Microbial Manipulation of Phagocyte Function During Infection and Health

Christopher Bruce Hergott
University of Pennsylvania, hergott@mail.med.upenn.edu

Follow this and additional works at: http://repository.upenn.edu/edissertations

Part of the Allergy and Immunology Commons, Immunology and Infectious Disease Commons, Medical Immunology Commons, and the Microbiology Commons

Recommended Citation
Hergott, Christopher Bruce, "Microbial Manipulation of Phagocyte Function During Infection and Health" (2015). Publicly Accessible Penn Dissertations. 1760.
http://repository.upenn.edu/edissertations/1760

This paper is posted at ScholarlyCommons. http://repository.upenn.edu/edissertations/1760
For more information, please contact libraryrepository@pobox.upenn.edu.
Microbial Manipulation of Phagocyte Function During Infection and Health

Abstract
Phagocytic cells comprise a central component of the inflammatory response to pathogens, particularly against extracellular bacteria that proliferate on mucosal surfaces. Mounting evidence suggests that microbes can manipulate phagocyte function dynamically to shape the persistence and efficacy of antibacterial defenses. Successful pathogens often restrain inflammatory responses to evade clearance and promote dissemination within the host. In contrast, commensal bacterial communities have been shown to bolster the functional capacity of phagocytes throughout the body. Despite the critical role of microbe-phagocyte interactions in maintaining health and dictating infection outcome, the mechanisms underlying this influence remain incompletely understood. Here, we examined the impact of pathogenic and commensal microbes on the functions of neutrophils, monocytes, and macrophages, three phagocyte subsets indispensable for antibacterial host defense. Using a mouse model of upper airway infection, we found that the bacterial pathogen Streptococcus pneumoniae (the pneumococcus) exploits molecular mimicry to disarm responding neutrophils. Phosphorylcholine (ChoP) moieties displayed on the exterior of the pneumococcus and within the inflammatory phospholipid platelet-activating factor (PAF) allow the microbe to leverage its ChoP-remodeling enzyme, Pce, to remove PAF from the airway. Neutrophils deprived of PAF signaling fail to eliminate bacteria effectively, allowing the pneumococcus to persist, disseminate systemically, and transmit efficiently between hosts. We found that the pneumococcus also manipulates mononuclear phagocyte responses by stimulating the liberation of macrophage migration inhibitory factor (MIF), a cytokine responsible for retaining macrophages at sites of inflammation. MIF-driven macrophage responses accelerate pneumococcal clearance from the upper airway. However, MIF signaling provokes damaging inflammation and impairs bacterial control during pneumococcal pneumonia, underscoring the tight regulation of phagocyte responses required for effective host defense. Finally, we studied the impact of signals from the intestinal microbiota on systemic phagocyte lifespan, a key component of cellular fitness at homeostasis. We found that a neomycin-sensitive cohort of commensal bacteria augments the survival and circulating lifespan of neutrophils and inflammatory monocytes. This stimulation required signaling through the intracellular peptidoglycan sensor Nod1 and liberation of the pro-inflammatory cytokine IL-17A. Together, these data demonstrate that bacteria modulate phagocyte physiology during infection and health, influencing host readiness and response to pathogenic threats.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
Jeffrey N. Weiser

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/1760
MICROBIAL MANIPULATION OF PHAGOCYTE FUNCTION 
DURING INFECTION AND HEALTH

Christopher B. Hergott

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2015

Supervisor of Dissertation

____________________

Jeffrey N. Weiser

Professor Emeritus of Microbiology and Pediatrics

Graduate Group Chairperson

____________________

Daniel S. Kessler, Associate Professor of Cell and Developmental Biology

Dissertation Committee

Igor E. Brodsky, Assistant Professor of Microbiology

Gary D. Wu, Professor of Gastroenterology

Dmitry I. Gabrilovich, Professor of Pathology and Laboratory Medicine

George Hajishengallis, Professor of Microbiology
ABSTRACT

MICROBIAL MANIPULATION OF PHAGOCYTE FUNCTION
DURING INFECTION AND HEALTH

Christopher B. Hergott
Jeffrey N. Weiser

Phagocytic cells comprise a central component of the inflammatory response to pathogens, particularly against extracellular bacteria that proliferate on mucosal surfaces. Mounting evidence suggests that microbes can manipulate phagocyte function dynamically to shape the persistence and efficacy of antibacterial defenses. Successful pathogens often restrain inflammatory responses to evade clearance and promote dissemination within the host. In contrast, commensal bacterial communities have been shown to bolster the functional capacity of phagocytes throughout the body. Despite the critical role of microbe-phagocyte interactions in maintaining health and dictating infection outcome, the mechanisms underlying this influence remain incompletely understood. Here, we examined the impact of pathogenic and commensal microbes on the functions of neutrophils, monocytes, and macrophages, three phagocyte subsets indispensable for antibacterial host defense. Using a mouse model of upper airway infection, we found that the bacterial pathogen *Streptococcus pneumoniae* (the pneumococcus) exploits molecular mimicry to disarm responding neutrophils. Phosphorylcholine (ChoP) moieties displayed on the exterior of the pneumococcus and
within the inflammatory phospholipid platelet-activating factor (PAF) allow the microbe to leverage its ChoP-remodeling enzyme, Pce, to remove PAF from the airway. Neutrophils deprived of PAF signaling fail to eliminate bacteria effectively, allowing the pneumococcus to persist, disseminate systemically, and transmit efficiently between hosts. We found that the pneumococcus also manipulates mononuclear phagocyte responses by stimulating the liberation of macrophage migration inhibitory factor (MIF), a cytokine responsible for retaining macrophages at sites of inflammation. MIF-driven macrophage responses accelerate pneumococcal clearance from the upper airway. However, MIF signaling provokes damaging inflammation and impairs bacterial control during pneumococcal pneumonia, underscoring the tight regulation of phagocyte responses required for effective host defense. Finally, we studied the impact of signals from the intestinal microbiota on systemic phagocyte lifespan, a key component of cellular fitness at homeostasis. We found that a neomycin-sensitive cohort of commensal bacteria augments the survival and circulating lifespan of neutrophils and inflammatory monocytes. This stimulation required signaling through the intracellular peptidoglycan sensor Nod1 and liberation of the pro-inflammatory cytokine IL-17A. Together, these data demonstrate that bacteria modulate phagocyte physiology during infection and health, influencing host readiness and response to pathogenic threats.
# TABLE OF CONTENTS

ABSTRACT ................................................................................................................................. ii

LIST OF TABLES ........................................................................................................................ vii

LIST OF FIGURES ........................................................................................................................ viii

CHAPTER 1: Introduction ........................................................................................................ 1

Introduction to Phagocytes in Anti-Bacterial Defense .............................................................. 2
Neutrophils .................................................................................................................................. 3
Monocytes and Macrophages ..................................................................................................... 9
Principles of Phagocyte Evasion by Bacteria ........................................................................... 12
Introduction to *Streptococcus pneumoniae* ............................................................................ 15
Murine Models and the Host Response to *S. pneumoniae* ......................................................... 17
Pneumococcal Strategies for Phagocyte Evasion .................................................................... 19
Phagocyte Function at the Mucosa: The Roles of PAF and MIF ............................................ 22
The Microbiota and Systemic Professional Phagocyte Responses ........................................ 23

DISSERTATION AIMS ................................................................................................................ 26

CHAPTER 2: Bacterial exploitation of phosphorylcholine mimicry suppresses inflammation to promote airway infection .............................................................. 29

Abstract ................................................................................................................................... 30
Introduction .............................................................................................................................. 31
Results ....................................................................................................................................... 34
Discussion ................................................................................................................................. 45
CHAPTER 3: Macrophage migration inhibitory factor (MIF) promotes clearance of pneumococcal colonization

Abstract.........................................................................................................................82
Introduction......................................................................................................................83
Methods..........................................................................................................................86
Results.............................................................................................................................90
Discussion.......................................................................................................................96
Figure Legends...............................................................................................................100
Figures.............................................................................................................................105

CHAPTER 4: Brief Report: Macrophage migration inhibitory factor (MIF) is detrimental in pneumococcal pneumonia and a target for therapeutic immunomodulation

Abstract..........................................................................................................................113
Introduction....................................................................................................................113
Methods.........................................................................................................................114
Results.............................................................................................................................117
Discussion.......................................................................................................................119
Figure Legends...............................................................................................................122
LIST OF TABLES

Table 2.S1. List of primers used.................................................................66
LIST OF FIGURES

Figure 2.1. Pce-deficient pneumococci exhibit impaired persistence in the upper airway and elicit the recruitment of more activated, viable, and durable neutrophils to the nasal lumen........................................................................................................................................67

Figure 2.2. Pce promotes invasive pneumococcal disease and bacterial transmission between mice.................................................................................................................................68

Figure 2.3. Pce prevents accumulation of PAF in the lumen of the upper respiratory tract, and the absence of Pce stimulates transcription of genes important for PAF signaling........................................................................................................................................69

Figure 2.4. Pce is dispensable for pneumococcal persistence in the absence of infiltrating neutrophils or intact PAF signaling in the upper airway........................................................................70

Figure 2.5. Pce esterase hydrolyzes ChoP from conjugated substrates and directly inhibits PAF-mediated stimulation of neutrophil activation and function in vitro........71

Figure 2.6. Exogenous stimulation of neutrophils in situ overwhelms Pce-mediated immune evasion........................................................................................................................................72

Figure 2.7. H. influenzae GlpQ hydrolyzes ChoP and contributes to evasion of PAF-mediated neutrophil defense of the airway........................................................................................................73

Figure 2.S1. Clearance of Pce-deficient pneumococci on day 14 p.i. and upper airway luminal gating strategy........................................................................................................................................74

Figure 2.S2. Assessment of the direct effects of PCA-4248, PAF, and fMLP on pneumococcal growth and viability in vitro........................................................................................................75
Figure 2.S3. Quantification of complement deposition, capsule expression, and ChoP levels by bacterial flow cytometry

Figure 3.1. MIF is important for the clearance of pneumococcal colonization

Figure 3.2. Pneumococcal colonization leads to local upregulation and systemic production of MIF in WT mice

Figure 3.3. MIF is required for the macrophage influx and MCP-1 upregulation in response to pneumococcal colonization

Figure 3.4. Pneumolysin mediates the effect of MIF on pneumococcal colonization

Figure 3.5. Human macrophages differentiated from PBMCs were infected with 23F pneumococci

Figure 3.6. MIF promotes the generation of adaptive responses to pneumococcal colonization

Figure 3.7. Nasopharyngeal treatment with rMIF recovers the MIF-dependent defect in macrophage recruitment and pneumococcal clearance

Figure 4.1. MIF is detrimental to survival and increases lung pathology in a mouse model of pneumococcal pneumonia

Figure 4.2. MIF regulates inflammation, influencing both bacterial load and survival in pneumococcal pneumonia

Figure 5.1. Antibiotic treatment accelerates turnover of circulating phagocytes from the bloodstream
Figure 5.1. Antibiotic treatment accelerates turnover of circulating phagocytes from the bloodstream.........................................................152

Figure 5.2. Antibiotic treatment corresponds with impaired phagocyte survival ex vivo.................................................................153

Figure 5.3. Metagenomic analysis of the murine intestinal microbiota upon treatment with neomycin..................................................154

Figure 5.4. The intracellular peptidoglycan receptor Nod1 is necessary and sufficient to mediate the microbial influence on phagocyte lifespan......................................157

Figure 5.5. Neutralization of IL-17A abrogates the impact of the microbiota and Nod1 on phagocyte lifespan........................................158

Figure 5.S1. Gating strategies for neutrophils and inflammatory monocytes.................................................................159

Figure 5.S2. Effects of neomycin treatment on apoptosis among lymphocytes, endothelial cells, fibroblasts, and macrophages.................................160

x
CHAPTER 1:

Introduction

Christopher B. Hergott

Department of Microbiology, University of Pennsylvania,

Philadelphia, PA, 19104, USA
Introduction to Phagocytes in Anti-Bacterial Defense

In the 133 years since Ilya Metchnikoff’s seminal discovery of immune cells capable of engulfing foreign threats, phagocytes have become a central focus in the study of inflammatory responses to bacterial infection (323). While most cells in the body bear a modest capacity for microbial uptake, a specialized cadre of leukocytes—including neutrophils, monocytes, and macrophages—serve particularly crucial roles in clearing the host of bacterial pathogens. Termed “professional phagocytes” collectively, these cells react swiftly to stimulation by diverse pathogen-associated molecular patterns (PAMPs) and employ a potent array of bactericidal mechanisms to digest ingested microbes (114). Critically, host protection by phagocytes can commence without recognition of pathogen-specific antigens or instruction by the adaptive immune system (189). These cells therefore provide essential early control of bacterial growth and impede systemic dissemination before subsequent phases of immunity develop.

While each subset of professional phagocytes serve distinct roles in fighting infection, they share several fundamental characteristics. Most circulating phagocytes arise continuously from hematopoietic myeloid precursors in the adult bone marrow (88). The cells egress from the marrow in response to homeostatic cytokine signals from the periphery, circulate through the bloodstream, and seed tissues for residence and turnover (289). Infection perturbs each component of the phagocyte life cycle: myeloid precursors proliferate to spawn more mature phagocytes from the marrow; the lifespan of circulating phagocytes extends to enhance their persistence in combating pathogens; and inflammatory signals augment the recruitment and bactericidal capacity of phagocytes responding to infectious foci (94, 185, 215, 247). Despite these common
properties, the functions and regulation of neutrophils, monocytes, and macrophages
differ substantially and warrant individual examination.

**Neutrophils**

Neutrophils, also known as polymorphonuclear phagocytes (PMNs), comprise the most abundant leukocyte subset in the human bloodstream. Released from the bone marrow at a rate of \(\sim 10^{11}\) cells per day, these terminally differentiated phagocytes constitute more than half of all steady-state hematopoietic output (224). Neutrophils are recruited from the circulation upon bacterial infection by a diverse set of pro-inflammatory cytokines and chemokines (e.g. tumor necrosis factor α, interleukins 1β and 6, CXCL1 and -2) liberated by tissue-resident cells at sites of inflammation (25). Upon reaching the focus of infection, neutrophils engulf and destroy invading bacteria, release a multitude of antimicrobial proteins from stored granules into the tissue environment, and extrude their chromatin in the form of neutrophil extracellular traps (NETs) to impede microbial dissemination (38). These mechanisms cooperate to form a vital component of antibacterial defense. Accordingly, individuals suffering from defects in neutrophil abundance or function exhibit profound immunodeficiency, with increased risks of systemic infection by a wide range of bacterial pathogens, sepsis, and death (96, 293).

The life cycle of neutrophils begins with their development from hematopoietic progenitors in the bone marrow. While they share a common cellular origin with monocytes (termed granulocyte/monocyte progenitors, GMPs), neutrophils diverge from the monocytic lineage through the action of granulocyte colony-stimulating factor (G-
CSF), the key cytokine guiding neutrophil development (200). The maturation of granules bearing antimicrobial mediators delineates the subsequent steps in terminal neutrophil differentiation. Primary granules, which contain defensins, elastase, serine proteases, and myeloperoxidase, form at the early pro-myelocyte stage. Secondary granules form in the myelocyte stage that follows, bearing key components of the NADPH oxidase enzyme responsible for the bactericidal “respiratory burst,” along with lactoferrin and lysozyme. Last, tertiary granules containing cathepsin and gelatinase form during the “band stage” directly preceding the emergence of fully mature, segmented neutrophils (38-40). It is important to note that the sequence in which these granules develop does not reflect the sequence in which they act upon ingested microbes. The contributions of granule contents to antimicrobial defense are discussed below.

