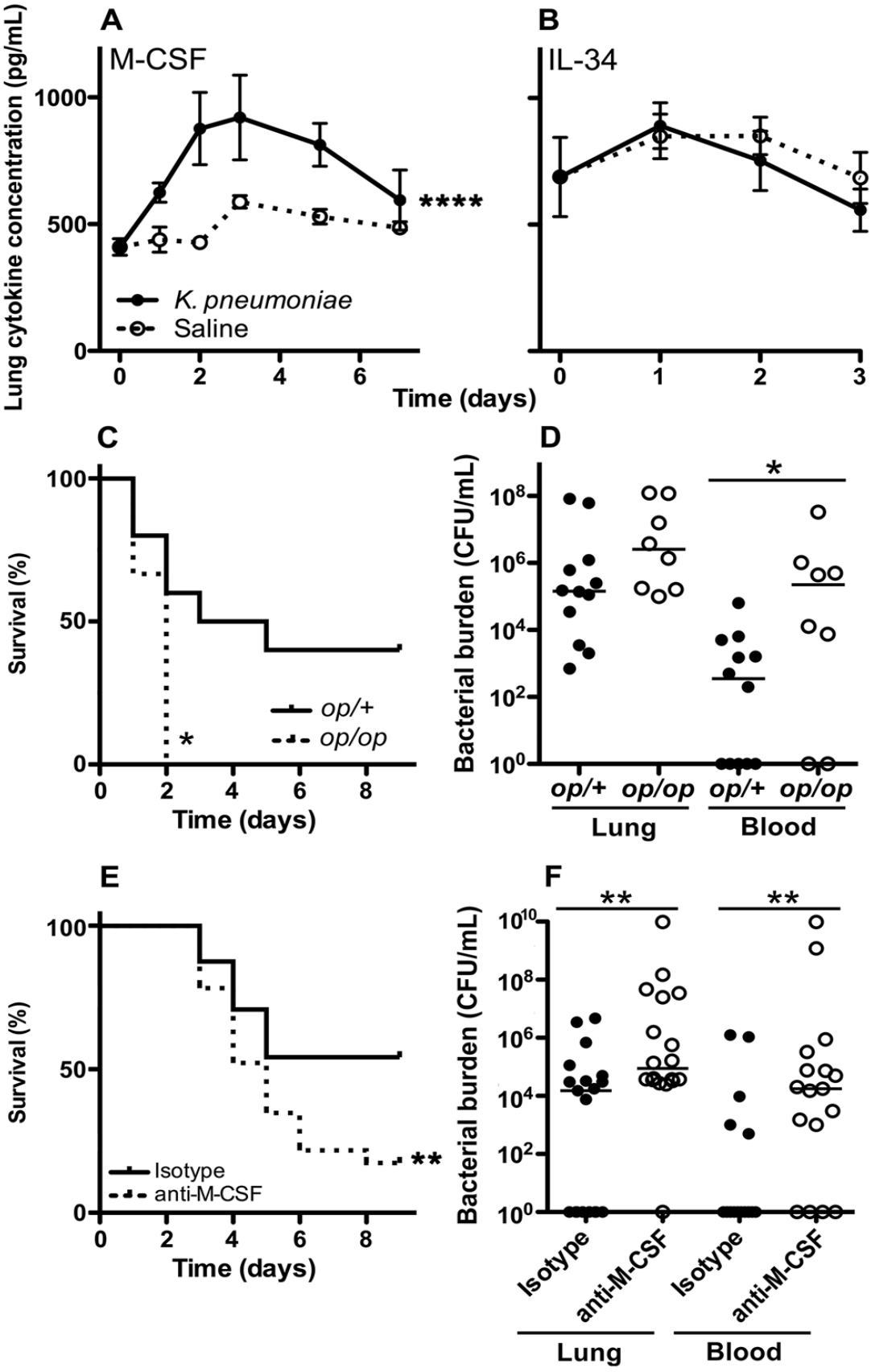

A
M-CSF
B
IL-34
Lung cytokine concentration (pg/mL)
1000
500
0
****
K. pneumoniae
Saline
0
2
4
6
0
1
2
3
Time (days)
C
Survival (%)
100
50
0
*
op/+
op/op
0
2
4
6
8
Time (days)
D
Bacterial burden (CFU/mL)
*
10^8
10^6
10^4
10^2
10^0
op/+
op/op
op/+
op/op
Lung
Blood
E
Survival (%)
100
50
0
**
Isotype
anti-M-CSF
0
2
4
6
8
Time (days)
F
Bacterial burden (CFU/mL)
**
**
10^{10}
10^8
10^6
10^4
10^2
10^0
Isotype
anti-M-CSF
Isotype
anti-M-CSF
Lung
Blood

Figure 2.1. M-CSF level increases in the lungs of mice with pneumonia and is required for host defense. Time series of mean +/- SEM of lung M-CSF (A) and IL-34 (B) protein in mice infected with *K. pneumoniae*; n = 5 to 6 per time point per group from two experiments. Time 0 represents uninfected animals. ****p < 0.0001, two-way ANOVA. (C) Outcome of pneumonia in op/op mice and littermate controls. n = 9 to 10 per group from two experiments (*p < 0.05, log-rank test). (D) Bacterial burden in lungs and blood of infected op/op mice and littermate controls on day 1 of infection. Each data point represents one animal, and horizontal lines indicate medians. Data from two experiments (*p < 0.05, Mann–Whitney). (E) Outcome of pneumonia in animals treated with anti–M-CSF or isotype control Ab; n = 23 to 24 per group from two experiments (**p < 0.01, log-rank test). (F) Bacterial burden in lungs and blood of animals with pneumonia on day 3 of infection in animals treated with anti–M-CSF or isotype control Ab. n = 17–19 per group; data shown are representative of two experiments (**p < 0.01, Mann–Whitney). In (D) and (F), samples with no recoverable bacteria are depicted as 1 CFU on the logarithmic scale.

The role of M-CSF during development of the mononuclear phagocyte system results in a number of well documented phenotypic abnormalities in *op*/*op* mice, including reduced number of alveolar macrophages in juvenile, but not adult, animals and spontaneous production of matrix metalloproteinases by mutant alveolar macrophages (250). To discriminate between the role of M-CSF induction during bacterial pneumonia and its role during development, we tested the effect of immunoneutralization of M-CSF starting at the onset of the infection. Wild-type mice receiving anti–M-CSF–neutralizing Ab had increased mortality, increased lung bacterial burden, and markedly increased incidence and severity of bacteremia as compared with animals receiving an isotype control Ab (Fig. 2.1E, 2.1F). We found similar results with antagonism of CSF1R during the infection. The administration of a neutralizing CSF1R Ab to uninfected animals did not influence the number of blood Ly6C^{hi} monocytes or lung mononuclear phagocytes but lead to a reduction in blood Ly6C^{lo} monocytes (Fig. 2.2A, 2.2B), consistent with prior reports that, in steady state, M-CSF mediates differentiation of blood Ly6C^{hi} monocytes to their Ly6C^{lo} counterparts (43,251). In the context of infection, administration of anti-CSF1R Ab resulted in a similar increase in mortality and bacterial burden as noted with M-CSF neutralization (Fig. 2.2C, 2.2D). These data indicate that the M-CSF–CSF1R interaction is necessary for host defenses during bacterial pneumonia independent of their role in development.

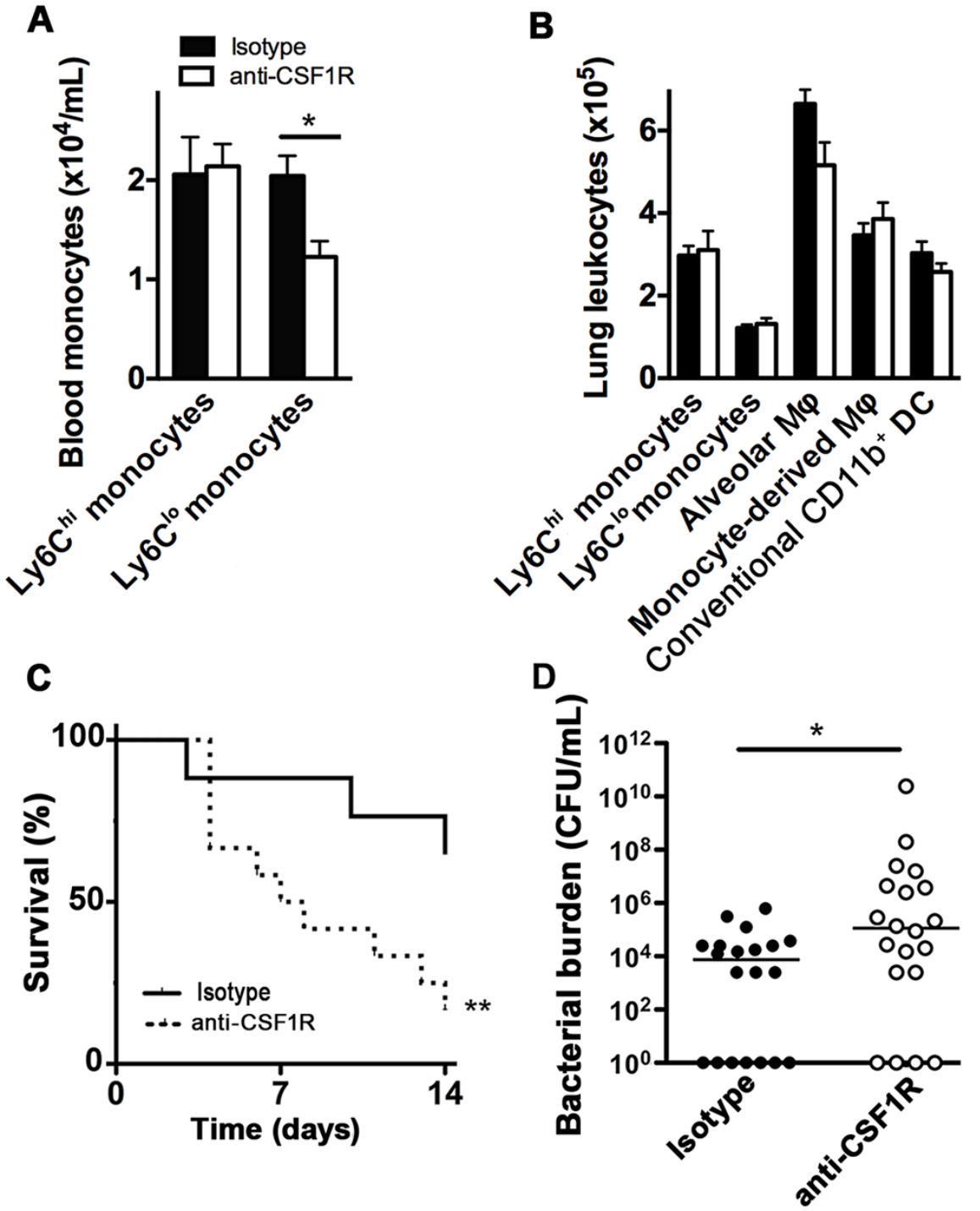

A
Isotype
anti-CSF1R
Blood monocytes (x10^4/mL)
*
Ly6C^hi monocytes
Ly6C^lo monocytes
B
Lung leukocytes (x10^5)
Ly6C^hi monocytes
Ly6C^lo monocytes
Alveolar Mφ
Monocyte-derived Mφ
Conventional CD11b^+ DC
C
Survival (%)
Isotype
anti-CSF1R
**
Time (days)
D
Bacterial burden (CFU/mL)
*
Isotype
anti-CSF1R

Figure 2.2. CSF1R is required for host defense in pneumonia. Concentration of circulating monocytes (A) and lung mononuclear phagocytes (B) in uninfected mice after administration of daily CSF1R Ab or isotype control for 7 d using the gating strategy depicted in Fig. 3A; n = 4 to 5 per group (*p < 0.05, Mann–Whitney). (C) Outcome of pneumonia in animals treated with anti-CSFR1 or isotype control Ab; n = 23–24 per group from two experiments (**p < 0.01, log-rank test). (D) Bacterial burden in lungs of animals with pneumonia treated with anti-CSFR1 or isotype control Ab on day 3 of infection. Data from two experiments. Samples with no recoverable bacteria are depicted as 1 CFU. *p < 0.05, Mann–Whitney. Mφ, macrophages.

Mechanism of M-CSF–mediated host defense during pneumonia

To begin to address how M-CSF mediates its beneficial effects during bacterial pneumonia, we examined the effect of M-CSF neutralization on lung mononuclear phagocyte subsets in the lungs in the context of pneumonia (Fig. 2.3A). M-CSF neutralization resulted in a significant reduction in the numbers of cells of monocyte lineage, namely Ly6C^{hi} and Ly6C^{lo} monocytes, monocyte-derived macrophages, and monocyte-derived inflammatory DC, in the lungs of mice during pneumonia (Fig. 2.3B–D). In addition, M-CSF neutralization caused a reduction in the number of alveolar macrophages and conventional CD11b+ DC, but not other DC subsets or neutrophils (Fig. 2.3E–J), similar to our prior report in mice with CCR2 deficiency (50).

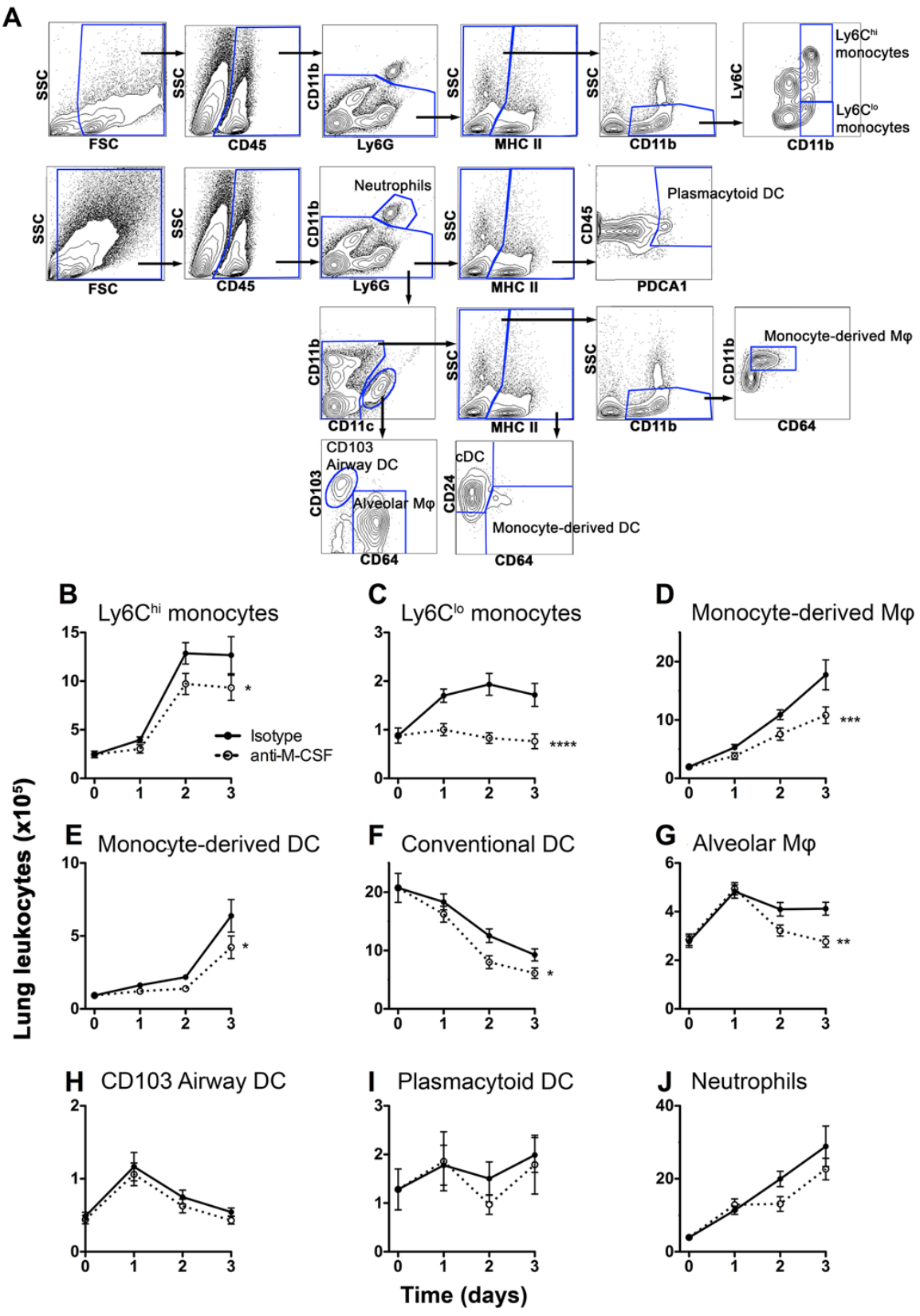

A
SSC
FSC
SSC
CD45
CD11b
Ly6G
SSC
MHC II
SSC
CD11b
Ly6C
Ly6C^hi monocytes
Ly6C^lo monocytes
CD11b
Neutrophils
CD45
PDCA1
Plasmacytoid DC
CD11c
Monocyte-derived Mφ
CD64
CD103
Airway DC
Alveolar Mφ
CD24
cDC
Monocyte-derived DC
B Ly6C^hi monocytes
C Ly6C^lo monocytes
D Monocyte-derived Mφ
E Monocyte-derived DC
F Conventional DC
G Alveolar Mφ
H CD103 Airway DC
I Plasmacytoid DC
J Neutrophils
Isotype
anti-M-CSF
Lung leukocytes (x10^5)
Time (days)

Figure 2.3. M-CSF is required for the maintenance of lung mononuclear phagocytes during pneumonia. (A) Representative flow cytometry plots showing gating strategy for lung leukocytes. Cells were enumerated in single-cell suspensions from whole lung. (A–J) Time-series of mean +/- SEM of indicated lung leukocyte populations; n = 20–24 per time point per group from four experiments. Time 0 represents uninfected animals. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, two-way ANOVA. cDC, conventional CD11b+ DCs; FSC, forward light scatter; Mφ, macrophages; MHC II, MHC class II; PDCA1, plasmacytoid DC Ag-1; SSC, side scatter.

To understand how the M-CSF–CSF1R axis contributes to the numbers of lung mononuclear phagocytes during *Klebsiella* pneumonia, we reasoned that the observed reduction in lung monocyte-derived cells, and possibly alveolar macrophages and conventional CD11b+ DC, may be attributable to impairments in monocytopoeisis, trafficking between the bone marrow, blood, and lung compartments, or survival of leukocytes that have arrived in the lungs. We found that neutralization of M-CSF had no effect on the concentration of Ly6C^{hi} and Ly6C^{lo} monocyte numbers in bone marrow or blood (Fig. 2.4A, 2.4B). Consistent with this, we found no difference in the proliferation of bone marrow macrophage-DC progenitor or the recently described committed-monocyte progenitor (4) after neutralization of M-CSF (Fig. 2.4C, 2.4D). These data indicate that M-CSF is dispensable for the generation of monocytes from progenitor cells in the bone marrow during bacterial pneumonia. M-CSF can be chemotactic for mononuclear phagocytes (252,253). To assess for this possibility, we pulsed uninfected mice with the thymidine analog, BrdU, thereby labeling populations of proliferating cells that were in S-phase at the time of the pulse and then quantified the number of labeled leukocytes in various compartments after the onset of infection. As expected, we found fewer BrdU-positive bone marrow neutrophils and a marked increase in labeled neutrophils in the blood and lung of infected mice compared with uninfected controls that was independent of M-CSF neutralization (Fig. 2.5A–C). M-CSF neutralization also did not influence the numbers of labeled Ly6C^{hi} monocytes in the bone marrow, blood, or lungs during infection (Fig. 2.5A–C), suggesting that

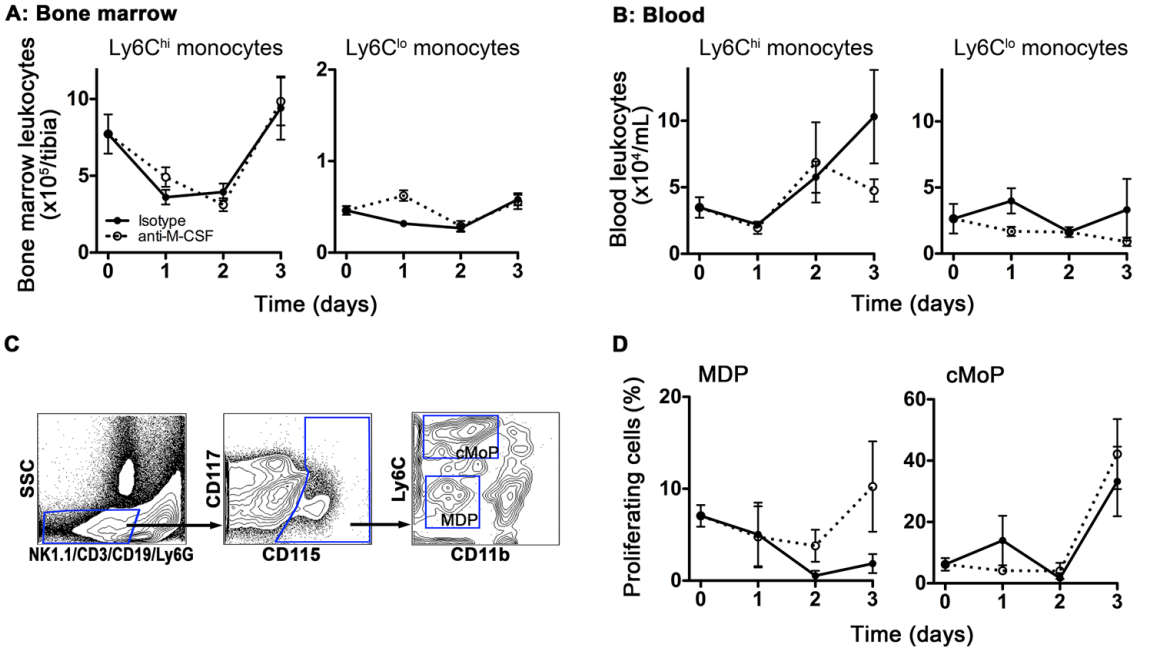

A: Bone marrow
Ly6C^hi monocytes
Ly6C^lo monocytes
Bone marrow leukocytes (x10^5/tibia)
Isotype
anti-M-CSF
Time (days)
B: Blood
Ly6C^hi monocytes
Ly6C^lo monocytes
Blood leukocytes (x10^4/mL)
Time (days)
C
SSC
NK1.1/CD3/CD19/Ly6G
CD117
CD115
Ly6C
CD11b
cMoP
MDP
D
MDP
cMoP
Proliferating cells (%)
Time (days)

Figure 2.4. M-CSF is dispensable for maintenance of bone marrow and blood monocytes and progenitor proliferation during pneumonia. (A and B) Time series of mean +/- SEM of indicated leukocyte populations in the blood and bone marrow. n = 10–12 from two experiments. (C) Representative flow plots of CD45+ bone marrow cells, showing gating strategy of bone marrow to identify progenitor cells. (D) Time series of mean +/- SEM of percent Ki67+ bone marrow monocyte progenitors. n = 6–12 per group per time point from two experiments. Time 0 represents uninfected animals.

We next assessed the role of M-CSF on survival of mononuclear phagocytes during infection, because the M-CSF–CSF1R axis can mediate survival of mononuclear phagocytes under homeostatic conditions (254). We addressed this question by measuring the proportion of dead cells in the bronchoalveolar lavage fluid of infected animals to minimize the artifact of ex vivo cell death that inevitably occurs during processing of samples. M-CSF neutralization resulted in a significant increase in the proportion of dead Ly6C^{hi} monocytes in the lungs of infected animals (Fig. 2.6A), potentially implicating M-CSF as a survival factor for lung monocyte-derived cells during pneumonia. To directly examine the role of M-CSF in mediating monocyte survival during infection, we also assessed the survival of bone marrow–derived macrophages co-incubated with *K. pneumoniae*. As compared with cells incubated with bacteria in the presence of M-CSF, monocyte-derived macrophages incubated with bacteria in the absence of M-CSF had reduced survival (Fig. 2.6B). In addition, the surviving monocyte-derived macrophages had impaired phagocytosis of *K. pneumoniae* in the absence of M-CSF (Fig. 2.6C), suggesting that M-CSF promotes the survival of mononuclear phagocytes during infection and also contributes to the antimicrobial functions of these cells independent of its effect on the number of leukocytes.

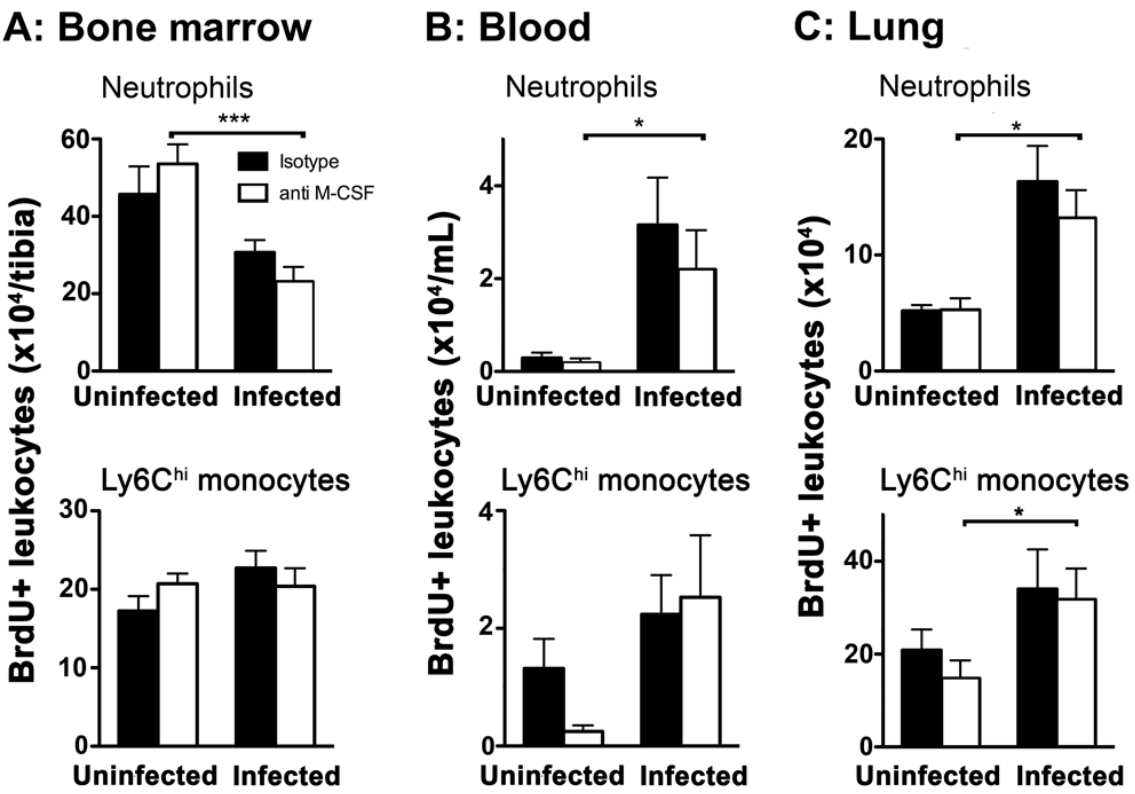

A: Bone marrow
B: Blood
C: Lung
Neutrophils
Ly6C^hi monocytes
BrdU+ leukocytes (x10^4/tibia)
BrdU+ leukocytes (x10^4/mL)
BrdU+ leukocytes (x10^4)
***
*
Isotype
anti M-CSF
Uninfected
Infected

Figure 2.5. M-CSF is dispensable to the influx of mononuclear phagocytes during pneumonia. Mean +/- SEM of BrdU-positive leukocyte populations in the bone marrow (A), blood (B), and lungs (C). n = 6–12 per group per time point from two experiments. *p < 0.05, ***p < 0.001, one-way ANOVA with Dunn posttest.