Following maturation, counterpoised chemokine signals regulate the release of neutrophils from the bone marrow. CXCL12, acting through its receptor CXCR4, mediates the retention of mature neutrophils in the marrow to maintain a central pool of mature phagocytes available to fight infection (38, 100, 145). Opposing this force is CXCR2, through which CXCL1 and CXCL2 signal to liberate neutrophils from the marrow at homeostasis (99). The balance of these competing influences promotes steady peripheral abundance of cells at homeostasis. In contrast, systemic inflammation disrupts the equilibrium of neutrophil mobilization to promote trafficking of phagocytes into the periphery en masse. Systemic infection with *Listeria monocytogenes* or signaling through Toll-like receptors (TLRs) 2 and 4 have been shown to deplete central neutrophil stores, indicating that direct detection of pathogens can accelerate phagocyte egress (36, 248). Moreover, systemic infections upregulate the circulating levels of
cytokines that enhance neutrophil mobilization, including G-CSF, GM-CSF, and IL-6 (215). While inflammatory mobilization of neutrophils augments the pool of phagocytes actively engaged in fighting pathogens, bone marrow exhaustion and host demise can result if this response fails to swiftly control bacterial dissemination.

While neutrophils serve an important role in controlling disseminated infections, their protective role begins most often in response to localized infections within host tissues. Phagocyte trafficking to sites of inflammation requires targeted, efficient exit from the vasculature. For neutrophils, this process is governed by sequential interactions with endothelial cells lining the blood vessels of inflamed tissue. First, the speed of neutrophil transit within the vasculature is slowed by low-affinity interactions with the endothelium, causing the cells to roll along the luminal vascular surface. Neutrophil rolling is initiated primarily by the binding of E- and P-selectins displayed on the endothelia to ligands expressed on the neutrophil surface, including E-selectin ligand 1 (ESL-1) and P-selectin ligand 1 (PSGL-1) (38, 50, 56). Together, these interactions trigger reorganization of neutrophil cytoskeletal structure to enhance display of the $\beta_2$ integrins LAF-1 and Mac-1 on the neutrophil surface. Binding of these integrins to their ligands on endothelia, Intercellular Adhesion Molecules (ICAM)-1 and -2, permits firm adhesion of neutrophils and mediates their transit through the vascular wall and into inflamed tissue (110, 358).

After trafficking to infected tissues, neutrophils deploy a plethora of bactericidal mechanisms to eliminate the pathogens with which they are now in close proximity. These defenses can be divided into two general categories: those requiring pathogen
uptake and degradation (phagocytic) and those requiring release of antimicrobial mediators into the extracellular environment (non-phagocytic).

Phagocytic uptake begins with the binding of pathogens to receptors expressed on the surface of the neutrophil. Engulfment is accelerated dramatically if microbes are first coated with soluble inflammatory factors, such as antibody or complement. These bind to antigens exposed on the exterior of microbes and provide a conserved target of recognition for uptake receptors in a process termed opsonization (97). Accordingly, opsonin receptors comprise the most well-studied molecules governing microbial uptake into neutrophils and are upregulated readily by microbial products and inflammatory cytokines (186). Uptake of many bacterial pathogens relies predominantly on complement Receptor 3 (CR3, Mac-1)—which recognizes complement component C3b—and Fc receptors (FcRs), which exhibit specificity for the conserved, basal component of antibodies that are bound to microbial surfaces (194). The mechanistic details of opsonization are beyond the scope of this discussion and are reviewed extensively elsewhere (272, 286). However, despite the diversity of opsonins involved in bacterial uptake, engagement of their receptors on neutrophils yields a common result: invagination of the neutrophil plasma membrane to engulf microbes in an internal compartment called the phagosome.

Phagosome formation within neutrophils is followed swiftly by its fusion with membrane-bound granules bearing the enzymes and toxic mediators responsible for bacterial killing. In contrast to macrophages and other professional phagocytes, bacterial elimination within neutrophils does not require acidification of the phagosomal
environment (159). Neutrophils instead rely on a combination of oxidative and non-oxidative means to destroy ingested bacteria at neutral pH (292).

Oxidative killing requires the liberation of reactive oxygen species (ROS) within the phagosome to attack and degrade microbial structures. After assembly at the phagosome membrane, the multi-subunit enzyme NADPH oxidase begins this “respiratory burst” by generating superoxide anions capable of disrupting microbes directly and serving as substrates for the generation of other ROS. Downstream production of hydrogen peroxide (through dismutation of superoxide anions), reactive halides (catalyzed by myeloperoxidase enzymes), and other oxygen radical species provides the neutrophil with a diverse repertoire of bactericidal molecules with which to attack microbial structures (74, 292). While the means by which ROS destroy bacteria remain under active investigation, proposed mechanisms include disruption of bacterial membrane integrity, degradation of essential bacterial enzymes, and damage to bacterial DNA (108). Patients who suffer from a congenital absence of NADPH oxidase components (termed chronic granulomatous disease, CGD) illustrate the central importance of the respiratory burst to bacterial control. Neutrophils lacking functional NADPH oxidase activity exhibit markedly impaired ROS production, resulting in defective microbial killing (105). Accordingly, these patients show markedly enhanced susceptibility to infections by diverse bacterial pathogens, including many that rarely cause disease in immunocompetent individuals (e.g. Klebsiella species, *E. coli*, *S. aureus*, *Nocardia* species) (354).
In addition to oxidative mechanisms, neutrophils kill ingested bacteria through the action of antimicrobial proteins, introduced to the phagosome through successive fusions with cytoplasmic granules. First, secondary granules introduce lysozyme and defensins that rupture bacteria, along with proteins like lactoferrin and lipocalin that deprive the microbe of requisite nutrients (20, 54). Shortly thereafter, primary granules bring pore-forming defensins and serine proteases to the phagosome. These factors disrupt the osmotic balance of bacteria and degrade microbial proteins to accelerate microbial death (261, 351). Lastly, cathepsins and gelatinase in tertiary granules provide further bactericidal pressure through microbial proteolysis (39).

More recently, neutrophils have been shown to kill extracellular bacteria without phagocytosis through the liberation of neutrophil extracellular traps (NETs). NETs are formed upon neutrophil activation by pathogens through the extrusion of fibers composed of neutrophil chromatin, proteases, and anti-microbial peptides. Diverse gram-negative and gram-positive bacteria associate with and become enmeshed within this NET lattice, inhibiting their growth and accelerating microbial clearance (45). The mechanisms driving NET formation in vivo remain incompletely understood, but are thought to involve an orchestrated de-condensation of neutrophil chromatin governed by the enzymes peptidyl arginine deaminase type IV (PAD4) and neutrophil elastase (184). Further study has revealed that neutrophils can remain vital and exert phagocytosis in vivo despite extruding their genomic content in NETs (363). Thus, neutrophils appear to employ multiple, parallel levels of defense against invading bacteria from both within and outside the phagocyte cell body.
**Monocytes and Macrophages**

The mononuclear phagocyte system (MPS) comprises another subset of professional phagocytes essential for antibacterial defense. The MPS is composed of monocytes, macrophages, and dendritic cells, defined originally by common hematopoietic origins in the bone marrow (162). While dendritic cells (DCs) engulf microbes efficiently and are considered professional phagocytes, they function primarily to present microbial antigens to the adaptive immune system rather than killing bacteria directly (238). Therefore, while reviewed extensively elsewhere (237), dendritic cells will not be discussed in detail here.

Monocyte and macrophage homeostasis in adults has traditionally been defined as a continuum of hematopoietic development, systemic circulation of monocytes, differentiation into macrophages, and deposition of macrophages into tissues as resident phagocytes (162). However, recent findings challenge this classical dogma. Resident macrophages from a number of tissues have been shown to arise from progenitors deposited during embryonic life, proliferating locally and without input from the hematopoietic system in adult life (127). In turn, monocytes can exit from the circulation and survey host tissues without differentiating into macrophages (158). While our understanding of the MPS continues to develop and evolve, it remains clear that the classical monocyte-macrophage axis continues to replenish macrophages populations from many tissues (e.g. the gastrointestinal tract) in adults and is the primary mechanism by which these cells proliferate during inflammation (22). The discussion below will therefore focus primarily upon this paradigm of ontogeny.
Monocytes develop in the bone marrow as part of normal hematopoiesis. Arising from a progenitor cell shared with neutrophils (granulocyte-monocyte progenitors, GMP), monocytes differentiate under the guidance of M-CSF, the cardinal cytokine of monopoiesis (125). Accordingly, mice lacking M-CSF or its receptor (M-CSFR) exhibit profound defects in the systemic abundance of monocytes and macrophages (63).

Monocytic lineages differentiate from that of dendritic cells through at least two parallel pathways: M-CSF-guided divergence from a precursor cell shared with DCs (monocyte-dendritic cell precursors, MDPs) and development from independent, committed precursor cells called common monocyte progenitors (cMOPs) (116, 146). Mature monocytes emigrate from the bone marrow at homeostasis under the guidance of the chemokine CCL2, permitting entry of the cells into systemic circulation (106). Notably, CCL2 also drives the accelerated egress of monocytes upon bacterial infection and systemic inflammation (295).

Circulating monocytes are divided into two primary subsets, distinguished by differential receptor expression and functional roles. In mice, “inflammatory” monocytes express high levels of the surface markers Ly6C and CCR2 (the chemokine receptor for CCL2) and are the predominant subtype recruited from the marrow in both homeostatic and inflammatory conditions. These cells exhibit a brief circulating half-life (18–24 hours) and turn over rapidly during health, but also comprise the primary subtype amplified and recruited to sites of inflammation during bacterial infection (124). “Classical” or “patrolling” monocytes do not express high levels of Ly6C or CCR2, instead upregulating the chemokine receptor CX3CR1. Classical monocytes survey blood vessel barriers at homeostasis to intercept invading pathogens and guard vascular integrity (16).
Exhibiting a longer half-life (5-7 days) than inflammatory monocytes, these cells also migrate from the vasculature and are thought to form the immediate precursors to resident macrophages and/or an independent population of tissue-resident monocytes (124). Interestingly, recent studies have established that classical, Ly6C\textsuperscript{lo} monocytes arise chiefly from trans-differentiation of inflammatory, Ly6C\textsuperscript{hi} monocytes in the peripheral circulation (364). Thus, unlike neutrophils, monocytic lineages appear to continue differentiating after egress from the bone marrow at homeostasis.

During infection, a network of chemokines recruit monocytes from the bloodstream and into inflamed tissues. Among bacterial pathogens, this process has been studied most closely in the setting of systemic infection by *Listeria monocytogenes*. Animals lacking the chemokine receptor CCR2 exhibit a profound impairment in monocyte recruitment, accelerated bacterial outgrowth, and enhanced mortality during *Listeria* infection, indicating that CCR2\textsuperscript{+} inflammatory monocytes are required for optimal bacterial clearance (190, 296). Subsequent work demonstrated that these defects are caused mainly by ineffective mobilization of the mature monocyte pool from bone marrow stores (295). Other chemokines and cytokines, including CCL7 (a CCR2 ligand) and TNF\textalpha, act in cooperation with CCL2 to govern optimal recruitment of inflammatory monocytes to sites of infection (302). Classical, CX\textsubscript{3}CR\textsubscript{1+} monocytes also augment this process. Selective depletion of CX\textsubscript{3}CR\textsubscript{1+} cells reduces systemic classical monocyte abundance and diminishes the recruitment of CCR2\textsuperscript{+} inflammatory monocytes responding to *Listeria*-infected tissues. (17).
Upon arriving at sites of infection, monocytes can contribute to bacterial killing while maintaining monocytic identity through production of reactive oxygen/nitrogen species and the release of pro-inflammatory cytokines (302, 311). However, macrophages govern the bulk of bacterial phagocytosis. While the mechanisms governing microbial uptake by macrophages are broadly similar to those of neutrophils, macrophage phagosome dynamics after pathogen engulfment are distinct. Macrophage phagosomes mature and acquire bactericidal factors through successive fusions with cytoplasmic vesicles and lysosomes rather than granules (114). Also in contrast to neutrophils, macrophages acidify phagolysosomes to accelerate bacterial killing. Membrane-bound V-ATPase enzymes pump protons into the luminal environment of the phagolysosome in an ATP-dependent manner to damage microbial structures, inhibit bacterial growth, and optimize the function of bactericidal proteases (114, 150). In addition to the reactive oxygen species described for neutrophils, macrophages more readily produce reactive nitrogen species (RNS) like nitric oxide (NO). Generated by the inducible nitric oxide synthase enzyme (iNOS, NOS2), NO attacks microbial proteins, lipids, and nucleic acids to accelerate bacterial degradation (319). Macrophages also deploy a number of the antimicrobial peptides and proteases described above for neutrophils, though many of these proteases function optimally at low pH in adaptation to the acidic environment of the macrophage phagolysosome (319).

**Principles of Phagocyte Evasion by Bacteria**

Successful microbes must evade phagocytic clearance to persist at mucosal surfaces and within host tissues. Accordingly, most bacterial pathogens have evolved a complex collection of virulence factors tasked with eluding or disarming each step of phagocyte
function. While phagocyte evasion will be discussed in more detail using *Streptococcus pneumoniae* as a model bacterial pathogen, a number of general principles governing this process can be illuminated by examining common tactics used by a diversity of pathogenic microbes. These include subversion of bacterial recognition by phagocytes, uptake into cells, and degradation by phagocyte bactericidal mechanisms.

Bacterial evasion of phagocyte detection often relies upon masking surface structures or other immunogenic factors against which the host has evolved sensors or opsonins. Many bacteria produce thick polysaccharide capsules to provide this shield. Used with particularly high frequency among respiratory pathogens like *S. pneumoniae, N. meningitidis, H. influenzae*, and *P. aeruginosa*, capsules provide a physical barrier to the access of cell wall components and proteins on the exterior of the microbe (285). Capsules provide especially potent protection against opsonization by complement, which would otherwise facilitate microbe recognition by conserved uptake receptors. Complement activation fragments (e.g. C3b) bind poorly to capsular polysaccharide lattices and capsules provide suboptimal platforms for the activation of complement activation cascades (170). Accordingly, mutations in capsule production among normally encapsulated bacteria accelerate complement deposition and bacterial clearance (151). Many microbes also secrete proteins that bind to complement and antibodies, thereby inhibiting their deposition or activation (e.g. *S. aureus, S. pyogenes*). Others produce proteases that cleave complement and immunoglobulins directly (e.g. *Neisseria spp., H. influenzae, S. pneumonia, S. sanguis*) (285). The breadth and depth of anti-opsonization strategies among diverse microbes underscore the central importance of this step in facilitating phagocytic clearance.
In addition to blocking opsonization, many bacteria modify their surface structures to evade sensing by pattern recognition receptors (PRRs) and thereby inhibit the recruitment and activation of responding phagocytes. *S. typhimurium, P. gingivalis,* and *H. pylori* have all been shown to remodel surface lipopolysaccharide structures to evade detection by its host receptor, Toll-like receptor 4 (TLR4) (71, 75, 171). *C. jejuni* and *H. pylori* evade TLR5 similarly through modifications to their flagellar structures (11, 285). Together, these mechanisms serve to dampen the inflammatory response to infection, thereby reducing the numbers of recruited, activated phagocytes with which they must contend.