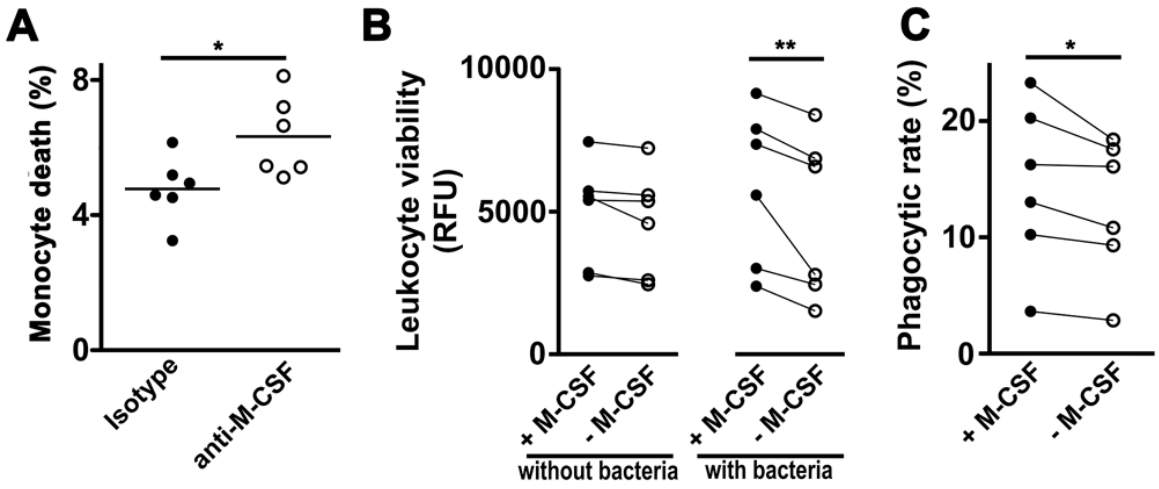

A
Monocyte death (%)
8
4
0
*
Isotype
anti-M-CSF
B
Leukocyte viability (RFU)
10000
5000
0
**
+ M-CSF
- M-CSF
+ M-CSF
- M-CSF
without bacteria
with bacteria
C
Phagocytic rate (%)
20
10
0
*
+ M-CSF
- M-CSF

Figure 2.6. M-CSF is required for the survival and function of mononuclear phagocytes during pneumonia. (A) Percentage of 7-AAD+ lung Ly6C^{hi} monocytes on day 3 of infection. n = 6 per group from two experiments (*p < 0.05, Mann–Whitney). (B) Effect of M-CSF on viability of bone marrow–derived macrophages incubated with or without *Klebsiella* in vitro, as measured in relative fluorescence units (RFU). Each data point represents an independent biological replicate paired to its respective control (**p < 0.01, two-way repeated-measures ANOVA). (C) Effect of M-CSF on phagocytosis of CypHer5E-labeled *Klebsiella* by bone marrow–derived macrophages. Each data point represents an independent biological replicate paired to its respective control (*p < 0.05, Wilcoxon matched-pairs signed-rank test).

Role of M-CSF in controlling liver injury during bacterial pneumonia

In the course of the above studies, we noticed that M-CSF neutralization during pneumonia was associated with gross visual evidence of liver injury. To investigate this, we assessed the effect of M-CSF neutralization on the histology of lung and liver during experimental pneumonia. The lung histology did not differ between pneumonic mice treated with anti–M-CSF and isotype controls; in contrast, M-CSF neutralization during pneumonia resulted in large areas of sharply demarcated hepatocellular necrosis, associated with prominent hepatocyte microvesicles (Fig. 2.7A). Consistent with this, neutralization of M-CSF resulted in elevations in plasma concentrations of aspartate transaminase and alanine transaminase during pneumonia (Fig. 2.7B). Because M-CSF neutralization resulted in increased incidence and extent of bacteremia (Fig. 2.1), we next assessed whether the observed hepatocellular injury was associated with increased hepatic bacterial content after the liver had been perfused to displace its blood content. We found that, in mice with pneumonia, M-CSF neutralization resulted in increased liver bacterial burden (Fig. 2.7C), potentially explaining the increased liver injury. To assess whether the hepatocellular injury is merely a reflection of multi-organ dysfunction in the context of sepsis, we also quantified other parameters of end-organ dysfunction. As expected, M-CSF neutralization resulted in increased lung injury, as measured by albumin concentration in the bronchoalveolar lavage fluid (Fig. 2.8A). In contrast to its effect on the liver, however, M-CSF neutralization did not affect renal function, as measured by the concentration of blood urea nitrogen, nor cause significant end-

organ hypoperfusion, as quantified by plasma lactate concentration (Fig. 2.8B, 2.8C), suggesting that M-CSF neutralization results in specific injury to the liver in addition to the lungs.

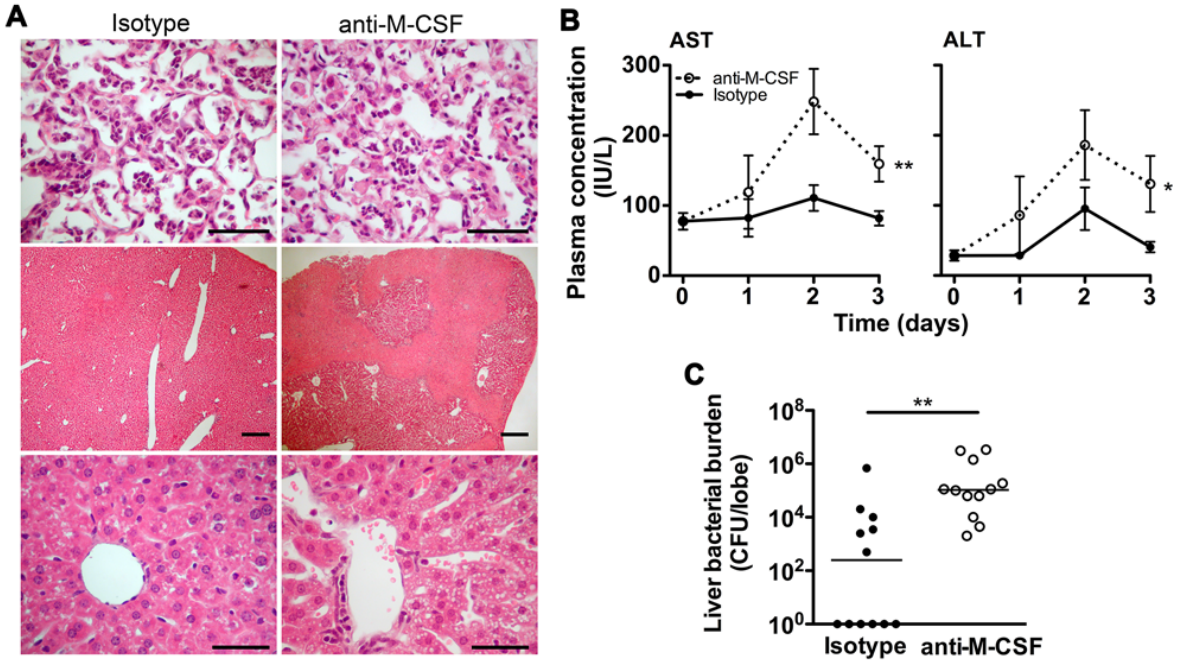

A
Isotype
anti-M-CSF
B
AST
ALT
anti-M-CSF
Isotype
Plasma concentration (IU/L)
300
200
100
0
**
*
0
1
2
3
Time (days)
C
Liver bacterial burden (CFU/lobe)
10^8
10^6
10^4
10^2
10^0
**
Isotype
anti-M-CSF

Figure 2.7. M-CSF is required for controlling bacterial dissemination and secondary liver damage during pneumonia. (A) H&E stain of lung (top panels) and liver sections (middle and bottom panels) on day 3 of infection in animals treated with anti–M-CSF or isotype control Ab. Top and bottom panels, Scale bars 40 μm, original magnification X 400; middle panels, scale bars 200 μm, original magnification X40. Representative data from three mice per group. Top panels show alveolar infiltration by acute inflammatory cells. Middle panels show areas of necrosis with anti–M-CSF treatment (bright pink areas). Bottom panels show central hepatic veins and surrounding lobule; the hepatocytes in mice treated with anti–M-CSF show prominent microvesicles. (B) Time series of mean +/- SEM of plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) during pneumonia, as measured in IUs per liter. n = 10–12 from two experiments. *$p < 0.05$, **$p < 0.01$, two-way ANOVA. (C) Bacterial burden in the right lobe of the liver in mice treated with anti–M-CSF or isotype control Ab. Each data point represents one animal, and horizontal lines indicate medians; samples with no recoverable bacteria are depicted as 1 CFU on the logarithmic scale. Data from two experiments (**$p < 0.01$, Mann–Whitney).

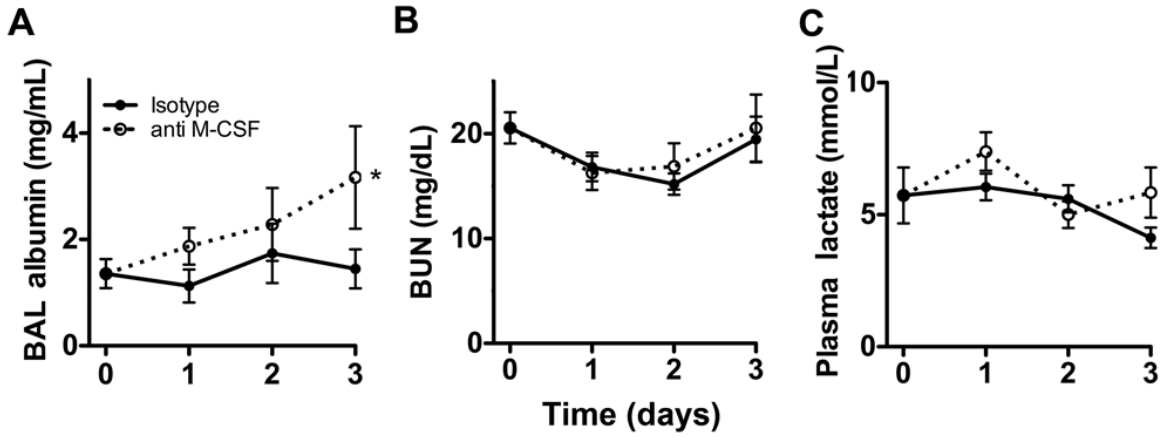

A
B
C
Isotype
anti M-CSF
BAL albumin (mg/mL)
BUN (mg/dL)
Plasma lactate (mmol/L)
Time (days)
*
0
1
2
3
4
10
20
5

Figure 2.8. M-CSF neutralization causes lung injury but not renal failure or tissue hypoperfusion during pneumonia. Time series of mean +/- SEM of bronchoalveolar lavage (BAL) albumin concentration (A), blood urea nitrogen (BUN; B), and plasma lactate (C) in mice with pneumonia, treated with anti–M-CSF or isotype Ab. n = 8–12 per group per time point from two experiments. Time 0 represents uninfected animals. *$p < 0.05$, two-way ANOVA.

In order to investigate the contribution of M-CSF in the liver during bacterial pneumonia, we assessed the liver levels of M-CSF protein, because the plasma concentrations of M-CSF were undetectable during the infection. The liver contained high concentrations of M-CSF protein at baseline that did not change significantly during the infection (Fig. 2.9A). We therefore tested the hypothesis that, similar to our findings in the lungs, liver M-CSF mediates the survival of liver mononuclear phagocytes during the infection, leading to better clearance of bacteria from the liver. Consistent with this and analogous to the lungs, we found that liver monocyte-derived macrophages and F4/80+ CD11b+ resident Kupffer cells were reduced in number with M-CSF neutralization during pneumonia (Fig. 2.9B, 2.9C). Also similar to our findings in the lungs, M-CSF neutralization resulted in increased death of liver Ly6C^{hi} monocytes during pneumonia (Fig. 2.9D). Taken together, these data indicate that M-CSF is also required for mononuclear phagocyte-mediated defense in the liver during bacterial pneumonia.

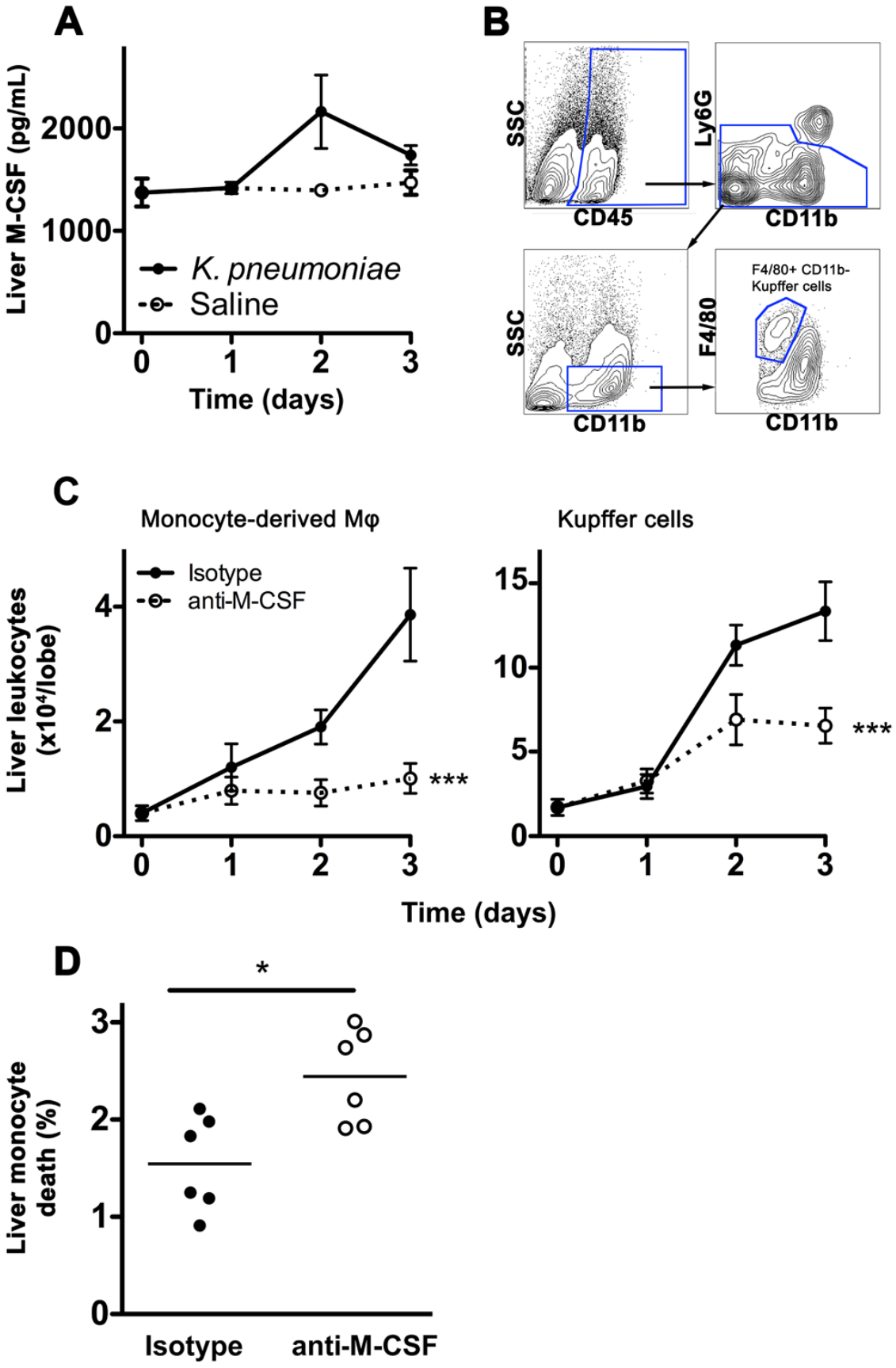

A
Liver M-CSF (pg/mL)
2000
1000
0
K. pneumoniae
Saline
0
1
2
3
Time (days)
B
SSC
CD45
Ly6G
CD11b
SSC
CD11b
F4/80
F4/80+ CD11b-
Kupffer cells
CD11b
C
Monocyte-derived Mφ
Kupffer cells
Isotype
anti-M-CSF
Liver leukocytes
(x10⁴/lobe)
4
2
0
***
15
10
5
0
***
0
1
2
3
Time (days)
D
*
Liver monocyte
death (%)
3
2
1
0
Isotype
anti-M-CSF

Figure 2.9. M-CSF is required for the maintenance of mononuclear phagocytes in the liver during pneumonia. (A) Time series of mean +/- SEM of M-CSF protein in the left liver lobe in mice with pneumonia. n = 6–16 per time point per group from two experiments. (B) Representative flow cytometry plots showing gating strategy for Kupffer cells. (C) Time series of mean +/- SEM of indicated mononuclear phagocytes in the median liver lobe. Monocyte-derived macrophages were identified as depicted in Fig. 3A. n = 10–12 per group per time point from two independent experiments. ***$p < 0.001$, two-way ANOVA. (D) 7-AAD–positive Ly6C^{hi} liver monocytes on day 3 of infection; data from two independent experiments (*$p < 0.05$, Mann–Whitney). Time 0 represents uninfected animals. Mφ, macrophages; SSC, side scatter.

Discussion

The mononuclear phagocyte system has been implicated in host defense against bacterial pneumonia (50,89,105,255), but the mechanisms that generate and maintain these cells during the infection have not been defined. In the current study, we report that M-CSF, but not IL-34, is induced in the lung during the infection. M-CSF was essential to the survival of the host as a critical survival signal for mononuclear phagocytes in infected tissues, but appeared to be dispensable for the generation, recruitment, and differentiation of mononuclear phagocytes during the infection. The absence of M-CSF thus resulted in reduced numbers of both monocyte-derived cells and resident macrophage populations in the lungs and the liver, resulting in increased bacterial burden and injury to these organs and increased incidence and extent of bacteremia.

M-CSF is produced by many cell types, including mononuclear phagocytes themselves, and has been documented to have a multitude of effects on mononuclear phagocytes in different experimental conditions, including development during embryogenesis, monocytopoeisis in adulthood, proliferation, differentiation, chemotaxis, and survival (as reviewed in Ref. 11). M-CSF has not been studied extensively in the context of infections, but has been associated with host defense in intracellular infections caused by *Listeria monocytogenes* (238,256) and influences macrophage phenotype in experimental tuberculosis and schistosomiasis (257,258). We report that M-CSF appeared to be dispensable for the proliferation and recruitment of monocytes and their progenitors during pneumonia, similar to recent findings in sterile peritonitis

(251). Our studies did not directly examine the role of M-CSF in the differentiation of mononuclear phagocytes in the lungs in vivo, but the intact recruitment of monocytes to the lungs, together with the role of M-CSF in maintaining the numbers of both lung monocytes and monocyte-derived cells during infection indicates that the differentiation of monocytes into other mononuclear phagocytes during pneumonia remained intact despite M-CSF neutralization. In contrast, M-CSF was essential in maintaining lung and liver monocytic phagocyte populations by promoting their survival. M-CSF has been shown to be critical to survival of mononuclear phagocytes during development and under homeostatic conditions (259-262) but, to our knowledge, has not been shown to mediate mononuclear phagocyte survival in the setting of infection or inflammation. In this context, macrophage survival is a known regulatory factor in the context of infection: alveolar macrophage apoptosis, for example, is both antimicrobial and anti-inflammatory during pneumococcal pneumonia (149,263), whereas macrophage necroptosis induces lung damage during Gram-negative and *S. aureus* pneumonia (70,264).

In addition, we found that M-CSF mediates enhanced phagocytosis of bacteria by surviving mononuclear phagocytes. In this context, macrophages from op/op animals have been shown to have impaired phagocytosis against bacteria but not parasites (265,266), but it is difficult to differentiate whether any effect in op/op macrophages is attributable to developmental abnormalities of the cells or lack of exposure to M-CSF during acute infection. Similar to our findings, however, M-CSF results in enhanced phagocytosis of fungal conidia by human

monocyte-derived macrophages in vitro (267). Taken together, these data suggest that locally produced M-CSF can enhance antimicrobial properties of mononuclear phagocytes in target organs.

An unexpected finding in our study was the role of M-CSF in protecting the liver from injury during experimental pneumonia. The propensity of Gram-negative bacteria, specifically *Klebsiella*, to infect the liver is recognized clinically (268). In the context of experimental pneumonia, the liver is responsible for a protective acute-phase response, but is also susceptible to TNF-mediated injury (269-271). M-CSF has recently been shown to mediate proliferation of liver macrophages and recruitment of monocytes to the injured liver (272). Our data add to this literature, indicating that endogenous production of M-CSF during pneumonia mediates the accumulation of both monocyte-derived macrophages and Kupffer cells and protects the liver from disseminated infection. The present work has several implications for future research. First, our data suggest that M-CSF is required not only for maintaining the number of monocyte-derived cells, but also alveolar macrophages and CD24+ [and thus pre-DC–derived (37)] CD11b+ DC in the lungs as well as Kupffer cells in the liver. This finding is reminiscent of our prior report in CCR2-deficient mice (50) and suggests either that Ly6C^{hi} monocytes repopulate other lineages of mononuclear phagocytes in the acute setting, as has been shown previously (42,273), or that M-CSF is necessary to the maintenance of all three lineages of mononuclear phagocytes in the setting of acute injury. Second, we found M-CSF to have a marked role in the accumulation of Ly6C^{lo} monocytes in the lungs, but any role of these cells in the

setting of acute infection remains undefined. Third, our work documents the critical role of M-CSF during bacterial pneumonia but does not preclude an additional contribution for IL-34 or its second receptor, protein tyrosine phosphatase-ζ, in the context of pneumonia. Finally, our findings may be relevant to efforts to inhibit M-CSF signaling as a therapeutic strategy in cancer (274), because such strategies may be expected to predispose patients to more severe pneumonia.

Chapter III

Intestinal colonization with Segmented Filamentous Bacteria influences host defense against invasive pulmonary aspergillosis

ABSTRACT

Invasive pulmonary aspergillosis is a severe and life-threatening infection caused by the saprophytic fungus *Aspergillus fumigatus*. The host immune response is a key determinant in whether the host will clear the organism or develop disease. The immunosuppressed host is susceptible to infection with *A. fumigatus*, but not all immunosuppressed hosts will develop disease. Emerging knowledge of the role of the gut microbiota in the context of immunity in peripheral tissue led us to hypothesize that the composition of the gut microbiota influences host defense against invasive pulmonary aspergillosis. In the context of immunosuppression by neutropenia, we show for the first time that the presence of Segmented Filamentous Bacteria in the gut microbiota influences host defenses against invasive pulmonary aspergillosis. We report increased mortality during infection between C57bl/6 mice bred and maintained at The University of Virginia compared to C57bl/6 mice purchased from The Jackson Laboratory and show that this difference is conferred in Jackson mice by fecal transplantation from mice from The University of Virginia. We additionally show that susceptibility of Jackson mice to infection is unaffected by antibiotic treatment, suggesting that the protection conferred in mice from the University of Virginia is due to the presence of a beneficial microorganism rather than the absence of deleterious microorganisms. We additionally show that mice from the

University of Virginia contain Segmented Filamentous Bacteria in their gut microbiota. Finally, we show that the specific addition of Segmented Filamentous Bacteria to the gut microbiota of Segmented Filamentous Bacteria-negative Jackson mice results in host defense during invasive pulmonary aspergillosis. Therefore, we report for the first time that intestinal colonization with Segmented Filamentous Bacteria is sufficient for host defense against invasive pulmonary aspergillosis.

INTRODUCTION

Invasive pulmonary aspergillosis is a severe pneumonic infection that is associated with a high mortality rate and a growing pool of susceptible hosts. This illness is most commonly caused by the fungus *Aspergillus fumigatus*, which is ubiquitous in the environment and one of the most prevalent molds in the world (77-79). Despite this, disease incidence in the community setting is only 40 cases per million. However, invasive aspergillosis is the second most common cause of health-care associated fungal infections and carries the greatest incremental hospitalization cost of other systemic fungal infections (167,168). Even with treatment, mortality rates for those infected are between 30-50% (169-171), underscoring the imminent need for uncovering new risk factors associated with the onset of disease, as well as the key immune mediators involved in host defenses during infection.

Known risk factors associated with the onset of invasive aspergillosis include diseases of immune cells, cancer treatments resulting in chemotherapy, bone marrow and whole organ transplantation, and long-term disease treatments utilizing corticosteroids (83,275,276). These risk factors are strongly linked to host susceptibility to invasive aspergillosis due to qualitative or quantitative neutrophil defects and impaired T cell mediated immunity. These risk factors underscore the critical role of the host response in infection: an immunocompetent host will clear inhaled fungal spores called conidia, but a neutropenic or otherwise immunosuppressed host will fail to clear the fungus;

inhaled conidia will germinate and form long branching multicellular hyphae that invade lung tissue and cause severe pneumonia and death (84).