Upon contact with phagocytes, many bacterial pathogens employ strategies to inhibit their uptake into the cells. These include disruptions in host cell signaling essential for receptor-mediated internalization or reorganization of the phagocyte cytoskeleton to block phagosome formation. For example, *Yersinia* and *Salmonella* species inject phosphatases into phagocytes to inhibit host tyrosine phosphorylations essential for early phagocytic signaling (12, 168). *Yersinia* and *P. aeruginosa*, among others, express mimetics of host GTPase-activating proteins to terminate intracellular signaling important for actin fiber remodeling and phagosome formation (34, 126). Another tactic used commonly to evade internalization is eliminating the phagocytes entirely. A number of bacteria, including *S. pneumoniae, S. aureus,* and *S. pyogenes,* secrete toxins that form pores in the membranes of responding phagocytes, often triggering programmed or necrotic cell death (204).
Even upon successful internalization, many pathogenic bacteria retain virulence strategies to promote their prolonged survival within the intracellular environment. One tactic, used by *M. tuberculosis* and *L. pneumophila*, relies on reprogramming the intracellular trafficking of phagosomes to inhibit their fusion with lysosomal compartments and subsequent maturation (114). *L. monocytogenes* uses a pore-forming toxin, lysteriolysin O, to escape the phagosome altogether, transiting swiftly into the phagocyte cytosol (27). Still others, including *Salmonella* species and *C. burnetti*, persist within mature phagolysosomes, using acidification as a trigger for the deployment of virulence factors (8, 223). Collectively, these findings have made clear that well-adapted bacterial pathogens mount evasion strategies against each step in phagocytic clearance.

**Introduction to *Streptococcus pneumoniae***

*Streptococcus pneumoniae*, also known as the pneumococcus, was among the first bacterial pathogens studied on a molecular level and among the first for which phagocyte evasion strategies were described (345). The pneumococcus is a gram-positive, extracellular bacterium that exists as both a common microbial resident of the human upper respiratory tract and a highly invasive pathogen capable of dissemination to the lungs, bloodstream, and meninges (143). It remains a leading cause of otitis media, pneumonia, sepsis, and meningitis worldwide, especially among children, the elderly, and immunocompromised individuals. Each year, invasive pneumococcal disease is responsible for over 1 million deaths worldwide and accounts for approximately one-tenth of all deaths among children under five years old (252). In the United States, the pneumococcus remains a leading cause of community-acquired bacterial pneumonia among adults, causing thousands of deaths annually (283). The historic and ongoing
clinical burden exacted by \textit{S. pneumoniae} has long made it a subject of intensive investigation, yielding mechanistic discoveries that have illuminated fundamental principles of bacterial virulence and immune evasion.

All pneumococcal infections begin with asymptomatic colonization of the upper respiratory tract (348). In addition to serving as the platform from which invasive infections arise, pneumococcal colonization is a prerequisite for transmission between hosts via respiratory secretions and droplets (244). Carriage is disproportionately prevalent in children, where levels can exceed 50% at its peak between ages two and five. While the prevalence of colonization in adults is lower (~10%), these rates increase among adults in frequent contact with young children (257). Pneumococcal isolates circulating in populations are classically characterized by serotype, reflecting variants of the polysaccharide capsule that envelops nearly all virulent pneumococcal strains (131). Serotypes are delineated by differential reactions with antisera targeted against particular groupings of pneumococcal strains, with matched capsule-antisera mixtures leading to a characteristic swelling of the capsular polysaccharide upon microscopic examination (termed the Quellung reaction) (139). At present, more than 90 pneumococcal serotypes have been discovered and independently described (143).

Decades of epidemiologic monitoring have revealed that capsular serotypes exhibit vastly different invasive capacities and propensities for systemic disease, underscoring the critical importance of the capsule in determining pneumococcal virulence (142). Accordingly, vaccines developed to combat the pneumococcus use capsular polysaccharide from virulent strains as their immunogen. Two variants of vaccines
currently hold licenses: the pneumococcal polysaccharide vaccine (PPV) and the pneumococcal conjugative vaccine (PCV). Both confer protection through the elicitation of systemic antibody responses, facilitating opsonophagocytic clearance of pneumococci bearing capsule serotypes present in the vaccine (306, 330). PPV vaccines provide effective protection against invasive disease by 23 pneumococcal serotypes, but the poor stimulation of immunologic memory inherent to isolated polysaccharide antigen and suboptimal efficacy in preventing pneumococcal transmission has reduced the use of this vaccine to elderly adults (>65) and immunocompromised individuals (346). In contrast, the protein-conjugated PCV vaccine has become the standard of care in vaccinating children and provides robust protection against disease, colonization, and transmission between hosts (264). The PCV vaccine is presently formulated to include 13 pneumococcal serotypes, with expansion under active development. Despite the efficacy of these vaccines, pneumococcal infections continue to exact a clinical toll worldwide, necessitating the need for a mechanistic understanding of how the microbe elicits and evades host immunity.

**Murine Models and the Host Response to S. pneumoniae**

While humans comprise the sole natural host for *Streptococcus pneumoniae*, mouse models permit mechanistic dissection of host-pathogen interactions and recapitulate the cardinal host responses to pneumococcal infection (225). Within 24 hours after the acquisition of pneumococcal colonization, the bacteria adhere to the surfaces of the nasopharynx and stimulate pro-inflammatory signaling at the mucosa (166). Subsequent recruitment of phagocytes to the nasal spaces requires host detection of pneumococcal lipoproteins/lipoteichoic acid and peptidoglycan by TLR2 and nucleotide-binding
oligomerization domain-containing protein 2 (Nod2), respectively. Mice lacking either or both of these pattern recognition receptors exhibit delayed bacterial clearance from the upper airway (337). Neutrophils comprise the first wave of recruited phagocytes, reaching maximal abundance ~3 days after acquisition. Despite entering the nasal spaces and making contact with colonizing pneumococci, these cells are wholly ineffective at killing colonizing pneumococci. Systemic depletion of neutrophils from colonized mice confers no change in the kinetics of pneumococcal clearance, suggesting that these phagocytes are evaded efficiently by the microbe (222). Mice rendered deficient in complement component C3 or lacking antibody-producing B cells also exhibit normal clearance kinetics, reinforcing that acute inflammation plays little role in protection against pneumococcal colonization (227).

Facing little bactericidal pressure by the acute neutrophil response, colonizing pneumococci persist in the nasopharynx for 3-4 weeks. Eventual removal of the microbe from the upper airway is driven by a sustained influx of macrophages into the nasal lumen, and localized depletion of macrophages significantly impairs the kinetics of pneumococcal clearance (366). Optimal macrophage recruitment depends upon host detection of pneumococcal ligands by Nod2 and TLR2 as described above, the development of a robust, IL-17A-producing CD4 T cell response (Th17), and liberation of the monocyte/macrophage chemokine CCL2 (87, 366). The repertoire of Th17 cells generated during colonization forms the basis of immunologic memory to subsequent episodes of pneumococcal colonization, with rapid, IL-17A-driven neutrophil responses more swiftly clearing pneumococci upon repeated exposures (205).
In comparison with the host response to upper airway colonization, pneumococcal pneumonia elicits inflammation that is both more robust and more deleterious to host tissues. First, resident alveolar macrophages provide early phagocytic clearance of bacteria, though this response is quickly overwhelmed in the context of abundant or prolonged pneumococcal burden (93). Within hours, neutrophils and inflammatory monocytes are recruited to the lungs in large numbers, attracted by TNFα, IL-1β, CXCL1, and CXCL2 released by alveolar macrophages and epithelia (130, 217). The effectiveness of this inflammatory response in controlling bacterial abundance governs the balance between recovery and systemic dissemination, with the latter leading to host demise within days. Intriguingly, neutrophils have been shown to promote bacterial dissemination during pneumococcal pneumonia rather than conferring protection, and neutropenic mice exhibit reduced rates of sepsis (216). This deleterious impact has been attributed to liberation of cytotoxic mediators by the abundant neutrophil response, resulting in profound damage to pulmonary tissues. If the host survives this initial inflammation and clears the pneumococcus, robust Th17 cellular immunity and antibody-dependent clearance contribute to protection from subsequent re-infection of the lung (353).

**Pneumococcal Strategies for Phagocyte Evasion**

The success of *Streptococcus pneumoniae* as a respiratory pathogen in the face of a robust host defense suggests the microbe has developed well-adapted strategies for eluding clearance by phagocytes. The most well-studied among these is the pneumococcal polysaccharide capsule. Forming a 200-400 nm thick barrier around the exterior of the microbe, the capsule inhibits the deposition of opsonins and prevents
phagocytosis by neutrophils and macrophages in the absence of serotype-specific antibodies (166). Pneumococcal virulence during invasive infection is directly proportional to the thickness of the polysaccharide capsule surrounding the microbe, reinforcing the central significance of this protective mechanism (208). However, the pneumococcus also employs a number of surface-exposed virulence factors in addition to capsule expression, suggesting that capsule alone is likely insufficient to explain the totality of phagocyte evasion by the microbe.

The pneumococcus is among the many bacterial pathogens that employ secreted toxins to inhibit phagocyte responses. Pneumolysin, the pore-forming toxin produced by the pneumococcus, is conserved among nearly all pneumococcal isolates (166). Upon binding to neutrophils and macrophages, pneumolysin monomers oligomerize to form a barrel-shaped ring capable of forming large pores in the phagocyte membrane (328). This can result in direct lysis of responding phagocytes or disruption of migration and respiratory burst capacity if the toxin is present at sub-lytic concentrations (259). Pneumolysin expression is especially critical for pneumococcal survival in the lungs and systemic circulation, where abundant phagocytes readily come into contact with the microbe. Bacterial mutants lacking pneumolysin are rapidly cleared from these sites (165).

Another paradigm of pneumococcal phagocyte evasion relies upon the decoration of its surface with the small molecule phosphorylcholine (ChoP). ChoP display is a widely conserved feature of extracellular bacteria in the respiratory tract, often used to mask underlying structures from deposition of antibodies or complement that would otherwise
hasten uptake by phagocytes (67). On the pneumococcus, ChoP is covalently linked to teichoic and lipoteichoic acids emanating from the gram-positive cell wall. In addition to aiding evasion of phagocytosis, ChoP can serve to anchor pneumococci to the mucosal surface through binding to host platelet-activating factor receptor (PAFR), mimicking the phosphorylcholine moiety also present on the receptor’s cognate host ligand, platelet-activating factor (76). Pneumococci lacking ChoP exhibit drastically reduced survival during infection of both the upper and lower respiratory tract, reinforcing its central role in bacterial virulence (172).

ChoP also serves as a substrate platform to which a number of surface virulence proteins adhere (termed choline-binding proteins). Mutation of many of these proteins, including LytA, PspA, and Pce, result in fitness defects during the course of pneumococcal infection, though the mechanisms driving this accelerated clearance remain largely unknown (129). Some evidence suggests choline-binding proteins function to inhibit phagocyte responses. LytA functions to auto-lyse the pneumococcal cell wall, potentially liberating pneumolysin to attack responding phagocytes (166). PspA, in turn, is thought to inhibit complement deposition on the pneumococcal exterior, thereby inhibiting opsonization and phagocytic uptake (161). Pce functions to hydrolyze phosphorylcholine molecules from the pneumococcal cell wall in a process of continuous turnover to also inhibit ChoP-directed opsonin deposition (340). These choline-binding proteins may function cooperatively to inhibit phagocytic clearance and promote pneumococcal persistence in colonization and disease.
Phagocyte Function at the Mucosa: The Roles of PAF and MIF

While many investigations into factors governing mucosal phagocyte responses focus on their effects on cell recruitment, a growing number of inflammatory mediators have been shown to alter phagocyte function predominantly after their arrival at sites of inflammation. Here, we will discuss two secreted factors, platelet-activating factor (PAF) and macrophage migration inhibitory factor (MIF), which stimulate mucosal neutrophil and macrophage function respectively and play central roles in phagocyte responses to bacterial pathogens.

Platelet-activating factor (PAF) is a secreted, pro-inflammatory phospholipid first discovered as a mediator of systemic platelet aggregation (65, 140). Subsequent investigations revealed that PAF potently stimulates neutrophil activity after recruitment to sites of inflammation (188, 299). PAF is secreted from neutrophils, epithelial cells, and macrophages in response to inflammatory stimuli, including bacterial infection (269, 303). Through binding to its cognate receptor (PAFR) on activated neutrophils, PAF triggers diverse intracellular signaling pathways (e.g. PI3K, PLC, PLA2) to stimulate cell viability, upregulation of bacterial uptake receptors, and enhanced phagocytic capacity (48, 101, 232, 298). Accordingly, PAFR knockout mice are highly susceptible to numerous bacterial pathogens against which neutrophil responses form a key component of defense, including *K. pneumoniae, P. aeruginosa*, and *S. pneumoniae* (310, 334, 338).
Macrophage migration inhibitory factor (MIF) is a mammalian cytokine and potent activator of macrophage responses (246). Secreted by macrophages and lymphocytes in response to diverse inflammatory stimuli, MIF acts in an autocrine and paracrine manner to bind its cognate receptors (composed of diverse complexes of CXCR2, CXCR4, and CD74) on the surface of monocytes and macrophages (26). While initially characterized by its effects on macrophage mobility in vitro (35), subsequent animal studies have revealed a central role for MIF in governing innate immunity against bacterial pathogens. Mice lacking MIF exhibit enhanced susceptibility to a diverse set of infections, including *S. typhimurium, K. pneumoniae*, and polymicrobial peritonitis (183, 265, 278). The mechanism of MIF-mediated macrophage stimulation appears to be multi-factorial. Signaling downstream of MIF receptors upregulates the expression of pattern recognition receptors (e.g. TLR4) essential for detection of bacterial pathogens (277). MIF also suppresses p53 signaling within target cells to prolong the lifespan of macrophages recruited to sites of infection and stimulates the capacity of macrophages to engulf foreign particles (240, 256). Collectively, these pathways yield macrophages that are more activated, persistent, and bactericidal in response to pathogenic threats.

**The Microbiota and Systemic Professional Phagocyte Responses**

The evasion or disarmament of professional phagocytes has long been known to be essential for the persistence of pathogenic bacteria. In contrast, it has only recently become clear that the commensal bacterial flora of the gastrointestinal tract provides systemically disseminated signals during health to enhance phagocyte function upon infection (69). This suggests that disruptions in the community structure of the gut
microbiota, termed dysbiosis, can impair neutrophil, monocyte, and macrophage responses to pathogens throughout the body.

Mice raised in the absence of commensal microbes (germ-free) exhibit dysfunctions in nearly every component of neutrophil homeostasis. These mice are neutropenic in the steady state, explained at least in part by impaired granulopoiesis in the bone marrow (53, 173). Neutrophil migration to sites of inflammation is also impaired because of reduced levels of chemoattractant peptides like serum amyloid A (169). Upon arriving at infected tissues, neutrophils from germ-free animals exhibit functional defects, including impaired phagocytic capacity and reactive oxygen species production (70, 255). Accordingly, germ-free mice exert sub-optimal control over a number of extracellular bacterial pathogens during infection, including *S. pneumoniae*, *S. aureus*, and *E. coli* (70, 91).