It is increasingly evident that there are other risk factors associated with the development of disease that are currently undefined, as evidenced by the fact that not all immunocompromised hosts will develop disease. Clinically, it has been shown that patients receiving broad-spectrum antibiotics as prophylactics prior to bone marrow transplantation are more susceptible to gastrointestinal infection with *Clostridium difficile* due to altered gut microbiota associated with this broad-spectrum antibiotic treatment (277,278). The concept that gut microbiota influence local intestinal immunity has been well defined (190,279), and in some cases, specific microorganisms have been identified and implicated to play specific roles in various disease states and infections. For example, the Gram-positive, obligate anaerobe Segmented Filamentous Bacteria (SFB), have been shown to colonize the terminal ileum of certain cohorts of mice and not others (195,198), and these bacteria specifically induce Th17 cells (201,280), which provide mucosal protection against a variety of intestinal pathogens (195,204).

While the ability of gut microbiota to influence intestinal immunity has been well defined, the hypothesis that the gut microbiota influences immunity in peripheral tissue has only been studied in recent years (281,282). Altered gut microbiota resulting in microbial dysbiosis has been implicated in disease states ranging from allergy to autism (283,284). Within this emerging field, the concept that the composition of the gut microbiota can influence peripheral immunity to an

infectious agent has not been well defined, and furthermore, the specific identity of intestinal microorganisms that influence host defense in lung infections is widely unknown. Therefore, we sought to test the hypothesis that specific microorganisms in the gut microbiota influence host defenses against invasive pulmonary aspergillosis.

MATERIALS & METHODS

Animals & *in vivo* procedures

We used a previously characterized mouse model of invasive pulmonary aspergillosis (246,285,286). For some experiments, C57bl/6 mice used were bred and maintained under pathogen-free conditions at the University of Virginia (UVa), in compliance with institutional animal care regulations. For other experiments, C57bl/6 mice were purchased from The Jackson Laboratory, Bar Harbor, ME or The Jackson Laboratory, Sacramento, CA. Age- and sex-matched 6- to 8-wk-old mice were used in all experiments. In all experiments, neutrophil depletion was achieved by administering one single intraperitoneal injection of anti-Ly6G antibody (clone 1A8) one day before administration of *A. fumigatus* conidia (BioXCell, West Lebanon, NH).

Antibiotic treatment

We used a previously established method of antibiotic treatment to alter the gut microbiota of Jackson mice (287,288). Briefly, mice received a cocktail of 4 antibiotics including colistin, gentamicin, vancomycin, and metronidazole in their drinking water for 3 days, followed by autoclaved, antibiotic-free water for 2 days. One day prior to infection with *A. fumigatus*, mice received a single intraperitoneal injection of clindamycin (10 mg/kg).

SFB mono-associated feces

SFB mono-associated stool was obtained from Dr. Ivaylo Ivanov for collaboration purposes. Gnotobiotic mice mono-associated with SFB as

previously described (193,194) are maintained at The Rockefeller University in New York, NY in compliance with institutional animal care regulations. Feces from SFB mono-associated mice are collected and stored at -80˚C for future use.

Co-housing and fecal transplantation

For co-housing experiments: Mice purchased from The Jackson Laboratory were placed in cages containing feces collected from mice bred at UVa for a total of 14 days prior to neutrophil depletion and infection with *A. fumigatus*.

For fecal gavage experiments: Feces from mice bred at UVa were collected and weighed. Two fecal pellets (40 mg feces) were ground in a petri dish (BD Falcon, Franklin Lakes, NJ) and resuspended in 1 mL of sterile 1X PBS or saline. Each mouse received an oral gavage of 200 µL by 20G gavage needle (Cadence Inc., Cranston, RI), as previously described (289). This procedure was repeated 7 days after the initial gavage for a total of 2 gavages. Mice were inoculated with *Aspergillus* 14 days after the initial gavage. Control mice were purchased from The Jackson Laboratory and given a fecal gavage of their own feces. In experiments where Segmented Filamentous Bacteria (SFB) mono-associated stool was used, mice from The Jackson Laboratory arrived and were assessed by qPCR for SFB colonization. SFB negative Jackson mice received a total of 9 pellets of SFB mono-associated stool over the course of 24 hours. Briefly, SFB mono-associated stool was ground in a petri dish and resuspended in sterile saline. Each mouse received an oral gavage of a volume equaling 3 pellets; 600 µL. Four to eight hours after this initial gavage, mice received

another gavage of 3 pellets, and received a final gavage of 3 pellets 16-18 hours following this second gavage. Feces were collected 5-8 days after this final gavage and assessed by qPCR for presence of SFB. SFB-colonized mice were inoculated with *Aspergillus* 10 days after the final gavage. Control mice were also purchased from The Jackson Laboratory and given fecal gavages of their own SFB-negative feces.

Preparation and administration of Aspergillus

Aspergillus fumigatus strain 13073 (American Type Culture Collection, Manassas, VA) was grown for 10-18 days at 37˚C on Sabouraud's dextrose agar plates. Conidia were harvested in 0.1% Tween-80 in PBS, filtered through sterile gauze, and counted using a hemacytometer. Conidia were administered intratracheally in inocula ranging between 0.8 to 2.3 x 10^7 in 30 µL per mouse.

Fecal DNA extraction and Quantitative PCR

DNA was extracted from fecal samples using the ZR fecal DNA MiniPrep commercial kit (Zymo Research, Irvine, CA). SFB colonization was measured as previously described (204). SFB Ct values were normalized to the total bacterial ribosomal subunit 16S (EUB) using the $2^{-\Delta\Delta Ct}$ method. Jackson stool was normalized to SFB mono-associated stool using the $2^{-\Delta\Delta Ct}$ method. Primer sequences: SFB forward, 5'-GACGCTGAGGCATGAGAGCAT-3'; SFB reverse, 5'GACGGCACGGATTGTTATTCA-3'; EUB forward, 5'-ACTCCTACGGGAGGCAGCAGT-3'; EUB reverse, 5'-ATTACCGCGGCTGCTGGC-3'.

Statistical analysis

Data were analyzed using Prism statistical software (version 5.0d; GraphPad Software, San Diego, CA). Data from survival experiments were analyzed using the log-rank test. Comparisons between two groups at a single time were performed using the Student's t-test or the nonparametric Mann–Whitney U test. The p values < 0.05 were considered statistically significant.

RESULTS

The gut microbiota influence host defense during invasive pulmonary aspergillosis

Using a previously described model of murine invasive pulmonary aspergillosis, in which the host is transiently neutrophil-depleted prior to inoculation, we tested the hypothesis that the composition of the gut microbiota influences susceptibility to infection. We began by comparing the susceptibility of C57bl/6 mice bred and maintained at the University of Virginia (UVa) to C57bl/6 mice purchased from The Jackson Laboratory (Jackson). Mice bred and maintained at UVa did not survive significantly more in response to invasive pulmonary aspergillosis compared to mice purchased from Jackson (Fig. 3.1*A*), but because these cohorts of mice are bred and maintained in different facilities, it is possible that susceptibility to infection could be due to environmental disparities or slight genetic drift between mouse strains rather than gut microbiota. Thus, to further assess susceptibility of different mouse cohorts to invasive pulmonary aspergillosis, mice purchased from Jackson were housed in cages containing feces collected from UVa mice and compared to Jackson mice housed in cages containing Jackson feces prior to neutrophil depletion and infection with *A. fumigatus*. We found that Jackson mice co-housed with feces from UVa mice survived significantly more than Jackson mice housed in cages containing Jackson feces (Fig. 3.1*B*). These results suggest that differences in susceptibility during invasive pulmonary aspergillosis are not due to environmental differences or genetic drift between mice from different facilities,

so we next sought to directly assess the contribution of the gut microbiota in host defense against invasive pulmonary aspergillosis by fecal transplantation.

To directly assess the contribution of the gut microbiota in host defense during invasive pulmonary aspergillosis, Jackson mice were gavaged with fecal matter from UVa mice and compared to Jackson mice gavaged with Jackson fecal matter prior to neutrophil depletion and infection with *A. fumigatus*. During infection, we found that Jackson mice receiving UVa fecal transplantation survived significantly more than Jackson mice receiving control Jackson fecal transplantation (Fig. 3.1*C*), suggesting that the gut microbiota influence host defense during invasive pulmonary aspergillosis. We next sought to assess the contribution of an altered microbiota on susceptibility to invasive pulmonary aspergillosis. To assess this, we used a previously described model in which broad spectrum antibiotics are administered to induce host susceptibility to *Clostridium difficile* infection (287,288). Interestingly, we found that altering the microbiota by broad spectrum antibiotics did not influence susceptibility to invasive pulmonary aspergillosis (Fig. 3.1*D*), suggesting that the influence of the gut microbiota during invasive pulmonary aspergillosis is not due to general microbial dysbiosis resulting in the absence of deleterious bacteria and repopulation by other microorganisms (213,290,291), but is perhaps instead caused by the presence of specific intestinal microorganisms. Altogether, these results suggest that the gut microbiota contribute to host defenses during invasive pulmonary aspergillosis, and that this contribution is not due to the absence of certain gut microbiota resulting from antibiotic treatment.

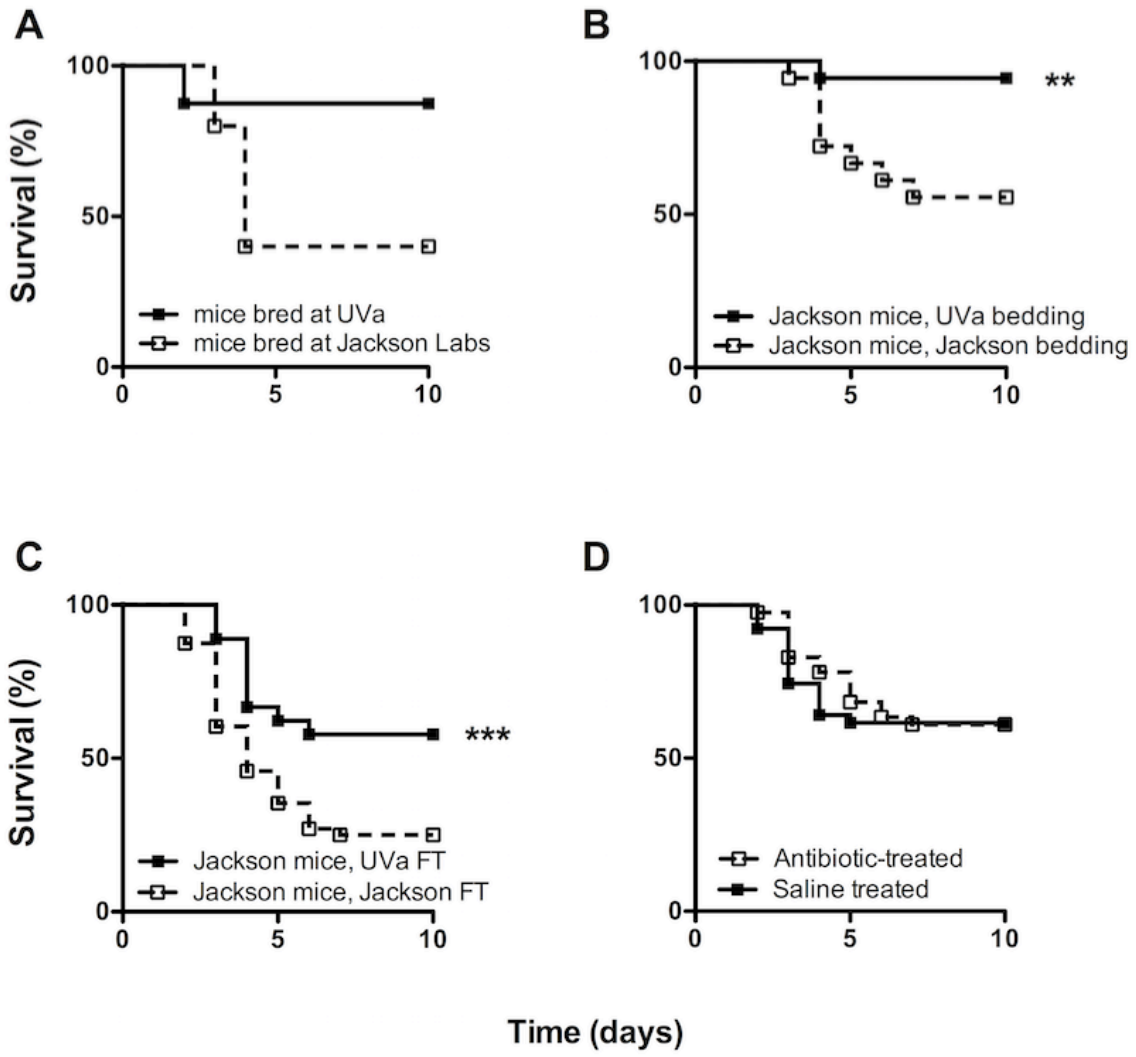

A
B
C
D
Survival (%)
100
50
0
0
5
10
mice bred at UVa
mice bred at Jackson Labs
Jackson mice, UVa bedding
Jackson mice, Jackson bedding
**
Jackson mice, UVa FT
Jackson mice, Jackson FT
***
Antibiotic-treated
Saline treated
Time (days)

Figure 3.1. The composition of the gut microbiota influences host defense during invasive pulmonary aspergillosis. (*A*) Outcome of invasive pulmonary aspergillosis in UVa C57bl/6 mice compared to Jackson C57bl/6 mice. *n* = 7-15/group; data shown from 1 experiment. (*B*) Outcome of invasive pulmonary aspergillosis in Jackson mice housed in cages with feces collected from UVa mice. *n* = 18/group; data shown from 1 experiment. (*$p < 0.05$, Log-rank test). (*C*) Outcome of Jackson mice receiving UVa fecal transplant compared to Jackson mice receiving Jackson fecal transplant. *n*= 45-48/group; data are pooled from 3 independent experiments. (***$p < 0.001$, Log-rank test). (*D*) Outcome of invasive aspergillosis in Jackson mice treated with broad spectrum antibiotics compared to saline-treated. *n* = 40 per group; data are pooled from 3 independent experiments. (Log-rank test).

Segmented Filamentous Bacteria are present in the UVa gut microbiota

We next sought to determine differences in the composition of gut microbiota of UVa mice and Jackson mice that might confer protection against invasive pulmonary aspergillosis. Because Segmented Filamentous Bacteria (SFB) have been shown to induce specific immune responses in the terminal ileum and are absent from Jackson mice (195), we hypothesized that the UVa gut microbiota contain SFB. We found that the UVa gut microbiota contain SFB and that SFB were not detectable in the Jackson microbiota (Fig. 3.2), consistent with previously published reports (195). Additionally, we found that SFB present in the UVa gut microbiota could be transferred to SFB-negative Jackson mice by housing these mice in cages containing feces collected from SFB-positive UVa mice. Taken together, these data suggest that the UVa gut microbiota contain SFB and that SFB can be transferred to SFB-negative Jackson mice.

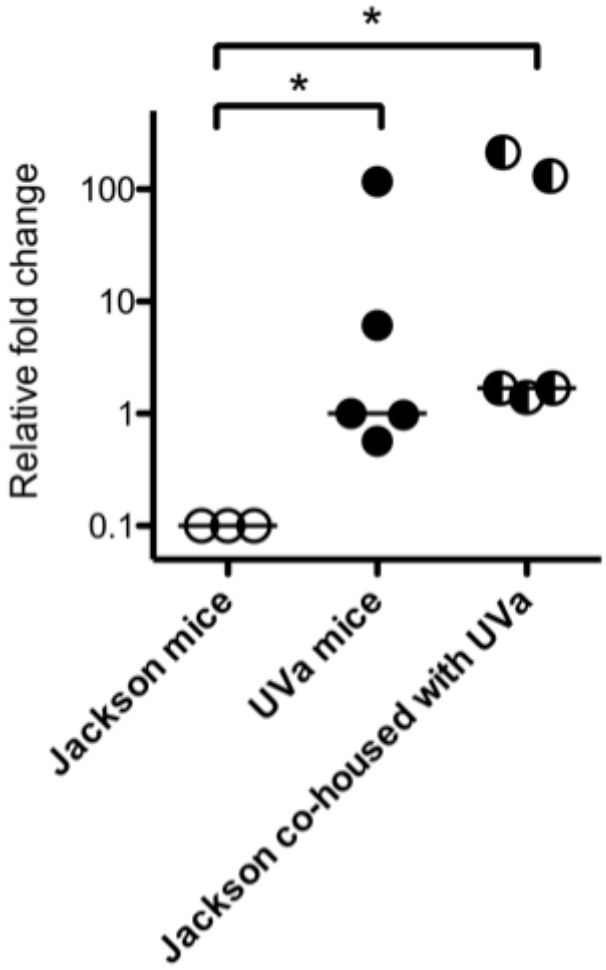

*
*
Relative fold change
100
10
1
0.1
Jackson mice
UVa mice
Jackson co-housed with UVa

Figure 3.2. Segmented Filamentous Bacteria are present in the UVa gut microbiota. Relative expression of SFB in the feces of UVa mice compared to Jackson mice. Jackson mice were housed with UVa feces for 14 days prior to fecal collection. n = 3-5 per group. (*$p < 0.05$; Mann-Whitney).

Segmented Filamentous Bacteria are sufficient to confer protection against invasive pulmonary aspergillosis

Because the UVa gut microbiota contain a variety of microorganisms in addition to SFB, we next sought to assess the SFB-specific contribution to host defense during invasive pulmonary aspergillosis by colonizing SFB-negative Jackson mice with SFB mono-associated feces. To test this, we colonized SFB-negative Jackson mice with SFB mono-associated feces (Fig. 3.3*A*) and compared the survival of these mice to SFB-negative Jackson mice receiving SFB-negative Jackson feces during invasive pulmonary aspergillosis. We found that SFB-colonized Jackson mice survived significantly more compared to SFB-negative Jackson controls during invasive pulmonary aspergillosis (Figure 3.3*B*). These data suggest that intestinal colonization with SFB is sufficient to confer protection against invasive pulmonary aspergillosis.

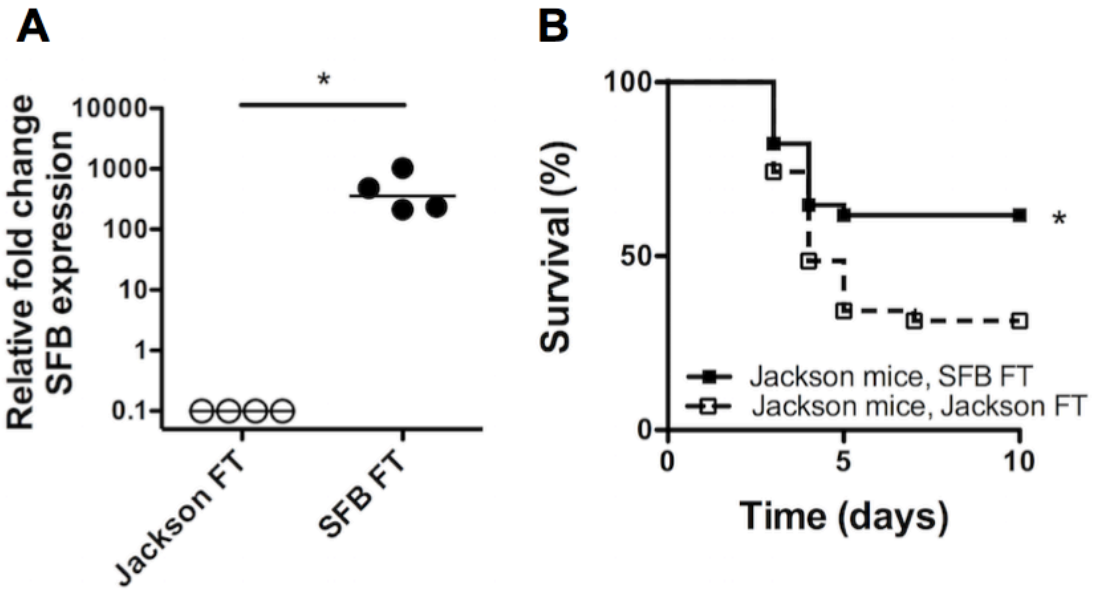

A
B
Relative fold change SFB expression
10000
1000
100
10
1
0.1
*
Jackson FT
SFB FT
Survival (%)
100
50
0
0
5
10
Time (days)
Jackson mice, SFB FT
Jackson mice, Jackson FT
*

Figure 3.3. Intestinal colonization with Segmented Filamentous Bacteria is sufficient to confer protection against invasive pulmonary aspergillosis. (*A*) Relative SFB expression of SFB-negative Jackson mice receiving SFB mono-associated fecal transplant (FT) compared to SFB-negative Jackson mice receiving SFB-negative Jackson FT. n = 4 per group (*$p < 0.05$, Mann-Whitney). (*B*) Outcome of SFB-negative Jackson mice receiving SFB mono-associated fecal transplant compared to SFB-negative control fecal transplant during invasive pulmonary aspergillosis. n = 34-35/group; data are pooled from 2 independent experiments (*$p < 0.05$, Log-rank test).

DISCUSSION

The composition of the gut microbiota has been implicated in influencing immunity in peripheral tissue, but the contribution of the gut microbiota during lung infection has not been completely described. Furthermore, the contributions of specific gut microbiota in lung infection are widely unknown. We report that the composition of the gut microbiota influences host defenses against invasive pulmonary aspergillosis, that the composition of the UVa gut microbiota is protective against infection compared to the gut microbiota of Jackson mice, and that this protection is transferable by fecal transplant. We additionally show that this disparity is not due to the presence of detrimental microorganisms in Jackson mice, as evidenced by antibiotic treatment, but is instead due to the presence of a beneficial microorganism in the UVa gut microbiota. We found that Segmented Filamentous Bacteria (SFB) are present in the gut microbiota of UVa mice and absent from Jackson mice, and furthermore, we show for the first time that the specific addition of SFB alone to the Jackson gut microbiota is sufficient to confer protection against invasive pulmonary aspergillosis.

The gut microbiota has only recently been shown to influence immunity in peripheral tissue, and to our knowledge, fewer than 10 papers have been published implicating a role of the gut microbiota in host defense against lung infections. The absence of the gut microbiota in a germ-free model of experimental murine *Klebsiella* pneumonia results in increased mortality and increased bacterial burden associated with elevated levels of IL-10, ultimately resulting in immune hyporesponsiveness (223). These data are difficult to

interpret due to the fact that germ free mice are completely uncolonized by microorganisms from birth and have been known to have aberrant immune responses as a result. However, in deletion experiments, others have shown that treatment with broad-spectrum antibiotics resulting in altered gut microbiota results in detrimental host responses during *Streptococcus* pneumonia and influenza A infection. These studies report increased bacterial dissemination, inflammation, organ damage, and mortality during *Streptococcus* pneumonia (224), and during influenza A infection, increased viral titers, reduced CD8+ T cell counts and IFNγ levels, which are rescued by TLR-agonism (225). In contrast to these studies, we report that the composition of the gut microbiota influences host defense during invasive pulmonary aspergillosis as evidenced by reduced mortality in Jackson mice compared to UVa mice, but importantly, we report no differences in mortality between antibiotic-treated and mock-treated mice, suggesting that gut dysbiosis alone is insufficient to influence host defense against *A. fumigatus*.

The addition of specific intestinal microorganisms and their impact on immunity to lung infections is currently being investigated. The few papers that have been published have shown that specific microorganisms can influence lung immunity during infection. In a model of experimental tuberculosis, researchers show that early intestinal colonization with *Helicobacter hepaticus* results in increased susceptibility to *Mycobacterium tuberculosis*, which is rescued by treatment with anti-IL-10 receptor antibody (226). In contrast to this, others have shown that the addition of specific intestinal microorganisms results

in protective host responses during infection. In a model of experimental *Klebsiella* pneumonia, researchers show that the addition of *Bifidobacterium longum* 5^{1A} to the gut microbiota of mice results in protective responses associated with ROS production and modulation of lung inflammation (227). In agreement with this, we report that the addition of SFB to the gut microbiota of the SFB-negative host results in a protective host response associated with decreased mortality during invasive pulmonary aspergillosis.

The present work has many implications for future research. First, our data show that the composition of the UVa gut microbiota results in increased host defense compared to Jackson microbiota in a model of experimental invasive pulmonary aspergillosis. With the advent of genomic deep sequencing, it is now possible to identify multiple key microorganisms present in gut microbiota that influence host defense to *A. fumigatus*. We additionally show that administration of broad-spectrum antibiotics does not influence mortality during invasive pulmonary aspergillosis, but instead requires the presence of specific microorganisms. Finally, we show that the specific addition of SFB to the gut microbiota of the SFB-negative host results in protective host responses associated with reduced mortality during infection, but the mechanism underlying host protection remains unknown. Given the well-defined ability of SFB to induce Th17 cells in the terminal ileum, it would be plausible to implicate a role of intestinal colonization with SFB and subsequent IL-17 production during invasive pulmonary aspergillosis. Additionally, the role of IL-17 during invasive pulmonary aspergillosis has been previously assessed, albeit with inconclusive results. One

group has shown that IL-17 produces a hyper-immune response associated with destructive inflammation (292,293) while another group has shown that administration of IL-17 results in a protective host response by dectin-1-dependent phagocytosis of conidia (294)

Finally, two groups have implicated SFB as the microorganism responsible for inducing the cytokines IL-17 and IL-22 in a model of *Staphylococcus* pneumonia and during infection with *Aspergillus* (196,197). Using SFB-negative mice from The Jackson Laboratory and SFB-positive mice from Taconic Farms, Gauguet et al. show that microbiota-induced production of IL-17 and IL-22 results in a protective host response by promoting neutrophilia during *Staphylococcus* pneumonia. During challenge with *Aspergillus*, McAleer et al. show that the gut microbiota can modulate the host immune response to *Aspergillus* by regulating IL-17 and IL-22 production, which they discovered by comparing mice from The Jackson Laboratory and Taconic Farms with or without oral vancomycin treatment. In contrast to our model of invasive pulmonary aspergillosis in the immunocompromised host, these results are from mixed models of fungal infection (neutropenic host) and challenge (immunocompetent host). Because the resulting immune response to *Aspergillus* is critically dependent on the immune-state of the host, it is unclear if these results and subsequent conclusions are operational during invasive pulmonary aspergillosis. Additionally, it is critical to note that neither group shows that the addition of SFB alone results in the findings concluded by the researchers. It has been previously shown that mice from Taconic Farms and The Jackson Laboratory differ not only

in SFB colonization, but that myriad differences exist between the gut microbiota of mice from each of these facilities (195). Thus, the results from these papers could be due to differences in the gut microbiota independent of the presence or absence of SFB. Furthermore, while our data show that the specific addition of SFB results in host defense during invasive pulmonary aspergillosis, it is unlikely that an SFB--IL-17--neutrophilia mechanistic axis is responsible for this protection, as suggested by results in *Staphylococcus aureus* pneumonia, as invasive pulmonary aspergillosis occurs in the context of neutropenia. Thus, our studies implicate a yet unknown mechanism of SFB-mediated mucosal immunity. Future work will interrogate the mechanistic contributions of SFB on protective host responses during invasive pulmonary aspergillosis.