The mononuclear phagocyte system is similarly perturbed in the absence of the gut microbiota. Systemic abundance of inflammatory monocytes and tissue resident macrophages are both reduced in germ-free mice, again correlated with impaired development in the bone marrow (69, 173). Tissue-resident macrophages exhibit defects in pro-inflammatory gene expression and liberation of inflammatory cytokines upon infection, reducing their ability to orchestrate immune responses to pathogens (2, 281). Together, these defects correspond with diminished control of infection by *L. monocytogenes*, against which the mononuclear phagocyte system is the predominant systemic defense (173). Impaired antibacterial responses extend to pulmonary infections, where reduced production of reactive oxygen species in alveolar macrophages
enhances susceptibility to \textit{K. pneumoniae} among mice with depleted commensal flora (68).

As the myriad defects in systemic phagocyte function following disruption of the microbiota become clear, attention has turned to understanding the signaling that connects commensal microbes with the phagocytes they instruct throughout the body. A number of pattern recognition receptors have been implicated in relaying gut microbial signals into systemic, low-level inflammatory responses during health. For example, TLR4 and downstream MyD88 signaling have both been shown to be important for microbial stimulation of steady-state granulopoiesis in the bone marrow (23, 53). Similarly, the cytosolic peptidoglycan receptor Nod1 is required for mediating the microbiota's role in augmenting neutrophil phagocytic capacity (70). Whether these PRRs are detecting microbial products at the intestine or after low-level, systemic circulation remains uncertain, though a recent report suggests the cytokine IL-17A serves as an intermediate messenger between commensal microbes and alterations of neutrophil homeostasis in infant mice (91). Further, the well-established connection between Nod1 signaling and the expansion of IL-17A-producing T cells within the intestinal lamina propria supports the paradigm of cytokine-dependent relays of microbe-driven signals (119). Despite these advances, the mechanistic underpinnings of communication between the gut microbiota and systemic professional phagocytes remain largely unknown, especially for mononuclear phagocytes.
Mounting evidence suggests that microbes can manipulate phagocyte function dynamically to shape the persistence and efficacy of antibacterial defenses. Successful bacterial pathogens often restrain inflammatory responses to evade clearance and promote dissemination within the host (114). In contrast, commensal bacterial communities have been shown to bolster the functional capacity of phagocytes throughout the body (281). Despite the critical role of microbe-phagocyte interactions in maintaining health and dictating infection outcome, the mechanisms underlying this influence remain incompletely understood. We hypothesized that continuous influence by microbial signals regulates the functional capacity of neutrophils and mononuclear phagocytes in both infection and health. To address this overarching hypothesis, we pursued the following specific aims:

**Aim 1: Determining whether Streptococcus pneumoniae disarms neutrophils via exploitation of phosphorylcholine mimicry.**

Infection by bacterial pathogen *Streptococcus pneumoniae* begins with asymptomatic colonization of the upper respiratory tract. Prolonged carriage depends upon efficient evasion of neutrophils recruited to the airway shortly after bacterial acquisition. Using a mouse model of upper airway infection, we found that *S. pneumoniae* exploits molecular mimicry of the host-derived molecule phosphorylcholine (ChoP) to disarm responding neutrophils and enhance bacterial survival. We determined that ChoP moieties displayed on both the bacterial cell wall and the inflammatory phospholipid platelet-activating...
factor (PAF) allowed the microbe use a hydrolytic surface enzyme, Pce, to remove PAF from the airway. Neutrophils deprived of PAF signaling exhibited impaired viability and phagocytic capacity, allowing S. pneumoniae to elude acute clearance, disseminate systemically, and transmit efficiently between hosts. Use of PAFR knockout mice and intranasal treatment with exogenous PAF affirmed that Pce-mediated PAF processing is absolutely necessary for neutrophil evasion and prolonged infection of the upper airway by S. pneumoniae.

**Aim 2: Determining whether Streptococcus pneumoniae governs macrophage responses through stimulation of macrophage migration inhibitory factor (MIF).**

While a sustained presence of macrophages in the respiratory tract is known to be necessary for clearance of Streptococcus pneumoniae, the mechanisms by which this response is maintained remained incompletely understood. We found that S. pneumoniae stimulates the liberation of macrophage migration inhibitory factor (MIF) in the upper airway to promote persistence of the luminal macrophages responsible for bacterial clearance. This stimulation required the action of the pore-forming toxin pneumolysin, and activation of intracellular p38-MAPK signaling. However, MIF-mediated macrophage persistence impaired bacterial clearance during infection of the lower respiratory tract, accompanied by enhanced neutrophil influx and damage to the lung parenchyma. Together, these results underscore the fine regulation necessary to efficiently promote bacterial clearance by mononuclear phagocytes.
**Aim 3: Determining whether the intestinal microbiota regulates the cellular lifespan of neutrophils and inflammatory monocytes at homeostasis.**

While the intestinal microbiota is known confer stimulation to phagocytes throughout the body, the mechanisms by which this occurs at homeostasis remains less clear. We found that a neomycin-sensitive cohort of commensal microbes prolongs the cellular lifespan of neutrophils and inflammatory monocytes throughout the body during health. Phagocytes deprived of microbial stimulation exhibited accelerated turnover from systemic circulation and enhanced rates of apoptosis when cultured *ex vivo*. Signaling through the cytoplasmic peptidoglycan receptor Nod1 was necessary and sufficient to detect the microbial signals responsible for regulating phagocyte lifespan, while the cytokine IL-17A was necessary to relay Nod1-dependent signals to circulating phagocytes. Together, these results suggest that steady-state signals from the intestinal flora modulate the homeostatic turnover of systemic phagocytes, a key component of maintaining fitness against infections and other inflammatory insults.

Together, these specific aims will elucidate the mechanisms by which a bacterial pathogen modulates phagocyte responses to promote persistence (Aims 1 & 2) and through which commensal microbes instruct systemic innate immunity in the absence of infection (Aim 3).
CHAPTER 2:

Bacterial exploitation of phosphorylcholine mimicry suppresses inflammation to promote airway infection.

Christopher B. Hergott¹, Aoife M. Roche¹, Nikhil A. Naidu¹, Clementina Mesaros², Ian A. Blair², and Jeffrey N. Weiser¹³⁴

¹ Departments of Microbiology,

² Systems Pharmacology and Translational Therapeutics, and

³ Pediatrics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

⁴ Department of Microbiology, New York University School of Medicine, New York, NY 10016, USA

This work was originally published in The Journal of Clinical Investigation, August 2015, Epub ahead of print, doi:10.1172/JCI81888
Abstract

Regulation of neutrophil activity is critical for immune evasion among extracellular pathogens, yet the mechanisms by which many bacteria disrupt phagocyte function remain unclear. Here, we show that the respiratory pathogen *Streptococcus pneumoniae* disables neutrophils by exploiting molecular mimicry to degrade platelet-activating factor (PAF), a host-derived inflammatory phospholipid. Using mass spectrometry and murine models of upper airway infection, we demonstrate that phosphorylcholine (ChoP) moieties shared by PAF and the bacterial cell wall allow *S. pneumoniae* to leverage a ChoP-remodeling enzyme, Pce, to remove PAF from the airway. PAF deprivation corresponds with impaired viability, activation, and bactericidal capacity among responding neutrophils. In the absence of Pce, neutrophils clear *S. pneumoniae* rapidly from the airway and impede invasive disease and transmission between mice. Abrogation of PAF signaling renders Pce dispensable, reinforcing that the enzyme deprives neutrophils of essential PAF-mediated stimulation. Accordingly, exogenous activation of neutrophils overwhelms Pce-mediated phagocyte disruption. *Haemophilus influenzae* also employs an enzyme, GlpQ, to hydrolyze ChoP and subvert PAF function, suggesting a common paradigm of mimicry-driven immune evasion among respiratory pathogens. These results uncover a novel mechanism by which shared molecular structures enable microbial enzymes to subvert host lipid signaling, suppress inflammation, and ensure bacterial persistence at the mucosa.
Neutrophils comprise an essential component of the acute inflammatory response to pathogens, particularly against extracellular bacteria which reside on mucosal surfaces (10). Typically first among leukocytes recruited to sites of infection, neutrophils exert potent bactericidal activity, impede microbial dissemination from epithelial barriers, and can inhibit bacterial transmission between hosts (214, 292, 304). In turn, opportunistic microbes have evolved strategies to evade the neutrophils they elicit, the mechanisms of which have long been the subject of intensive study (285, 333). However, as understanding of neutrophil biology has advanced, it has become clear that neutrophil bactericidal capacity is regulated dynamically and locally at inflamed sites (282) and that some pathogens manipulate phagocyte activation state directly to inhibit microbial clearance at sites of infection (211, 312). Our understanding of the mechanisms by which neutrophil phagocytic function is suppressed in vivo remains incomplete.

*Streptococcus pneumoniae*, known as the pneumococcus, was among the first pathogens for which neutrophil evasion mechanisms were proposed (180, 356, 357). A leading cause of gram-positive bacterial pneumonia, sepsis, and meningitis, the pneumococcus causes nearly a million deaths each year worldwide and is estimated to be responsible for more than 100,000 hospitalizations in the United States annually (135, 252). All pneumococcal infections begin with asymptomatic colonization of the upper airway, and clinical studies have observed that progression from benign carriage to invasive infection often occurs only a few days after acquisition of upper airway infection (132, 138, 307). This coincides with the peak of neutrophil influx to the airway lumen and implies that
Pneumococcus-neutrophil interactions in the upper airway may govern the balance between bacterial clearance and disease (225, 366).

Pneumococcal resistance against neutrophils has traditionally been ascribed to its thick polysaccharide capsule, which serves to shield the bacterium from opsonization with complement or antibodies that would otherwise hasten phagocytic uptake (128, 236). However, in contrast to that in the lungs or bloodstream (164), pneumococcal resistance to neutrophils in the upper airway does not rely upon antibody or complement evasion (227, 337). Further, colonizing pneumococci express markedly less capsular polysaccharide compared to blood and lung isolates, exposing cell wall components long known to be targets for immune recognition (175). Together, this suggests capsule-mediated anti-phagocytosis fails to fully explain pneumococcal neutrophil evasion in the upper airway. We sought to determine whether pneumococci instead disarm the phagocytic function of neutrophils recruited to the airway lumen, rendering them unable to mediate acute bacterial clearance.

Molecular mimicry is among the most widely conserved mechanisms by which bacteria evade immunity, exploiting the host’s inability to recognize self-derived molecular structures (316). The pneumococcus, like many other airway bacteria, capitalizes upon this vulnerability by displaying the host-derived small molecule phosphorylcholine (ChoP) on its surface. Decoration of pneumococcal cell wall components with ChoP is essential for bacterial fitness and is necessary to inhibit bacterial opsonization (67). However, ChoP moieties are found also within a wide range of mammalian phospholipids, including those that function as secreted, pro-inflammatory lipid
mediators (113). The receptor for one such host lipid, platelet-activating factor (PAF), has been shown to be a docking site for pneumococcal ChoP during epithelial cell adherence, highlighting that the PAF-PAFR axis may be a particularly important target for ChoP mimicry in the airway (76). PAF is secreted rapidly into the airway lumen in response to diverse inflammatory stimuli, including bacterial infection (303, 359). Though first identified as a mediator of systemic inflammation and platelet aggregation (66, 140), PAF was subsequently found to potently stimulate neutrophil phagocytic capacity and bactericidal function (178, 299). PAF binding to its G protein-coupled receptor on neutrophils triggers multiple intracellular signaling pathways (including PI3K, MAPK, PKA, PLC, and PLA₂) to stimulate cellular viability, surface expression of bacterial uptake receptors, and mobilization of bactericidal machinery (49, 102, 232, 298). Despite the central role PAF plays in regulating neutrophil function at mucosal surfaces, it remains unexplored whether ChoP mimicry can suppress PAF-mediated neutrophil activation.

Here, we use a murine upper airway infection model and primary human neutrophils to uncover a novel, enzymatic mechanism by which molecular mimicry can be exploited by bacteria to modulate innate immunity. In a previously reported screen, we identified pce, encoding a cell wall-bound phosphorylcholine esterase (also known as CbpE), as a pneumococcal gene potentially contributing to evasion of neutrophil-mediated killing (81). We now demonstrate that S. pneumoniae employs Pce to hydrolyze ChoP from host-derived PAF in the lumen of the airway. The absence of functional PAF deprives infiltrating neutrophils of stimulatory signals necessary for optimal phagocyte activation and effective bacterial clearance, allowing pneumococci to persist in the airway,
disseminate systemically, and transmit between hosts efficiently. Lastly, we find that this exploitation of molecular mimicry is functionally conserved among multiple ChoP-bearing airway microbes, as the gram-negative pathogen *Haemophilus influenzae* employs a surface-bound phosphodiesterase, GlpQ, to hydrolyze phosphorylcholine and subvert PAF-mediated stimulation of acute inflammation.

**Results**

**Neutrophils fail to contribute to mucosal defense during *S. pneumoniae* upper airway infection**

We sought first to determine the contribution of acute inflammation to the control of pneumococcal upper airway infection. Mice treated every 4 days with either neutrophil-depleting (anti-Ly6G) (79) or IgG2a isotype control antibody were inoculated with a clinical isolate of *S. pneumoniae* (serotype 23F) and sacrificed at 4, 14, and 24 days post-inoculation (p.i.). Neutrophil depletion was verified by flow cytometry (data not shown) and pneumococcal CFUs were enumerated at each time point from nasal lavages. Consistent with previous observations during early infection (222), we found that neutropenic and control mice cleared pneumococci at equivalent rates over 24 days (Figure 1A). These results suggested that, despite their rapid influx into the airway lumen following acquisition of infection (222), neutrophils failed to exert significant bactericidal pressure against *S. pneumoniae* in the upper airway.
Pneumococcal Pce esterase inhibits bacterial clearance and neutrophil activation.

We asked next whether Pce esterase contributes to pneumococcal evasion of acute clearance from the airway. Mice inoculated with wild type (WT) or Pce-deficient (Δpce) pneumococci were sacrificed and lavaged for bacterial enumeration 1, 3, 4, and 7 days p.i., corresponding to the period during which neutrophilic inflammation is most prominent (366). While WT bacteria persisted at maximal density over the first week of infection, Δpce pneumococci exhibited a survival defect that exceeded 20-fold by day 7 p.i. (Figure 1B). Notably, WT and Δpce bacterial loads were equivalent at 1 day p.i., suggesting differential inoculum retention or impaired establishment of infection were unlikely to explain the mutant’s subsequent persistence defect. The poor survival of Δpce pneumococci was recapitulated with an independently constructed unmarked, in-frame deletion mutant, restored upon genetic correction (Figure 1C), and preserved in a distinct serotype 4 isolate (Figure 1D). By day 14 p.i., Δpce bacteria were effectively cleared while WT pneumococci persisted at high levels (Figure S1A). Collectively, these findings suggested that Pce is necessary for effective pneumococcal persistence during acute infection of the upper airway.

We then performed flow cytometry to characterize the acute inflammatory response to WT and Δpce bacteria. Neutrophils dominated the cellular infiltrates elicited by both WT and Δpce pneumococci (>95% of CD45+ events, Figure S1C). Despite lower bacterial density in the airway, mice inoculated with the Δpce mutant exhibited elevations in the maximum density and, more markedly, the duration of neutrophil influx (Figure 1E). Congruent with bacterial load measurements, neutrophils persisted in the airway lumen
through day 14 p.i. in Δpce-infected mice but were nearly absent in response to WT pneumococci (Figure S1B).

The more robust acute inflammatory response observed in the absence of Pce led us to hypothesize that the enzyme may perturb the qualitative capacity of neutrophils to persist and clear bacteria. Accordingly, we predicted that neutrophils recruited by Δpce pneumococci would exhibit greater activation and superior phagocytic capabilities. To assess antimicrobial capacity in vivo, we functionally characterized the neutrophils responding to WT vs Δpce bacteria by flow cytometry (Figure 1F). Compared to neutrophils elicited by WT pneumococci, those elicited by the mutant exhibited significantly elevated expression of CD11b (CR3) and CD64 (FcγRI)—which serve as both activation markers and bacterial uptake receptors (10, 176, 298)—and >2-fold enhanced shedding of CD62L (L-selectin), a marker for inflammation-induced neutrophil activation (177, 324). Luminal neutrophils elicited by Δpce bacteria included approximately twice as many viable cells compared to those responding to WT, a finding consistent with their enhanced persistence in the airway lumen. Lastly, Δpce-elicited neutrophils exhibited a ~5-fold increase in reactive oxygen species (ROS) production per cell (as detected by CM-H₂DCFDA dye staining) over those recruited to WT bacteria, underscoring their enhanced bactericidal capacity (292). Together, these findings implied that Pce esterase impairs the cellular viability and phagocytic functionality of neutrophils upon their arrival in the airway lumen.

**Pce promotes invasive pneumococcal disease and bacterial transmission between mice.**
A number of clinical and animal studies have found pneumococcal load in the upper airway to be associated strongly with both the onset and severity of subsequent invasive disease (7, 307) as well as the risk of pneumococcal transmission between hosts (138, 304). We asked whether the survival deficit exhibited by Δpce pneumococci during infection of the upper airway corresponded with differences in these clinically important outcomes. To gauge relative invasive disease risk, we inoculated mice intranasally with WT or Δpce pneumococci generated from a mouse-invasive strain (serotype 6A) and tracked survival over 9 days (Figure 2A). While approximately 60% of mice infected with WT bacteria were moribund within 5 days from pneumococcal sepsis, mice inoculated with the mutant were significantly protected, suffering less than 10% lethality over the course of the experiment. This difference in survival corresponded with substantial attenuation in bacterial invasion to the bloodstream among Δpce pneumococci (Figure 2B).

Δpce pneumococci were similarly impaired in an infant mouse model of pneumococcal transmission (274, 304). Within litters of mice, we inoculated 1-2 “index” pups with either WT or Δpce bacteria and quantified the acquisition and load of pneumococci among previously uninfected “contact” pups. In line with previous findings (274), litters exposed to WT pneumococci exhibited 64% transmission; however, only 6% of contact mice from litters exposed to Δpce pneumococci acquired bacteria (Figure 2C). This corresponded with >300-fold lower bacterial loads among index mice infected with the mutant. Taken together, these data provide evidence that the poor survival of Δpce pneumococci in the upper airway crosses a key threshold under which disease and transmission are nearly abrogated.
**Pce prevents accumulation of platelet-activating factor in the upper airway lumen.**

Next, we analyzed the mechanism by which Pce esterase impairs neutrophil function in the upper airway. Studies using purified, recombinant enzyme have shown that Pce is capable of hydrolyzing a wide range of molecules bearing phosphorylcholine (ChoP) in vitro, including PAF (144) (Figure 3A, diagram). To quantify directly the impact of Pce on airway luminal PAF levels, we inoculated mice with PBS (mock), WT, or Δpce pneumococci and pooled lavages from 5 mice per group on day 3 p.i.. After lipid extraction and liquid chromatography (LC) to purify PAF, we subjected samples from each condition to high-resolution electrospray ionization/mass spectrometry (ESI/MS). Lavage fluid from mice inoculated with Δpce bacteria harbored PAF at a concentration of approximately 3 nM while mock- and WT-infected mice secreted no PAF detectable by LC-ESI/MS (Figure 3A), demonstrating that Pce prohibits the accumulation of PAF in the upper airway lumen during pneumococcal infection.

We also examined the expression of genes essential for PAF signaling in the airway epithelium, as PAF is known to stimulate the transcription of its own synthetic enzymes and receptor via a positive feedback loop (243, 367). At 3 days p.i. with WT or Δpce bacteria, we obtained lavages with tissue lysis buffer and quantified mucosal transcripts by qRT-PCR in each condition relative to mock-infection. Transcript levels of the PAF synthetic enzyme lyso-PAF acetyltransferase (*lysoPAFAT*) and the PAF receptor (*Ptafr*) were markedly elevated among mice infected with Δpce pneumococci (Figure 3B). In contrast, transcription of neutrophil chemokines CXCL1 and CXCL2 trended lower in
mice inoculated with the mutant, likely a consequence of lower Δpce bacterial density compared to WT. Thus, elevation of PAF-related transcripts was not simply reflective of broad up-regulation of inflammatory mediators and Pce-mediated hydrolysis of PAF is sufficient to suppress the inflammatory feedback loop driven by PAF signaling.

**Neutrophil depletion or inhibition of PAF signaling renders Pce dispensable for *S. pneumoniae* persistence.**

Our data show that *S. pneumoniae* lacking Pce esterase exhibit a survival defect during acute infection of the airway which corresponds with elevated levels of PAF and enhanced activation of luminal neutrophils. We asked next whether neutrophils and PAF signaling play a causal role in driving the mutant’s rapid clearance. To determine if the poor survival of Δpce bacteria could be rescued in the absence of infiltrating neutrophils, mice were treated with neutrophil-depleting anti-Ly6G or IgG2a isotype control antibodies on days -1, +1, and +4 after inoculation with WT or Δpce pneumococci. Effective neutrophil depletion was verified in blood and nasal lavage fluid by flow cytometry (Figure 4A) and bacterial CFUs from neutropenic and control mice were enumerated from nasal lavages on day 7 p.i. While the Δpce mutant retained a ~20-fold survival defect in isotype control-treated animals, its survival was restored to WT levels in neutrophil-depleted mice (Figure 4B). This confirmed that neutrophils were required for the enhanced clearance of the mutant and that Pce esterase functions to impair neutrophil-mediated bactericidal function.
To assess whether local PAF signaling contributed to Pce-mediated neutrophil evasion, we performed the same 7-day infection with daily intranasal administration of PCA-4248, a selective antagonist of PAFR (111, 121, 220). Similar to our findings upon depletion of neutrophils, local PAFR antagonism restored survival of Δpce pneumococci to levels comparable to WT (Figure 4C). PCA-4248 has no direct effect on pneumococcal survival or growth in vitro (Figure S2B). Importantly, no additional increase in the density of Δpce bacteria was seen upon PCA-4248 treatment of neutrophil-depleted mice, underscoring that the predominant impact of elevated airway PAF in the absence of Pce is stimulation of neutrophil function. Lastly, we found that bacterial loads on day 7 p.i. were equivalent among WT and Δpce pneumococci in PAFR−/− mice (Figure 4D), providing independent evidence that augmented PAF signaling underlies the enhanced clearance of Pce-deficient bacteria. In sum, these results show that Pce esterase governs pneumococcal persistence by eliminating PAF from the airway lumen and preventing the PAF-mediated activation of neutrophils necessary for efficient pathogen clearance.

**Pce esterase hydrolyzes phosphorylcholine and abrogates PAF-mediated stimulation of neutrophil function in vitro.**

To further clarify the mechanism by which Pce inhibits PAF-mediated neutrophil stimulation, we turned to in vitro studies using mature neutrophils isolated from murine bone marrow or human peripheral blood. We began by using neutrophil killing assays (80) to determine whether Δpce pneumococci were more sensitive to neutrophils in the absence of PAF stimulation. After pre-incubation with complement, WT and Δpce bacteria were incubated with increasing concentrations of murine or human neutrophils and we measured their survival relative to no-neutrophil controls. At all neutrophil
concentrations tested, WT and mutant bacterial survival was equivalent (Figure 5A&B), providing evidence that the survival defects seen in vivo were not the result of non-specific sensitivity to neutrophil uptake. Similarly, WT and Δpce pneumococci were not significantly different in growth characteristics in vitro (Figure S2A), sensitivity to complement deposition (Figure S3A), capsule expression levels (Figure S3B), or cell surface phosphorylcholine (ChoP) accessibility (Figure S3C) as assessed by bacterial flow cytometry.

Using recombinant Pce esterase (rPce), we next confirmed that the enzyme bears efficient ChoP hydrolysis activity upon incubation with chromogenic substrate p-nitrophenylphosphorylcholine (pNPPC), which yields p-nitrophenol upon removal of its ChoP moiety (Figure 5C) (340). To assess whether rPce directly inhibits PAF-mediated stimulation of neutrophils, we pre-treated PAF at a range of physiologic concentrations centered around 3 nM (the concentration detected by ESI/MS) with rPce enzyme or PBS control. We applied the conditioned PAF media to the phagocytes and quantified up-regulation of CD11b and CD64 receptor expression on murine neutrophils by flow cytometry (Figure 5D). While increasing PAF concentrations correlated with up-regulation of both receptors in the PBS control condition, no such correlation was seen if PAF was pre-incubated with rPce. Similarly, pre-treatment of PAF with rPce abrogated the increased killing capacity conferred when PAF was applied to murine or human neutrophils (Figure 5D&E). Collectively, these findings reveal that Pce functions to hydrolyze ChoP from conjugated substrates and its processing of PAF directly inhibits neutrophil activation and phagocytic function.
Exogenous stimulation of neutrophils in situ overwhelms Pce-mediated immune evasion.

Having established that Pce inhibits acute clearance of *S. pneumoniae in vivo* by depriving neutrophils of an essential stimulatory ligand, we posited that this immune evasion mechanism could be overcome if luminal neutrophils were activated sufficiently. We tested this by performing 7-day WT and Δpce pneumococcal infection experiments with daily intranasal treatment with an excess of either PAF or N-formyl-Met-Leu-Phe (fMLP), a bacterial peptide which stimulates neutrophil chemotaxis and activation through pathways independent from that of PAF (120, 298). At day 7 p.i., mice inoculated with WT pneumococci and treated with PAF (Figure 6A) or fMLP (Figure 6D) exhibited significantly enhanced clearance—such that WT bacterial loads resembled that of Δpce—while the mutant’s survival was not substantially affected upon stimulant treatment. PAF and fMLP exerted no detectable effect on pneumococcal growth or viability in vitro (Figure S2C&D).

Critically, repeating these treatments after systemic neutrophil depletion restored survival of both pneumococcal strains to levels seen for WT bacteria in mice treated with vehicle controls (Figure 6A&D), reinforcing that neutrophils were responsible for the observed increase in clearance. Enhancement of WT pneumococcal clearance upon PAF treatment corresponded with an increase in the number of neutrophils in the airway lumen to levels similar to those seen during Δpce infection. In contrast, neutrophil numbers elicited by Δpce pneumococci were unaffected by PAF administration (Figure 5B). These results suggested that introduction of excess PAF ligand overwhelmed the capability of Pce esterase to mediate immune evasion for WT bacteria, yielding
neutrophil phenotypes and bacterial survival that mimicked infection in the absence of Pce. Consistent with its known role as a strong chemo-attractant (187), fMLP treatment stimulated a substantial elevation in airway neutrophil recruitment, but neutrophil numbers were again equalized among WT- and Δpce-infected mice (Figure 5E). Lastly, flow cytometric analysis of luminal neutrophils revealed that treatment with PAF or fMLP enhanced the relative expression of CD11b and CD64 on cells elicited by WT pneumococci to levels seen in those recruited to Δpce, abrogating the relative difference in activation observed in the absence of stimulation in situ (Figure 6C&F). Taken together, these results serve to reinforce that Pce promotes pneumococcal persistence through fine regulation of neutrophil activation and that clearance of WT bacteria can be substantially accelerated by stimulating neutrophil function.

Subversion of PAF signaling is conserved in Haemophilus influenzae, another ChoP-expressing pathogen of the airway.

We sought to determine whether exploitation of ChoP mimicry to suppress PAF represents a conserved mechanism among other ChoP-expressing bacterial pathogens. We focused on Haemophilus influenzae, as it displays phosphorylcholine on surface-exposed lipooligosaccharide chains (67), it remains an important cause of respiratory disease worldwide (284, 329), and could be investigated using a murine model of upper respiratory tract infection (368). No direct homologues for pneumococcal Pce were apparent in H. influenzae by sequence analysis. However, previous work from our laboratory suggested that the highly conserved, surface-bound phosphodiesterase lipoprotein GlpQ (also known as Protein D) bears the ability to hydrolyze ChoP efficiently from conjugated substrates (107). Previously ascribed a role in bacterial
acquisition of choline from host cells, GlpQ has long been known to be important for virulence during mucosal infection, though the nature of its contribution has remained incompletely understood (160). We hypothesized that this ChoP-binding enzyme functioned analogously to pneumococcal Pce esterase and contributed to the PAF evasion during airway infection.

To confirm previous reports that GlpQ contributes to hydrolysis of ChoP (107), we applied WT and ΔglpQ H. influenzae to chromogenic pNPPC assays and measured absorbance after 120 minutes compared to that generated by WT and Δpce pneumococci (Figure 7A&B). Note that GlpQ cleaves phosphorylcholine at the phosphoester bond proximal to choline and therefore requires exogenous alkaline phosphatase enzyme added to reactions for chromogenic activity in this assay (Figure 7A, arrows). Similar to the results for Δpce pneumococci, ChoP hydrolysis activity by H. influenzae was significantly impaired in the absence of GlpQ, though some residual activity remained (Figure 7B). These results affirmed that native GlpQ hydrolyzes ChoP efficiently.

We asked next whether GlpQ contributes to bacterial persistence in murine upper airway infection. Mice inoculated with WT or ΔglpQ H. influenzae were sacrificed on days 1 and 2 p.i. for bacterial enumeration. By day 2, ΔglpQ bacteria exhibited a survival defect exceeding 20-fold (Figure 7C). Flow cytometric analyses of lavage fluid suggested enhanced influx of neutrophils during acute infection with the ΔglpQ mutant compared to WT (Figure 7D) and neutrophils elicited by the mutant exhibited significantly elevated bacterial uptake receptor expression by day 2 p.i. (Figure 7E). To establish whether neutrophils drive ΔglpQ persistence defects, we repeated 2-day infections after anti-
Ly6G or IgG2a isotype antibody treatments on days -1 and +1 after inoculation. Akin to results observed with Δpce pneumococci, neutropenia rescued ΔglpQ survival such that it resembled that of WT H. influenzae (Figure 7F). ΔglpQ survival was also rescued in PAFR+/− mice while littermate control mice recapitulated the mutant defect seen previously, indicating that intact PAF signaling is essential for enhanced bacterial clearance in the absence of GlpQ (Figure 7G). Importantly, neutropenia did not lead to significantly enhanced bacterial survival in PAFR+/− mice, suggesting that PAF stimulation plays an important role in regulating neutrophil function during H. influenzae infection of the airway. Collectively, these findings reveal that GlpQ is critical for limiting neutrophil responses in vivo through inhibition of PAF signaling.