Chapter IV Conclusions and Future Directions

Conclusions

We undertook two studies to examine the role of host factors in bacterial and fungal pneumonia. We first assessed the role of M-CSF on mononuclear phagocytes during *Klebsiella* pneumonia. We found that M-CSF is dispensable for the proliferation and trafficking of mononuclear phagocytes, but that it is required for their survival and function during infection. In addition, we found that the absence of M-CSF results in bacteremia and tissue-specific liver damage, and that liver damage is associated with increased bacterial burden and reduced survival of liver mononuclear phagocytes during *Klebsiella* pneumonia.

Second, we examined the ability of gut microbiota to influence host defense against invasive pulmonary aspergillosis. We found that C57bl/6 mice bred and maintained at the University of Virginia (UVa) were more resistant to infection than C57bl/6 mice purchased from The Jackson Laboratory (Jackson), and we showed that Jackson mice could be protected from infection if given fecal transplantation from UVa mice. We additionally show that broad-spectrum antibiotic treatment of Jackson mice does not affect mortality during challenge with *Aspergillus fumigatus*. Lastly, we show that UVa mice contain Segmented Filamentous Bacteria (SFB) in their gut microbiota, and that the specific transfer of SFB mono-associated feces to SFB-negative Jackson mice is sufficient to confer protection against invasive pulmonary aspergillosis.

Future Directions

4.1 M-CSF and bacterial pneumonia

Molecular mechanism of mononuclear phagocyte death in Klebsiella *pneumonia*

We found a significant reduction in survival of mononuclear phagocytes in the absence of M-CSF *in vivo* and *in vitro*, but the mechanism underlying death in this context is unknown. M-CSF agonism of CSF1R has been shown to result in anti-apoptotic downstream signaling (259,261), which suggests that the absence of M-CSF would result in increased apoptosis of mononuclear phagocytes. We undertook an experiment to assess the mechanism underlying the death of mononuclear phagocytes. In *Klebsiella* infected mice treated with M-CSF neutralizing antibody or isotype control, bronchoalveolar lavage (BAL) was collected and alveolar macrophage cell death was assessed. Using Annexin V and 7AAD staining strategies, we found a significant increase in Annexin V-single positive alveolar macrophages in M-CSF neutralized mice, and an even greater difference in Annexin V and 7AAD double-positive alveolar macrophages in M-CSF neutralized mice (Fig. 4.1). These preliminary data suggest that alveolar macrophages undergo apoptosis in the absence of M-CSF, as evidenced by Annexin V single-positive staining, but also suggest that another form of cell death might be involved, as evidenced by Annexin V and 7AAD double-positive staining. Given recent literature, a plausible mechanism of non-apoptotic death in the context of *Klebsiella* pneumonia might be necroptosis, as previously suggested (295).

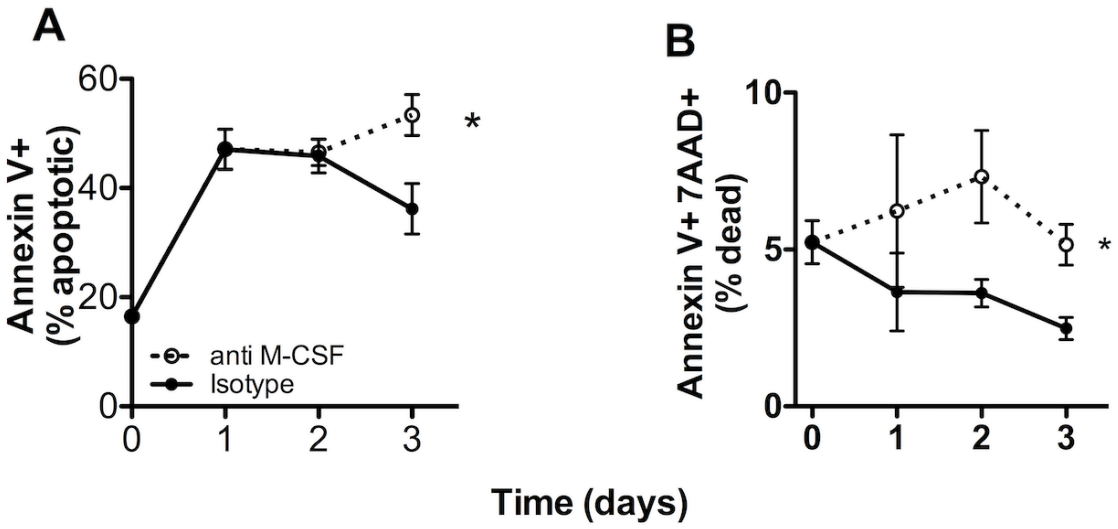

A
Annexin V+
(% apoptotic)
60
40
20
0
anti M-CSF
Isotype
*
0
1
2
3
B
Annexin V+ 7AAD+
(% dead)
10
5
0
*
0
1
2
3
Time (days)

Figure 4.1. M-CSF is required for the survival of alveolar macrophages during *Klebsiella* pneumonia. (*A*). Apoptotic alveolar macrophages (*B*) 7AAD+ Annexin V+ alveolar macrophages. Time series of mean +/- SEM of percent early apoptotic alveolar macrophages or dead alveolar macrophages. Death is represented by percent of 7AAD+ parent populations and apoptosis by Annexin V+ and exclusion of 7AAD. Bronchoalveolar lavage (BAL) was performed at time 0 through day 3 post-infection. n = 12 per time point per group, pooled from two experiments. (* $P < 0.05$, 2-way ANOVA).

Necroptosis is a form of programmed necrosis (reviewed in (296,297)) and has been interpreted as a mechanism of host defense against caspase inhibitor-expressing viruses (298). Recently, necroptosis has been identified as the major type of macrophage death in *Staphylococcal* pneumonia and *Listeriosis* (70,264,299). Necroptosis results from TNF-receptor activation of the receptor-interacting protein kinase 1 (Ripk1)-Ripk3 complex (297). It is well documented that apoptosis and necroptosis exist in a balance tipped by Caspase 8 activity (300), and that Caspase 8 prevents Ripk3-dependent necrosis without inducing apoptosis (300), which supports our preliminary data of alveolar macrophage death. Future studies are needed to further tease apart the mechanisms of mononuclear phagocyte death during *Klebsiella* pneumonia. Future experiments should utilize chemical inhibitors of apoptosis (z-VAD-fmk) and necroptosis (necrostatins, (301)) *in vitro* to assess the contribution of each cell-death pathway to mononuclear phagocyte death during *Klebsiella* pneumonia. Additional experiments can utilize mouse models unable to undergo apoptosis (Caspase knockout) and necroptosis (Ripk3 knockout) to further define the molecular mechanism of mononuclear phagocyte death during infection.

Recombinant M-CSF as a therapeutic

We found that the absence of M-CSF during *Klebsiella* pneumonia negatively influences host defenses. While the administration of exogenous M-CSF in the context of specific fungal infections has been shown to result in beneficial host responses (241,302), it is unknown if additional M-CSF administered to an M-CSF sufficient host could confer protection against *Klebsiella* pneumonia, ultimately resulting in improved host responses during infection or even host resistance to infection. To address this, we undertook preliminary experiments to assess the contribution of recombinant M-CSF on host defenses in M-CSF sufficient mice. Murine recombinant M-CSF was administered intraperitoneally one day prior to challenge with *Klebsiella pneumoniae* and the first two days following inoculation. Preliminary results suggest that administration of recombinant M-CSF intraperitoneally does not result in protection against *Klebsiella* pneumonia as measured by bacterial burden in the lungs, blood, and liver (Fig. 4.2). However, given our previous finding that M-CSF deficiency and neutralization both result in increased bacteremia during infection, it is possible that administration of M-CSF directly into the lungs would result in protective host responses culminating in reduced bacteremia and reduced mortality during infection. Thus, we propose the following model:

M-CSF is required for the survival and function of lung mononuclear phagocytes during infection. In the absence of M-CSF, lung mononuclear phagocytes die, while surviving cells fail to phagocytose and clear bacteria,

resulting in dissemination from the lungs to the blood. This bacteremia results in dissemination of Klebsiella to the liver, where mononuclear phagocytes likewise die in the absence of M-CSF. Whether by direct or indirect mechanisms, this M-CSF deficiency results in liver damage and hepatic necrosis. Altogether, absence of M-CSF results in reduced host defenses during bacterial pneumonia (Fig. 4.3).

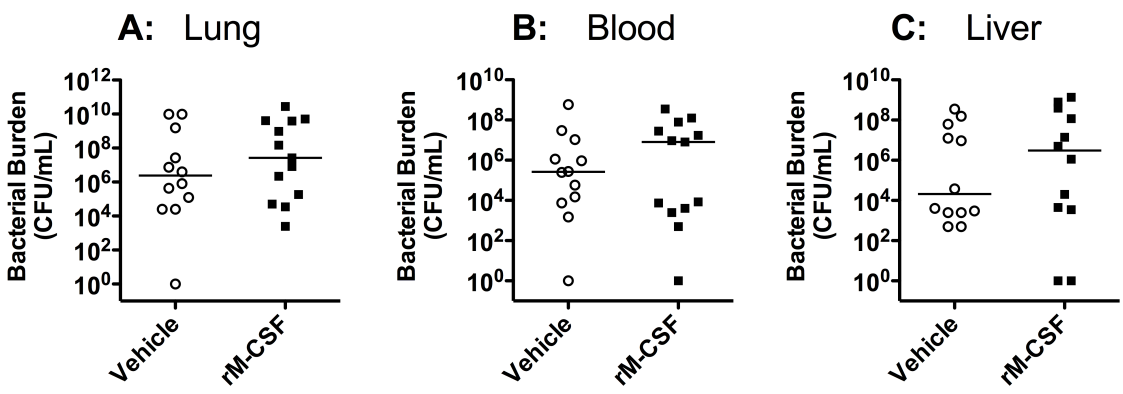
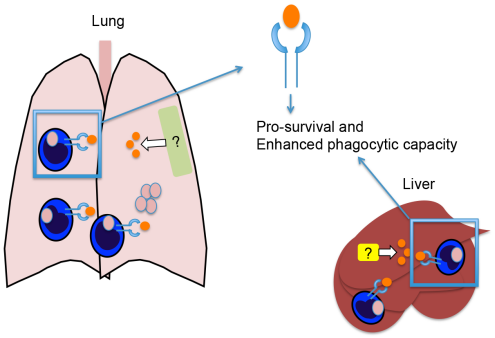
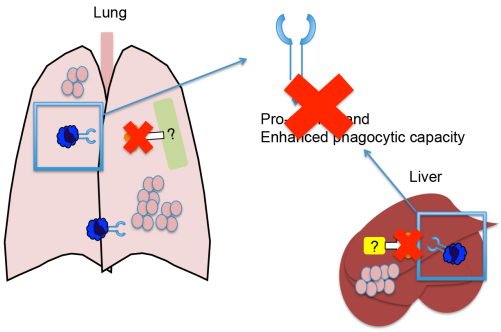

A: Lung
B: Blood
C: Liver
Bacterial Burden (CFU/mL)
10^{12}
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
Vehicle
rM-CSF

Figure 4.2. Murine recombinant M-CSF does not impact bacterial burden during *Klebsiella* pneumonia. (A) Bacterial burden in lungs of infected WT mice treated with murine recombinant M-CSF or treated with saline on day 3 post infection. (B) Bacterial burden in blood, and (C) Bacterial burden in left liver lobe. Each data point represents one animal, and horizontal lines indicate medians. Data shown are pooled from two independent experiments). *n* = 12 per group. Mice received 75 μg murine recombinant M-CSF 1 day prior to infection through day 2 post-infection. Samples with no recoverable bacteria are depicted as 1 CFU.