Lastly, we performed neutrophil bactericidal assays in vitro to assess whether pre-incubation of PAF with H. influenzae bearing GlpQ hindered its ability to stimulate neutrophil-mediated phagocytic function when compared to pre-incubation with ΔglpQ bacteria. We mixed neutrophils pre-treated with each enzyme-conditioned PAF solution with pre-opsonized WT H. influenzae and quantified bacterial survival over a range of PAF concentrations compared to no-PAF and no-neutrophil controls (Figure 7H). While evidence of PAF-mediated stimulation of phagocytosis remained evident after pre-treatment in either condition, this enhancement of killing was impaired significantly when neutrophils were pre-stimulated with WT bacteria (black bars) compared to ΔglpQ mutant (white bars). These results reinforce that glpQ inhibits the effects of PAF on neutrophil activity directly.

**Discussion**
Evasion of neutrophil-mediated phagocytosis is essential for the mucosal persistence of extracellular bacterial pathogens, and mounting evidence suggests direct suppression of neutrophil activation plays an important role in mediating microbial escape from acute inflammatory responses. Here, we showed that enzymatic degradation of PAF, driven by phosphorylcholine mimicry, underlies neutrophil subversion by *S. pneumoniae* and *H. influenzae* in the upper airway. As stable infection of the upper airway is a prerequisite for the development of pneumonia and sepsis for many respiratory pathogens, a mechanistic understanding of immune evasion in this niche is particularly important for developing interventions designed to prevent the transition from asymptomatic carriage to invasive disease (348).

We found that the surface-bound phosphorylcholine esterase Pce governed the exploitation of ChoP mimicry essential for pneumococcal persistence. The near-universal conservation of Pce among pneumococcal clinical isolates (90) and results from early animal studies (129) support that the enzyme contributes to bacterial fitness during airway infection, but the nature of its function in vivo has been less clear. Most studies characterizing Pce activity have focused on its ability to remodel the pneumococcal cell wall by hydrolyzing ChoP residues from teichoic acid chains, a function associated with enhanced adhesion to epithelial surfaces (144, 340). However, Pce-deficient pneumococci exhibit essentially unaltered epithelial adherence properties and the enzyme is unable to hydrolyze more than 30% of ChoP residues from these substrates, even in saturating conditions (129, 148). Our findings reveal that exploitation of shared ChoP moieties allows degradation of host-derived PAF, rather than modifications to the
cell wall, to be the major contribution of Pce to immune evasion during respiratory infection.

Using direct measurement of PAF by high-resolution LC-ESI/MS, we tested the hypothesis that Pce restricts PAF accumulation during infection. In upper airway lavage fluid, PAF was detectable only from mice infected with Pce-deficient bacteria, at a concentration of approximately 3 nM. Even if the dilution inherent to lavage is discounted, this concentration exceeded the dissociation constant of PAFR on leukocytes (49, 260) and mimicked PAF levels observed in the inflamed human airway (317). Together, this reinforced that PAF accumulated to a level sufficient to effectively stimulate neutrophils in the airway. The product of PAF hydrolysis by Pce, 1-O-hexadecyl-2-O-acetyl-sn-glycerol, was not detectable in any condition tested, possibly reflecting rapid degradation in vivo. Additionally, we showed that Pce directly suppressed PAF-mediated stimulation of human and murine neutrophil bactericidal function upon treatment ex vivo with physiologically relevant concentrations of PAF, further supporting that Pce limits PAF levels through enzymatic degradation.

We focused our investigations entirely on PAF C16 (1-O-hexadecyl-2-O-acetyl-sn-glyceryl-3-phosphorylcholine), the most abundant and well-characterized member of a diverse set of PAF-like lipids secreted during inflammation. While some PAF-like lipids can stimulate neutrophil bactericidal capacity, nearly all bind most avidly to receptors other than PAFR (361). A number of studies have shown that even small modifications to the molecular structure of PAF significantly disrupts its binding and signaling potency through its cognate receptor (267). Since we found that the enhanced neutrophil
activation observed during infection with Δpce pneumococci was abrogated entirely upon PAFR blockade in vivo or when using PAFR-deficient mice, we concentrated our analyses on PAF. Whether other PAF-like lipids are regulated similarly by Pce during pneumococcal infection remains a subject of ongoing investigation.

Our work demonstrated that Pce-mediated PAF degradation results in functional impairment of neutrophils responding to pneumococcal infection, rendering them unable to mediate efficient phagocytosis. Recent studies have suggested that, in addition to suppressing clearance, recruiting an ineffective acute inflammatory response can be directly beneficial for bacterial fitness. Increased mucus production can provide nutrients to stimulate bacterial growth at mucosal surfaces (305), inflammatory influxes can enhance bacterial shedding from the nasopharynx to promote transmission between hosts (274), and the recruitment of neutrophils can provide an inflammatory milieu which may neutralize bacterial competitors among the flora (211). These advantages rely upon phagocytes that alter the mucosal environment without presenting significant bactericidal pressure. For the pneumococcus, Pce-mediated neutrophil suppression may constitute a central mechanism through which inflammation can be utilized to the advantage of the microbe.

A wide range of extracellular bacteria display ChoP on their surfaces and express enzymes that govern ChoP hydrolysis and turnover, suggesting other pathogens may harbor unappreciated strategies for evading clearance through mimicry-driven degradation of PAF (67). Along with the pneumococcus, Haemophilus influenzae, Neisseria meningitidis, and Neisseria gonorrhoea carry ChoP as surface modifications
and most of these pathogens use ChoP to bind PAFR directly, reflecting a common strategy of passive, structural mimicry of PAF (24, 349, 350). Our work implies that active, enzymatic mechanisms exploiting ChoP mimicry may underlie the well-described resistance of these organisms to neutrophils and may expose a broadly applicable target for therapeutic intervention. To this end, we investigated neutrophil evasion by *H. influenzae* in the upper airway. Despite bearing no apparent homologues for Pce, we found that *H. influenzae* harbors an analogous mechanism for mimicry-driven, enzymatic subversion of PAF through ChoP hydrolysis by the surface-bound phosphodiesterase GlpQ. The functional similarity of these structurally unrelated esterases, employed by disparate pathogens occupying the same upper airway niche, suggests that PAF-mediated inflammation may have necessitated convergent evolution (316) aimed at manipulating and neutralizing PAF activity to achieve fitness in the airway environment. Our findings also suggest that GlpQ does not govern the entirety of ChoP hydrolytic activity mediated by *H. influenzae*, as some residual enzymatic activity persists among Δ*glpQ* mutants. The identity and functions of these other contributors remain under investigation.

The central importance of regulating PAF-mediated inflammation is exemplified further by microbes that do not express ChoP but disrupt PAF signaling by means other than molecular mimicry. *Staphylococcus aureus* has been shown to bind leukocytes and platelets and directly modulate PAFR signaling (149, 343). *Streptococcus pyogenes* uses a secreted enzyme to cleave acetyl groups from PAF ligand and inhibit neutrophil chemotaxis during invasive skin infection (202). It remains conceivable that additional mechanisms for PAF disruption exist for *S. pneumoniae* and *H. influenzae* independent
of ChoP hydrolysis activity. Together, this emphasizes that microbial manipulation of PAF signaling may be critical for successful immune evasion and that targeting convergent bacterial strategies to this end may be a promising avenue for antimicrobial interventions.

The tight regulation of PAF signaling mediated by the pneumococcus and other extracellular bacteria suggests that these neutrophil evasion mechanisms could be overcome if PAF levels are elevated sufficiently at the site of infection. Accordingly, we found that stimulating neutrophils in situ during pneumococcal infection with excess PAF restored their activation state and phagocytic capacity, resulting in enhanced bacterial clearance. This implies that a threshold concentration of PAF may define the balance between effective bacterial evasion and neutrophil-mediated clearance. While delivering PAF itself is unlikely to be a clinically tractable method to overcome such a threshold, targeting host-encoded negative feedback mechanisms that regulate PAF levels may be more promising. Lipoprotein-associated phospholipase A₂, also known as PAF acetylhydrolase, is secreted by the host, has been shown to mediate PAF hydrolysis, and can dampen PAF-mediated inflammation in vivo (231). It is released into the upper airway lumen during respiratory inflammation, has been shown to be upregulated during pneumococcal infection, and clinical trials have demonstrated that an antagonist, darapladib, is safe in humans (153, 294, 331). While studies aimed to assess the effectiveness of targeting PAF acetylhydrolase in combating pneumococcal and other bacterial infections are ongoing, this may serve as an important example of how mechanistic investigations of bacterial immune evasion may reveal rational targets for host-directed antimicrobial therapies.
Methods

Bacterial strains. *S. pneumoniae* strains P1121 (type 23F clinical isolate), TIGR4 (type 4 isolate), and P1547 (a mouse-virulent type 6A isolate) were grown in tryptic soy (TS) broth at 37° C to mid-log phase, as described previously (70, 225, 326). Mutants lacking phosphorylcholine esterase (Δpce) were derived for each strain from an insertion-duplication mutant (340) and used for all experiments except where specified. Independently, we created an in-frame, unmarked Δpce deletion mutant and a genetically corrected revertant (Δpce::pce) by previously described methods (81). Refer to Supplemental Methods for details and Table S1 for primers used in mutagenesis.

Spontaneously streptomycin-resistant isolates of *H. influenzae* Eagan (a type b encapsulated strain) and an isogenic mutant lacking the surface phosphodiesterase GlpQ (ΔglpQ) were used as described previously (107). *H. influenzae* was grown to mid-log phase shaking at 37° C in brain-heart infusion (BHI) broth supplemented with 2% Fildes enrichment (Thermo Scientific) and 2 μg/mL β-NAD (Sigma-Aldrich) (sBHI).

Murine model of upper airway infection. Six- to eight-week-old C57BL/6 mice were obtained from Jackson Laboratories. Platelet-activating factor receptor-deficient (*Ptafr*−/−) mice of C57BL/6 background (*Ptafr*tm1Eit) were a gift from Dr. Elaine Tuomanen of the St. Jude Children’s Research Hospital (47, 270). All knockout mice were bred from heterozygotes and compared to littermate controls. For upper airway infection with *S. pneumoniae* or *H. influenzae*, mice were inoculated intranasally with 10⁷ CFU of mid-log phase.
phase bacteria suspended in 10 µL of sterile PBS. To prevent aspiration of the inoculum from the upper airway to the lungs, mice were not anesthetized during inoculation. We obtained upper airway lavages upon sacrifice through tracheal cannulation and expression of 200 µL sterile PBS through the nares. Lavage samples from pneumococcal infections were plated on TS agar supplemented with 5-20 µg/mL neomycin and 5,000 U catalase per plate (Worthington Biochemical). To discern insertion-derived and in-frame deletion Δpce mutants from wild type pneumococci, plates were supplemented with 1 µg/mL erythromycin or 200 µg/mL streptomycin, respectively. Lavage samples from mice infected with *H. influenzae* were plated on sBHI agar supplemented with 100 µg/mL streptomycin. ΔglpQ mutants were discerned from wild type *H. influenzae* by supplementation with 20 µg/mL kanamycin. CFU counts were enumerated by quantitative culture after overnight incubation at 37° C in 5% CO₂ (305).

*Flow cytometry.* Nasal lavages were pelleted at 1,200g for 5 minutes and re-suspended in PBS with 1% BSA. FcR blocking was achieved with 1:100 dilution of anti-CD16/32 (BD Biosciences, Clone 2.4G2) and cell viability was assessed by staining with Fixable Viability Dye eFluor780 (eBioscience) according to manufacturer’s instructions. Neutrophils were immunophenotyped by staining with fluorophore-conjugated antibodies, diluted 1:150, against the following surface markers: CD45 (eBioScience, Clone 30-F11), Ly6G (BioLegend, Clone 1A8), CD11b (Biolegend, Clone M1/70), CD64 (BioLegend, Clone X54-5/7.1), and CD62L (BioLegend, Clone MEL-14). Neutrophil reactive oxygen species (ROS) were quantified after incubation with 10 µM CM-H₂DCFDA (Life Technologies) according to manufacturer’s instructions (193, 230).
cytometry was performed using a BD LSR II flow cytometer and analyzed with FlowJo software (Tree Star).

**Bacterial transmission model.** Infant mouse pneumococcal transmission experiments were performed as described previously (274, 304). At day 4 of life, we inoculated 1-2 “index” pups per litter (approximately 1 in 4) with 2000 CFUs of *S. pneumoniae* strain P1121 in 3 µL PBS. At day 8 of life, all pups were infected intranasally with $2 \times 10^4$ TCID$_{50}$ Influenza A strain HKx31, as influenza co-infection is required for pneumococcal transmission between mice (92). At day 14, all pups were sacrificed and upper airway bacterial loads were quantified among index and previously uninfected “contact” mice.

**LC-ESI/MS quantification of PAF from the murine upper airway lumen.** PAF-C16 was extracted and quantified from murine upper airway lavage fluid pooled from 5 mice per condition on day 3 of infection (performed in triplicate). Briefly, 0.5 mL of lavage fluid was spiked with 2 ng of deuterated PAF-C16-d$_4$ (Cayman Chemical) as a quantification standard, extracted in methanol with shaking, and resuspended in isopropanol/acetonitrile/water (3:5:2; v/v/v). Reversed phase separations of 3 µL injections were conducted using a nano-ACQUITY UPLC system and XBridge BEH130 C18 column (Waters Corp.) at 1.5 µL/minute. High-resolution LC-ESI/MS quantification was performed using a recently calibrated LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Scientific) in positive ion mode and with a Michrom captive spray ESI source. Data analysis was performed using Xcalibur software (Thermo Scientific) from raw mass spectral data.
**qRT-PCR.** RNA was extracted from the upper airway mucosa through lavage with 500 µL RLT lysis buffer (Qiagen) (30). After isolation of total RNA (Qiagen RNeasy Kit), cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and qRT-PCR was performed using 10 ng cDNA, 0.5 µM primers, and SYBR Green Master Mixes (Life Technologies). Differential RNA expression was quantified using the ΔΔCT method relative to GAPDH transcript levels. qRT-PCR primer sequences are listed in Table S1.

**Neutrophil depletion and intranasal drug treatments.** Systemic depletion of murine neutrophils was achieved by intraperitoneal injection of 250 µg anti-Ly6G antibody (BioXCell, Clone 1A8) or IgG2a isotype control antibody (Clone 2A3) at the indicated time points. Neutropenia was confirmed by flow cytometry and microscopic inspection using Shandon Kwik-Diff stains (Thermo Scientific). For intranasal drug treatments, 0.1 or 1 µg of PAF (Cayman Chemical), PAF receptor antagonist PCA-4248 (Tocris), or fMLP (Sigma-Aldrich) were instilled daily (days 1-6), suspended in 10 µL sterile PBS. Vehicle control experiments used dilutions of drug solvent (DMSO) in PBS identical to those performed for drug dilutions.

**Generation of recombinant Pce.** Recombinant Pce (rPce) protein was produced as described previously (340) with the following modifications. pce was cloned from the P1121 S. pneumoniae genome with primers which introduced flanking restriction sites for BamHI and SacI (New England BioLabs) (see Table S1). After restriction digest and
gel purification, the \textit{pce} fragment was cloned into pET28a (Novagen) using T4 DNA ligase (New England BioLabs) to generate a construct in which \textit{pce} was flanked with N- and C-terminal hexa-histidine tags. Ligated product was transformed into XL-1 Blue competent \textit{E. coli} (Agilent), amplified, and purified by plasmid miniprep (Sigma-Aldrich). After sequence confirmation, \textit{pce}-pET28a was transformed into BL21 (DE3)pLysS competent cells (Promega) and protein expression was induced according to manufacturer’s instructions with 1 mM IPTG. Native cell lysis was achieved by sonication on ice and lysates were applied to a Ni-NTA purification column (GE Life Sciences) by fast protein liquid chromatography (FPLC), followed by dialysis against 20 mM HEPES with 3 µM zinc sulfate.

\textit{Chromogenic assay for phosphorylcholine hydrolysis.} Pce catalytic activity was assessed by incubation of 4 µg enzyme with 21 µg of the chromogenic substrate p-nitrophenylphosphorylcholine (pNPPC, Sigma-Aldrich) at 37° C in 200 µL. Absorbance at 415 nm was monitored at the indicated time points to detect phosphorylcholine hydrolysis and resultant liberation of p-nitrophenol (340). For chromogenic assays using whole bacteria and isogenic mutants, 50 µL of OD$_{620}$ 1.0-normalized bacteria was re-suspended in potassium phosphate buffer (pH 8.0) and incubated with pNPPC as above for 120 minutes. For reactions testing \textit{H. influenzae} strains, 1 U of Shrimp Alkaline Phosphatase (Affymetrix) was included in the assays to cleave terminal phosphate groups remaining after GlpQ hydrolysis.

\textit{Isolation of neutrophils from murine bone marrow and human peripheral blood.} Mature murine neutrophils were flushed from murine femora and washed with Hank’s
Buffer with calcium (Mediatech) containing 0.1% gelatin before enrichment with a discontinuous gradient of Histopaque-1077 and -1119 (Sigma-Aldrich). Flow cytometry confirmed that >90% of CD45+ cells isolated were Ly6G+CD11b+. Human neutrophils were obtained from healthy donors and isolated by Polymorphprep (Axis Shield) gradient centrifugation as described previously (314).

**Neutrophil activation and bactericidal assays.** To assess the impact of Pce on PAF-stimulated neutrophil function, we performed assays using murine and human neutrophils ex vivo. Purified PAF (Cayman Chemical) was incubated in the presence or absence of 4 µg recombinant Pce in 100 µL of potassium phosphate buffer (100 mM, pH 7.4) for 20 minutes at 37°C. Reaction products were applied to 10^5 murine or 10^4 human freshly isolated neutrophils at a 1:1 dilution and allowed to incubate for 30 minutes. All values listed for PAF represent final concentrations after incubation with neutrophils. Murine neutrophil activation was assessed by flow cytometric analysis of CD11b and CD64 as described above. Assays for neutrophil opsonophagocytic killing were performed as described previously (70, 80); 10^2 bacteria were pre-opsonized for 30 minutes with baby rabbit serum (BRS) as a complement source and applied to PAF-treated neutrophils at the indicated bacterium:phagocyte ratios. Refer to Supplemental Methods for details on neutrophil bactericidal assays involving *H. influenzae*.

**Statistical analyses.** All data are presented as mean ± S.E.M. Data were analyzed using a 2-tailed Student’s *t* test for comparisons between 2 groups and ANOVA with Newman-Keuls post-test for all comparisons of >2 groups. Relative MFI measurements were
analyzed using pairwise one-sample $t$ tests relative to a null ratio of 1. For all analyses, $p < 0.05$ was considered statistically significant.

Study approval. All animal experiments were approved by and performed in strict accordance with the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee.

Author contributions. C.B.H. designed the research, performed the experiments, analyzed the data, and wrote the manuscript; A.M.R. and N.A.N. performed experiments; C.M. and I.A.B. contributed essential spectrometry techniques, performed experiments, and analyzed data; and J.N.W. designed the research, analyzed the data, and wrote the manuscript. All authors reviewed the manuscript.

Acknowledgements

We thank Dr. Jan Erikson (Wistar Institute) for providing influenza strains, Amanda Hay and Dr. Jun Zhu for assistance with kinetic bacterial growth assays, and Dr. Young Hwang, Jay Gardner and Dr. Michael Betts for assistance with FPLC and protein purification. This work was supported by NIH grants AI038446, AI105168, and AI060516 to J.N.W and P30CA016520 to I.A.B.
Figure Legends

Figure 1. Pce-deficient pneumococci exhibit impaired persistence in the upper airway and elicit more activated, viable and durable neutrophils to the nasal lumen.

(A) Bacterial clearance in mice inoculated with wild-type (WT) pneumococci, strain P1121 (Type 23F), with (open) or without (black) systemic neutrophil depletion (N = 4-5 mice per condition, Limit of detection = 2). (B) Survival of WT P1121 (black) or P1121Δpce (grey) pneumococci in the murine upper airway (N = 4-14). (C) Day 7 survival of P1121Δpce mutant generated by in-frame, unmarked deletion (Δpce) and with genetic correction (Δpce::pce) (N = 5). (D) Day 7 survival of WT and Δpce pneumococci in a Type 4 (T4) pneumococcal genetic background (N = 5). (E) Quantification of neutrophils (CD45+CD11b-Ly6G+) obtained from the upper airway lumen by nasal lavage before (N = 3) and after (N = 4-11) inoculation with WT (black) or Δpce (grey) pneumococci. (F) Flow cytometric characterization of luminal neutrophils elicited by infection with WT or Δpce pneumococci at day 4 p.i. (N = 6-8). MFI = Mean Fluorescence Intensity. Note that not all axes are continuous, and gaps in axes represent gaps in time. Dotted lines represent limits of detection. Statistical significance was assessed by one-way ANOVA with Newman-Keuls post-test for comparisons of >2 conditions (Panels A, B, & E), Student’s t test for 2-group comparisons (Panels C & D), and one-sample t test relative to null = 1 for relative MFI measurements (Panel F). NS = not significant, * = p < 0.05, *** = p < 0.001.
Figure 2. Pce promotes invasive pneumococcal disease and bacterial transmission between mice.

(A) Survival of adult mice inoculated intranasally with WT (black) or Δpce (grey) pneumococci of invasive strain P1547 (Type 6A) (N = 12 mice per condition from 3 independent experiments). Statistical significance was assessed by Mantel-Cox Test. (B) Enumeration of CFUs in the blood of mice infected with WT or Δpce P1547 pneumococci as above (N = 5, Limit of detection (LOD) = 14). (C) Infant murine transmission. Upper airway lavage CFUs enumerated from index (open circles, N = 3-4) and contact (closed circles, N = 14-18) pups on day 14 of life, after index mice were inoculated with WT (black) or Δpce (grey) pneumococci on day 4 of life and all pups were infected intranasally with influenza on day 8. Numerical values above contact mice columns represent percent acquisition (LOD = 10). Transmission data reflect 3 independent experiments. In Panels B & C, statistical significance was assessed by one-way ANOVA with Newman-Keuls post-test. ** = p < 0.01, *** = p < 0.001.

Figure 3. Pce prevents accumulation of platelet-activating factor in the lumen of the upper respiratory tract and its absence stimulates transcription of genes important for PAF signaling.

(A) Diagram of PAF with phosphorylcholine moiety (ChoP) labeled (and site of Pce-mediated hydrolysis marked with black arrow). Detection of PAF levels in the upper airway lumen by LC-ESI/MS (Limit of quantification = 0.066 nM, dashed line), quantified from pooled nasal lavages obtained from 5 mice at 3 days p.i. with PBS (open), WT (black), or Δpce (grey) P1121 pneumococci. Averages reflect 3 independent biological replicates of 5 pooled mice each. Statistical significance was assessed by one-
way ANOVA with Newman-Keuls post-test. For representative LC traces from lavage fluid of mock-, WT-, Δpce-infected mice: top row displays PAF detected at 8.6 min. retention, bottom row displays $^3$H$_4$-PAF C16 spiked control samples. (B) qRT-PCR analyses measuring relative gene expression of the PAF synthetic enzyme lysoPAFAT, PAFR (Ptafr), and chemokines Cxcl1 and Cxcl2 from nasal lavages obtained at 3 days p.i. from mice colonized with PBS, WT, or Δpce P1121 pneumococci (N = 6-10 mice per condition). All transcripts were normalized to GAPDH controls and are displayed relative to mice mock-infected with PBS (dotted lines). Statistical significance was assessed by Student’s t test. NS = not significant, * = p < 0.05

**Figure 4.** Pce is dispensable for pneumococcal persistence in the absence of infiltrating neutrophils or intact PAF signaling in the upper airway.

(A) Confirmation of neutrophil depletion. Mice were treated with neutrophil-depleting antibody (α-Ly6G, Clone 1A8) or IgG2a isotype control (250 µg, i.p.) on days -1, +1, and +4 post-inoculation (p.i.) with PBS (open), WT (black) or Δpce (grey) P1121 pneumococci (N = 3-4 mice per condition). On day 7 p.i., depletion was confirmed by flow cytometry of whole blood and nasal lavage. (B) Enumeration of WT (black) or Δpce (grey) pneumococcal CFUs obtained from nasal lavages on day 7 p.i after treatment of mice (N = 5-11 mice per group) with neutrophil-depleting α-Ly6G or IgG2a isotype control antibodies. (C) Enumeration of bacterial CFUs on day 7 p.i. after daily intranasal treatment with 0.1 or 1 µg of PAF receptor antagonist PCA-4248 (or 1% DMSO vehicle), from day +1 to +6 p.i (N = 5-12). The experiment was repeated with neutrophil depletion as described in (A). (D) WT and Δpce bacterial loads were enumerated in lavages obtained from PAFR$^{-/-}$ and littermate controls on day 7 p.i. (N = 5-11). Statistical
significance was assessed by one-way ANOVA with Newman-Keuls post-test for all panels. NS = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

**Figure 5.** Pce esterase hydrolyzes phosphorylcholine (ChoP) from conjugated substrates and directly inhibits PAF-mediated stimulation of neutrophil activation and function in vitro.

Killing of WT (black) or Δpce (grey) P1121 pneumococci in vitro by murine (A) or human (B) neutrophils, at the indicated neutrophil:bacterium ratios, after pre-opsonization with baby rabbit serum. HI = heat-inactivated. Bacterial survival was measured relative to control assays in the absence of neutrophils (dotted line). (C) Kinetic time-course of p-nitrophenol liberation (absorbance at 415 nm) after incubation of p-nitrophenylphosphorylcholine (pNPPC) with recombinant Pce enzyme (rPce, black line). Assays were repeated in the absence of Pce enzyme, pNPPC substrate, or in the presence of 250 mM EDTA. (D) Mean fluorescence intensity (MFI) quantification of bacterial uptake receptors CD11b and CD64 on murine neutrophils treated with PAF that was pre-incubated with rPce (black) or PBS (open). PAF stimulation assays were repeated with 10⁻⁵ M PAF receptor antagonist PCA-4248 as a specificity control. Killing assays using WT pneumococci were performed after PAF-mediated murine (D) or human (E) neutrophil stimulation in the presence (black) or absence (open) of rPce. Top dotted line denotes 100% bacterial survival; bottom dotted line denotes average survival in the absence of PAF. For all panels, data averages reflect at least 3 independent experiments (3-4 independent biological replicates for cellular assays). Statistical significance for all pairwise comparisons was assessed by Student’s t test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
Figure 6. Exogenous stimulation of neutrophils in situ overwhelms Pce-mediated immune evasion.

(A) Enumeration of WT (black) or Δpce (grey) pneumococcal CFUs obtained on day 7 p.i. and after daily (+1 to +6) intranasal treatments with 1 μg PAF or vehicle control (1% DMSO in PBS). The experiment was repeated after treatment with neutrophil-depleting α-Ly6G or IgG2a isotype control antibody (see Figure 4B) (N = 5-10 mice per condition). (B) Quantification of neutrophils elicited to WT (black) or Δpce (grey) P1121 pneumococci in nasal lavages of vehicle- or PAF-treated mice on day 7 p.i. (N = 5 per group). (C) Relative mean fluorescence intensity (MFI) of uptake receptor expression on luminal neutrophils elicited to WT or Δpce pneumococci (Δpce/WT) after daily treatment with vehicle (black) or 1 μg PAF (open), as described in (A) (N = 5). (D) Enumeration of bacterial CFUs as in (A), with and without daily intranasal treatments with 1 μg fMLP or vehicle (N = 5-7). (E) Quantification of elicited neutrophils as in (B) with or without treatment with fMLP (N = 5). (F) Relative uptake receptor MFI on neutrophils elicited to WT or Δpce pneumococci (Δpce/WT) after daily treatment with vehicle (black) or 1 μg fMLP (open), as in (C) (N = 5). Statistical significance was assessed by one-way ANOVA with Newman-Keuls post-test except for Panels C & F, wherein significance was assessed by one-sample t test relative to a value of 1. NS = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

Figure 7. Haemophilus influenzae GlpQ hydrolyzes phosphorylcholine and contributes to evasion of PAF-mediated neutrophil defense of the airway.

(A) Diagram of p-nitrophenylphosphorylcholine (pNPPC). Black arrow denotes site of
hydrolysis by *S. pneumoniae* Pce; white arrow indicates that by *H. influenzae* GlpQ. (B) P-nitrophenol liberation after incubating pNPPC with WT (black) or mutant (grey) *S. pneumoniae* (*Sp*) or *H. influenzae* (*Hi*) (*N* = 5). (C) Survival of WT (black) or ΔglpQ (grey) *Hi* during infection of the murine upper airway. (*N* = 5-8 mice per group, Limit of detection (LOD) = 4). (D) Quantification of neutrophils obtained from mice inoculated with WT or ΔglpQ bacteria (*N* = 4). (E) CD11b and CD64 relative MFI on neutrophils elicited by WT or ΔglpQ bacteria, d2 post-inoculation (p.i.) (*N* = 4). (F) Enumeration of day 2 WT or ΔglpQ *Hi* CFUs after treatment with α-Ly6G antibody or IgG2a isotype control. (*N* = 5, LOD = 4). (G) WT and ΔglpQ bacterial loads from PAFR-/- mice, littermate controls, or neutropenic PAFR-/- mice on day 2 p.i. (*N* = 3-9, LOD = 10). (H) Bacterial killing assay of WT *Hi* by 1000:1 murine neutrophils pre-treated with increasing concentrations of conditioned PAF media; PAF was pre-incubated with heat-killed WT (black) or ΔglpQ (white) bacteria before mixing with neutrophils prior to killing assays. Average values represent 3 biological replicates. Top dotted line: 100% bacterial survival; Bottom dotted line: average survival in the absence of PAF. Statistical significance was assessed by Student’s *t* test for pairwise comparisons (Panels B & H), one-way ANOVA with Newman-Keuls post-test for multi-group comparisons (Panels C, D, F, & G), and one-sample *t* test relative to null = 1 for relative MFI measurements (Panel E). NS = not significant, * = *p* < 0.05, ** = *p* < 0.01, *** = *p* < 0.001.

**Figure S1.** Clearance of Pce-deficient pneumococci on day 14 post-inoculation (p.i.) and upper airway luminal neutrophil gating strategy.

(A) Enumeration of WT (black) or Δpce (grey) P1121 pneumococcal CFUs obtained from nasal lavages on day 14 p.i. (*N* = 14). (B) Quantification of luminal neutrophils elicited
WT or Δpce pneumococci on day 14 p.i. (N = 5). (C) Gating strategy used to identify CD45+CD11b+Ly6G+ neutrophils from infiltrating immune cells during upper airway infection. Representative data represents neutrophils elicited by Δpce pneumococci on day 4 p.i. Statistical significance was assessed by Student’s t test. NS = not significant, *= p < 0.05.