A: *Klebsiella* infection with M-CSF



B: *Klebsiella* infection without M-CSF



C: Model of *Klebsiella* pneumonia in the absence of M-CSF

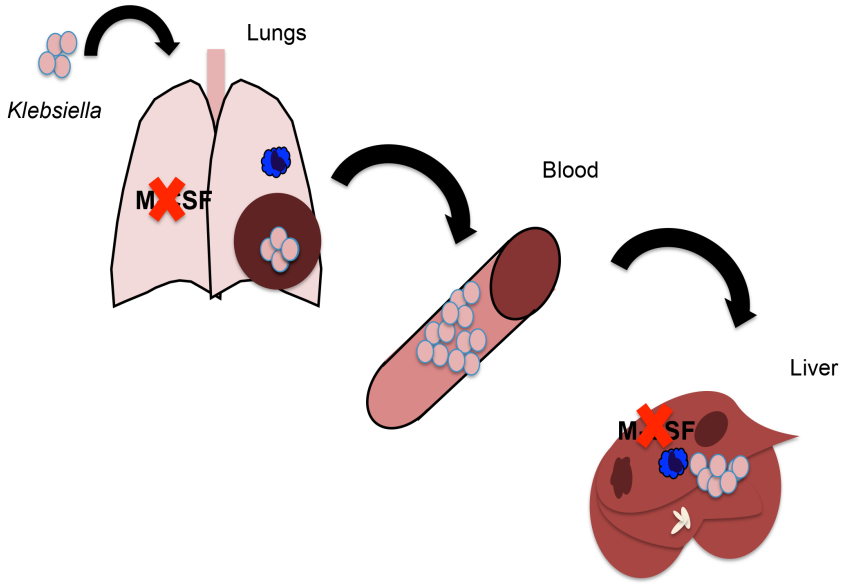


Figure 4.3. Models of M-CSF in mononuclear phagocyte-mediated host defense against bacterial pneumonia (*A*) Model of *Klebsiella* infection in the presence of M-CSF. M-CSF protein is produced by unknown cellular sources in the lungs and liver during infection and interacts with CSF1R on mononuclear phagocytes. M-CSF and CSF1R interaction results in pro-survival signals and enhanced phagocytic capacity in lung and liver mononuclear phagocytes. Pink circles, *K. pneumonia*; orange circles, M-CSF; green and yellow rectangles, cellular sources of M-CSF; blue circles, mononuclear phagocytes. (*B*) Model of *Klebsiella* infection in the absence of M-CSF. The absence of M-CSF results in mononuclear phagocyte death during infection and abrogates bacterial phagocytosis. (*C*) Model of *Klebsiella* pneumonia in the absence of M-CSF. *Klebsiella pneumonia* is aspirated into the lungs and replicates. In the absence of M-CSF, mononuclear phagocytes lose phagocytic capacity and die, resulting in reduced numbers of mononuclear phagocytes and dissemination of bacteria to the blood and liver, resulting in liver damage and hepatic necrosis.

The overall goals for this project in the future should be focused on determining the molecular mechanisms of M-CSF on the survival of mononuclear phagocytes during *Klebsiella* pneumonia. As discussed in the introduction, programmed cell death of mononuclear phagocytes may be beneficial or detrimental to the host response during bacterial pneumonia depending on the specific causative pathogen. In the absence of M-CSF, the mechanism underlying mononuclear phagocyte death during *Klebsiella* pneumonia is unknown. Additionally, M-CSF as a prophylactic versus a therapeutic should be assessed in *Klebsiella* as well as other models of bacterial pneumonia. The implications for the use of M-CSF as a therapeutic could lead to host-centric therapies for bacterial pneumonia in an era of increasing antibiotic resistance.

4.2. Gut microbiota and invasive pulmonary aspergillosis

We found that the gut microbiota influence host defense against invasive pulmonary aspergillosis in the context of neutropenia. We report two major findings: 1) the specific addition of SFB mono-associated feces to the SFB-negative recipient is sufficient to confer protection during invasive pulmonary aspergillosis, and 2) the composition of the UVa gut microbiota confers protection against invasive aspergillosis compared to the Jackson gut microbiota. To assess the role of SFB in invasive pulmonary aspergillosis, future studies should assess local lung inflammation and lung fungal burden, assess cytokine profiles of the lungs and terminal ileum that mediate protection during infection in SFB-colonized mice, and determine the composition of immune cells in the bone marrow, blood, lungs, and terminal ileum that mediate protection against infection in SFB-colonized Jackson mice**.** Additional work should define other components of the gut microbiota that mediate protection against invasive aspergillosis. To do this, future studies should identify specific mucosal microorganisms apart from SFB in UVa mice that are also sufficient to confer protection in Jackson mice during invasive pulmonary aspergillosis, and determine the specific mechanisms by which these microorganisms influence the host immune response during infection.

Impact of SFB on local lung inflammation and lung fungal burden

To further characterize the impact of SFB intestinal colonization during invasive pulmonary aspergillosis, future studies should assess lung histology between SFB-positive and -negative Jackson mice during infection. It is possible that intestinal colonization with SFB could influence immune cell phenotype or function, resulting in greater influx of immune cells as measured by histological analysis. Differences in immune cell function or composition might also influence phagocytosis and killing of *A. fumigatus*, and so lung fungal burden should also be assessed. Along these same lines, additional future experiments might consider assessing *in vitro* fungal killing by bone marrow-derived macrophages and dendritic cells harvested from SFB-colonized Jackson mice compared to SFB-negative Jackson mice. It is possible that colonization with SFB could result in enhanced antimicrobial functions of these immune cells in the presence of *A. fumigatus*, as suggested by results in other models of infection (204).

Cytokine profiles of SFB-colonized Jackson mice during invasive pulmonary aspergillosis

We performed preliminary experiments to begin to assess cytokine profiles in SFB-colonized Jackson mice compared to SFB-negative Jackson mice during infection. Preliminary data show low transcript levels of IL-17, IL-22, and GM-CSF in the lungs of mice infected with *A. fumigatus*, perhaps suggesting that these cytokines are not critical to host defense against invasive pulmonary aspergillosis in the lungs (Fig. 4.4*A*-*C*). Alternatively, it is possible that these cytokines are not transcribed in the lungs, but that the proteins are made elsewhere and then transported to the lungs. We have not yet assessed the levels of these cytokines in the terminal ileum, where SFB adheres and colonizes the host. Additionally, these data are representative of only one experiment, and so it is necessary that this be repeated before drawing conclusions. In the absence of evidence suggesting a role of the IL-17 family cytokines in SFB-mediated protection, future experiments should assess levels of Th1 and Th2 associated cytokines including IFNγ, IL-12p35, TNF-α, IL-25, IL-4, and IL-13. Additionally, it will be interesting to assess G-CSF levels in the lungs and terminal ileum of these mice, since a common mechanism of protection in SFB-colonized mice is neutrophilia (197,204). However, this is an unlikely result as our mouse model of infection necessitates neutrophil depletion. Regardless, SFB colonization might influence production of G-CSF and thus cause the repopulation of circulating neutrophils more quickly than in SFB-negative Jackson mice. This can be further assessed by quantifying numbers of neutrophil

precursors. Assessing the cytokine profiles of these mice will inform additional future studies that will assess the composition of immune cells during infection.

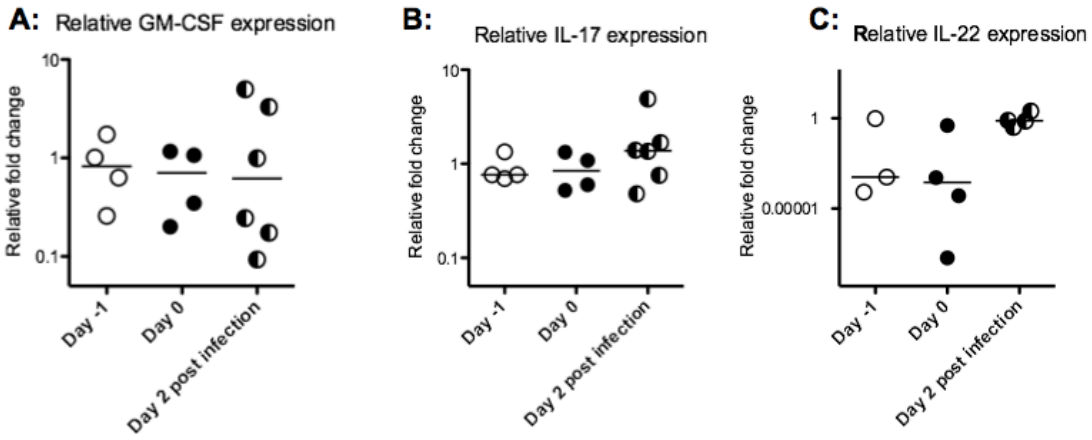

A:
Relative GM-CSF expression
Relative fold change
10
1
0.1
Day -1
Day 0
Day 2 post infection
B:
Relative IL-17 expression
Relative fold change
10
1
0.1
Day -1
Day 0
Day 2 post infection
C:
Relative IL-22 expression
Relative fold change
1
0.00001
Day -1
Day 0
Day 2 post infection

Figure 4.4. Relative expression of lung cytokine mRNA during invasive pulmonary aspergillosis. (A) Relative expression of GM-CSF in SFB+ Jackson vs. SFB- Jackson mice day 2 post-infection. (B) Relative expression of IL-17 in SFB+ Jackson vs. SFB- Jackson mice and (C) Relative expression of IL-22 in SFB+ Jackson vs. SFB- Jackson mice. n = 3-6 per group from one experiment. Each data point represents an independent biological replicate.

Immune cell composition in SFB-colonized Jackson mice during invasive pulmonary aspergillosis

We performed preliminary experiments to begin to assess the composition of immune cells in SFB-colonized mice compared to SFB-negative mice during infection. Because SFB-mediated protection occurs in the first 5 days after infection (Fig. 3.3), we focused on innate immune cells including neutrophils and monocytes. We assessed the numbers of bone marrow neutrophils and monocytes in SFB-colonized and SFB-negative Jackson mice at baseline and on day 2-post infection (Fig. 4.5 *A*-*C*). Preliminary data show no significant differences in bone marrow neutrophil and monocyte numbers between SFB-colonized or SFB-negative Jackson mice at baseline or during infection. These data might suggest that monocytes are dispensable to SFB-mediated host defense, and that SFB-mediated protection in this model is independent of neutrophilia. However, we have not yet assessed the numbers of these cells in the blood, lungs, or terminal ileum. Additionally, these preliminary data are representative of only one experiment, and so it is necessary that this be repeated before drawing conclusions. Future studies should also assess the contribution of innate lymphoid cells (ILCs), which have been implicated as key mediators to host defenses in various models of fungal and bacterial infections and are potent produces of IL-17 family cytokines (106,303,304). The potential model is that SFB induce IL-17 and GM-CSF production in ILCs (305) which activates monocytes and enhances monocyte-derived macrophage and DC

effector function, leading to inflammation associated with destruction of fungus and improved outcome.

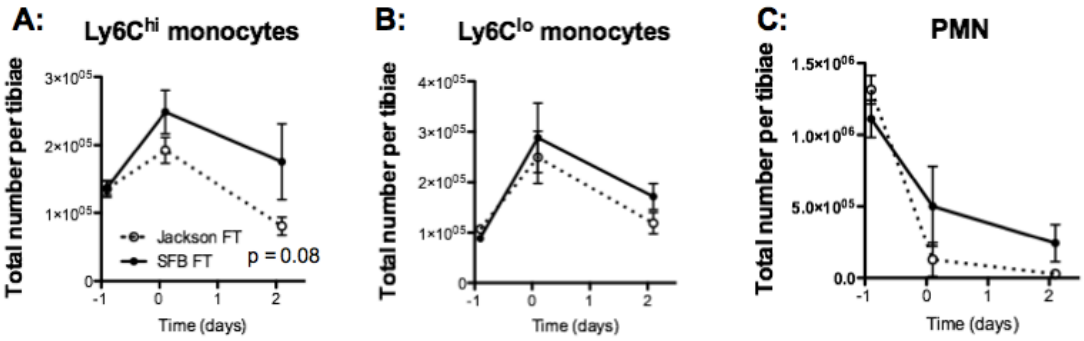

A:
Ly6C^hi monocytes
Total number per tibiae
3×10^05
2×10^05
1×10^05
0
Jackson FT
SFB FT
p = 0.08
-1
0
1
2
Time (days)
B:
Ly6C^lo monocytes
Total number per tibiae
4×10^05
3×10^05
2×10^05
1×10^05
0
-1
0
1
2
Time (days)
C:
PMN
Total number per tibiae
1.5×10^06
1.0×10^06
5.0×10^05
0.0
-1
0
1
2
Time (days)

Figure 4.5. Total numbers of bone marrow neutrophil and monocyte numbers in SFB+ Jackson mice compared to SFB- Jackson mice. (A) Time-series of mean +/- SEM of bone marrow Ly6C^{hi} monocytes, (B) Time series of mean +/- SEM of bone marrow Ly6C^{lo} monocytes, and (C) Time series of mean +/- SEM of bone marrow neutrophils. n = 4-6 per group per time point. Data shown are from one experiment.

Contributions of gut microbiota from UVa mice during invasive pulmonary aspergillosis

Additional future experiments should utilize genomic deep sequencing to identify additional microorganisms present in the UVa gut microbiota apart from SFB that contribute to host defenses against invasive pulmonary aspergillosis. These experiments should compare differences in 16S (bacterial) and 18S (fungal) sequences in the feces of Jackson mice compared to UVa mice. Utilizing a similar approach to Ivanov et al. 2009, these data can inform additional experiments that can then assess candidate-organism-directed approaches to define the composition of the gut microbiota that can contribute to host defenses during invasive pulmonary aspergillosis. These future experiments can closely mirror the experiments laid out for SFB-colonized mice that will assess the specific contributions of SFB to host defense.

Finally, additional and broad goals for this project should seek to assess how the intestine and lungs communicate: how do the gut microbiota signal to the immune system, and how does this signaling affect immunity in the lungs? To our knowledge, this has not been at all addressed in the literature. Additionally, studies should assess the ability of the gut microbiota to promote host defense in humans, as has been studied in the context of bone marrow transplantation and infection with *Clostridium difficile* (277,278). These studies might lead to the discovery of additional risk factors associated with invasive pulmonary aspergillosis and other infectious agents, ultimately paving the way for new therapeutic agents to combat infection.

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