**Figure S2. Assessment of the direct effects of PCA-4248, PAF, and fMLP on pneumococcal growth and viability in vitro.**

Growth curves (OD$_{620}$) of WT (black) and Δpce (grey) P1121 pneumococci in tryptic soy (TS) broth and grown either alone (A) or with increasing concentrations of PCA-4248 (B), PAF (C), or fMLP (D). Concentrations of drugs: PBS vehicle (circles), 2 nM (squares), 0.2 µM (diamonds), and 200 µM (triangles). Values represent three independent experiments. Statistical significance was assessed by Student’s t test; all comparisons were not significant.

**Figure S3. Quantification of complement deposition, capsule expression, and phosphorylcholine (ChoP) levels by bacterial flow cytometry.**

Graphs and representative histograms for mean fluorescence intensity (MFI) of: (A) Complement component C3 deposited on WT (black) and Δpce (grey) P1121 pneumococci after 30 minutes incubation with 66% murine serum. Experiments were also performed using heat-inactivated (HI) murine serum (white) and P1121Δcap lacking capsule expression (light grey). (B) P1121 capsule expression on WT and Δpce P1121 measured by deposition of anti-23F capsular antibody. (C) Accessibility of surface ChoP among P1121Δcap and P1121ΔpceΔcap pneumococci measured by deposition of anti-
phosphorylcholine antibody. Values represent duplicated independent experiments. Statistical significance was assessed by one-way ANOVA with Newman-Keuls post-test. NS = not significant, ** = p < 0.01.
<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence (5' to 3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pce amplification primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1, F</td>
<td>GGGGAATTCAGAATGAAAAGAAATTAAC</td>
<td>(340)</td>
</tr>
<tr>
<td>PCE-2, R</td>
<td>TATGGATCCCTACTGTCTGATTCCAGATTGT TTTTAC</td>
<td>(340)</td>
</tr>
<tr>
<td>Janus cassette insertion into pce</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP_0930 Upstream, F</td>
<td>TCTGCGTCCAGGGATTGTTC</td>
<td>This Study</td>
</tr>
<tr>
<td>SP_0930 Upstream, R</td>
<td>CATATTCCATAAAAATCAAACCATC</td>
<td>This Study</td>
</tr>
<tr>
<td>Janus Cassette, F</td>
<td>CAAGAAAGTGCCATGATGCGTGTATT</td>
<td>(321)</td>
</tr>
<tr>
<td>Janus Cassette, R</td>
<td>CACTCGTTTCAGACTTTATGCATTTCTG</td>
<td>(321)</td>
</tr>
<tr>
<td>SP_0930 Downstream, F</td>
<td>GTCCAAAGCATAGGAAGATCACAAGGC</td>
<td>This Study</td>
</tr>
<tr>
<td>SP_0930 Downstream, R</td>
<td>CAAGATGCTCTTGACCATCG</td>
<td>This Study</td>
</tr>
<tr>
<td>Unmarked pce deletion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP_0930 Upstream Del., F</td>
<td>GTCCAGGGATTTCTCACCGT</td>
<td>This Study</td>
</tr>
<tr>
<td>SP_0930 Upstream Janus Fusion, R</td>
<td>GAAGGCCCCTAAAACCTTCTTCTCTATCTTA</td>
<td>This Study</td>
</tr>
<tr>
<td>SP_0930 Downstream Janus Fusion, F</td>
<td>GGAACCTTGGAAAGATTTGAGAAAGTAAG</td>
<td>This Study</td>
</tr>
<tr>
<td>SP_0930 Downstream Del., R</td>
<td>CTTCACAAAGAGCCCTGC</td>
<td>This Study</td>
</tr>
<tr>
<td>pce cloning</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pce cloning primer, F</td>
<td>ATCGGATCGCACCTTGTAGGGCGTCTT</td>
<td>This Study</td>
</tr>
<tr>
<td>Pce cloning primer, R</td>
<td>ATCGAGCTCAACTCTGTTTCAGAGCTTTTG</td>
<td>This Study</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lyso-PAF Acetyltransferase, F</td>
<td>GGCACAGTGCTCTTCCAATGCT</td>
<td>(209)</td>
</tr>
<tr>
<td>Lyso-PAF Acetyltransferase, R</td>
<td>CCAACACCATGCATGGCGC</td>
<td>(209)</td>
</tr>
<tr>
<td>PAF Receptor, F</td>
<td>CTTAAGCTTGCACTAGGATTTG</td>
<td>(196)</td>
</tr>
<tr>
<td>PAF Receptor, R</td>
<td>GTACCTGCGATCTGTAAG</td>
<td>(196)</td>
</tr>
<tr>
<td>CXCL1, F</td>
<td>CTGCGAACGTCAACTCGATTACC</td>
<td>(245)</td>
</tr>
<tr>
<td>CXCL1, R</td>
<td>CAGGACGGAAGCCACCTGCTT</td>
<td>(245)</td>
</tr>
<tr>
<td>CXCL2, F</td>
<td>CGCTGCTATGCTGCAAAG</td>
<td>(245)</td>
</tr>
<tr>
<td>CXCL2, R</td>
<td>GGCCTGACACCTCAGATTTG</td>
<td>(245)</td>
</tr>
<tr>
<td>GAPDH, F</td>
<td>TGACACACACACTGCTT</td>
<td>(245)</td>
</tr>
<tr>
<td>GAPDH, R</td>
<td>GGATGCAAGGATGATTT</td>
<td>(245)</td>
</tr>
</tbody>
</table>
Figure 1

A

B

C

D

E

F
Figure 2
Figure 3

A

B

---

69
Figure 4
Figure 5

A

MURINE PMNs

![Graph A]

PMN: Bacterium Ratio

B

HUMAN PMNs

![Graph B]

PMN: Bacterium Ratio

C

![Graph C]

D

MURINE PMNs

![Graph D]

CD11b

E

HUMAN PMNs

![Graph E]

Bacterial Killing

[Graphs A, B, C, D, E show various experimental results related to PMN and bacterial interactions.]
Figure 6
Figure 7

A

B

C

D

E

F

G

H

pNPPC

PMN/mouse

CRU/mouse

Survival [%]

Bacterial Killing

[PAF] (M)

WT

ΔglpQ

WT

ΔglpQ

WT

ΔglpQ

WT

ΔglpQ

WT

ΔglpQ

WT

ΔglpQ

WT

ΔglpQ

WT

ΔglpQ

WT

ΔglpQ
Figure S2

A

No Drug

B

PCA-4248

C

PAF

D

fMLP
Figure S3
Supplemental Methods

*Generation of an insertion-duplication Δpce mutant.* A PCR product containing *pce* interrupted by an erythromycin resistance cassette was amplified from the R36AΔpce *S. pneumoniae* genome (a gift from Dr. Alexander Tomasz) using primers “P1” and “PCE-2” (see Table S1) (340). The product was transformed into the indicated wild type isolates, selected with 1 µg/mL erythromycin. Correct cassette insertion was verified by sequencing and lysates from all transformed mutants were incubated for 60 minutes with chromogenic p-nitrophenylphosphorylcholine to ensure no Pce enzymatic activity remained (see Methods).

*Generation and correction of an in-frame Δpce deletion mutant.* Deletion of *pce* was achieved through insertion and subsequent deletion of a bicistronic Janus construct bearing kanamycin resistance and streptomycin trans-sensitivity cassettes in spontaneously streptomycin-resistant bacteria, as detailed previously (81, 321). To insert the Janus cassette, an overlap-extension PCR product was created from three independent PCR reactions: First, a ~650 bp fragment upstream of *pce* was amplified from the pneumococcal genome using primers “SP_0930 Upstream, F&R”; the ~1.8 kb Janus cassette was amplified using primers “Janus Cassette, F&R”; and a ~640 bp fragment downstream of *pce* was amplified from the genome using primers “SP_0930 Downstream, F&R” (See Table S1). All three PCR products were then column-purified together (Qiagen) and extended using shared homology in a PCR reaction lacking primers. A final PCR using outside flanking primers “SP_0930 Upstream, F” and “SP_0930 Downstream, R” was performed to generate a product of approximately 3 kb. After purification, the product was transformed into wild type pneumococci and
transformants were selected with 500 µg/mL kanamycin before sequence verification. A similar procedure was performed to create a construct to delete the Janus-bearing pce gene, wherein 2 PCR products were generated by: 1) “SP_0930 Upstream Del., F” & “SP_0930 Upstream Janus Fusion, R”; and 2) “SP_0930 Downstream Janus Fusion, F” & “SP_0930 Upstream Del., R.” These products were sewn together as described above and used to transform Janus-bearing pce mutants. Clones were selected with 500 µg/mL streptomycin and sequenced to confirm in-frame deletion of pce. Lastly, a genetically corrected (Δpce::pce) strain was generated by transforming Janus-bearing Δpce mutants with a ~3 kb PCR product containing wild type pce using primers “SP_0930 Upstream, F” and “SP_0930 Downstream, R” before selection with 500 µg/mL streptomycin and sequence verification.

In vitro bacterial growth and toxicity assays. Kinetic bacterial growth assays were performed as described previously (89); 96-well plates bearing 190 µL of TS broth supplemented with 10 µL catalase per well (30,000 U/mL) (Worthington Biochemical) were inoculated with wild type or Δpce pneumococci at OD_{620} 0.1. Bacterial growth and viability were monitored at 15-minute intervals over 10 hours in the presence of PBS (vehicle control), PAF (Cayman Chemical), PAF receptor antagonist PCA-4248 (Tocris), or fMLP (Sigma-Aldrich) at the indicated concentrations.

Complement deposition assays. Complement component C3 deposition assays were adapted from Dalia et al., 2010 (80). Approximately ~10^6 PBS-washed, mid-log-phase bacteria were incubated at 37°C for 30 minutes in 66% fresh murine serum (in Hank’s Buffer with calcium). After a subsequent wash, bacteria were stained with 1:150 FITC-
conjugated, monoclonal anti-mouse complement component C3 antibody (Accurate Chem.) and detected by flow cytometry. Assays were repeated with serum heat-inactivated for 60 minutes at 65°C as negative controls and bacteria lacking capsule served as positive controls for C3 deposition.

Quantification of bacterial capsule and surface phosphorylcholine. To quantify pneumococcal polysaccharide capsule by flow cytometry, PBS-washed bacteria were incubated with 1:500 Type 23F polyclonal typing sera (Statens Serum Institut) for 30 minutes, washed, then stained with 1:150 AF647-conjugated anti-rabbit IgG (Life Technologies). Capsule-deficient pneumococci served as negative controls. To quantify surface phosphorylcholine, WT and Δpce bacteria lacking capsule (Δcap) were incubated as above with 1:500 monoclonal anti-phosphorylcholine IgA (TEPC-15, Sigma-Aldrich) before staining with 1:100 FITC-conjugated anti-mouse IgA (Sigma-Aldrich).

H. influenzae neutrophil bactericidal assays. Bacterial killing assays were performed to assess whether pre-incubation of PAF with GlpQ phosphodiesterase inhibits PAF-stimulated neutrophil bactericidal function. Increasing concentrations of PAF were pre-incubated for 60 minutes with 25 µL heat-killed WT or ΔglpQ H. influenzae re-suspended in PBS from OD₆₂₀ 1.0-normalized culture. Conditioned PAF solution was then centrifuged at 3,000g for 10 minutes to isolate supernatants, which were plated to confirm the absence of viable bacteria. Conditioned media from each condition was then applied to bone marrow-derived mature murine neutrophils, 10⁵ phagocytes per reaction. After an additional 30-minute incubation, 10² CFU wild type H. influenzae (pre-opsonized in 66% baby rabbit serum) was added to the treated neutrophils in each
reaction well. Bacterial survival was quantified in each condition by dilution plating after 45 minutes of incubation and compared to controls lacking neutrophils or PAF in pre-incubation steps.
CHAPTER 3:

Macrophage migration inhibitory factor (MIF) promotes clearance of pneumococcal colonization.

Rituparna Das¹, Meredith I. LaRose¹, Christopher B. Hergott¹, Lin Leng², Richard Bucala², Jeffrey N. Weiser¹

¹University of Pennsylvania School of Medicine, Philadelphia, PA,
²Yale School of Medicine, New Haven, CT

This work was originally published in The Journal of Immunology, July 2014, 193(2):764-72.
Abstract

Human genetic polymorphisms associated with decreased expression of macrophage migration inhibitory factor (MIF) have been linked to the risk of community-acquired pneumonia (CAP). Since *Streptococcus pneumoniae* is the leading cause of CAP and nasal carriage a precursor to invasive disease, we explored the role of MIF in the clearance of pneumococcal colonization in a mouse model. MIF-deficient mice (*Mif<sup>-/-</sup>*) showed prolonged colonization with both avirulent (23F) and virulent (6A) pneumococcal serotypes compared to wild-type animals. Pneumococcal carriage led to both local upregulation of MIF expression and systemic increase of the cytokine. Delayed clearance in the *Mif<sup>-/-</sup>* mice was correlated with reduced numbers of macrophages in upper respiratory tract lavages as well as impaired upregulation of monocyte chemotactic protein-1 (MCP-1/CCL2). We found that primary human monocyte derived macrophages as well as THP-1 macrophages produced MIF upon pneumococcal infection in a pneumolysin-dependent manner. Pneumolysin-induced MIF production required its pore-forming activity and phosphorylation of p38-MAPK in macrophages, with sustained p38-MAPK phosphorylation abrogated in the setting of MIF-deficiency. Challenge with pneumolysin-deficient bacteria demonstrated reduced MIF upregulation, decreased numbers of macrophages in the nasopharynx, and less effective clearance. *Mif<sup>-/-</sup>* mice also showed reduced antibody response to pneumococcal colonization and impaired ability to clear secondary carriage. Finally, local administration of MIF was able to restore bacterial clearance and macrophage accumulation in *Mif<sup>-/-</sup>* mice. Our work suggests that MIF is important for innate and
adaptive immunity to pneumococcal colonization and could be a contributing factor in
genetic differences in pneumococcal disease susceptibility.

Introduction

Despite the availability of vaccines and antimicrobial therapy, the burden of disease
caused by *Streptococcus pneumoniae*, or the pneumococcus, remains significant
worldwide. Among children <5 years, there are 14 million cases of serious pneumococcal
disease annually with almost 1 million deaths, concentrated largely in developing nations
(252). According to the Active Bacterial Core surveillance, 40,000 cases of invasive
pneumococcal disease (meningitis, bacteremia, and bacteremic pneumonia) occurred the
United States in 2010 (64). Additionally, the pneumococcus is estimated to be a
contributing organism in 20-60% of cases of community acquired pneumonia (CAP),
which leads to almost 600,000 annual hospitalizations, and is a leading cause of death
among persons >65 years (157).

Carriage of the pneumococcus in the nasopharynx is a risk factor for aspiration into the
lungs and a well-established to be a precursor to pneumonia and invasive disease with
the colonizing serotype (6, 37). Pneumococcal carriage begins within the first six months
of life and sequential colonization is prevalent throughout childhood (132). Immune
mediators of colonization, their contribution to disease, and whether they can be
assessed to understand disease risk and modulated in vaccination strategies are
important areas for study. Cell-mediated immunity appears to be more important than
humoral immunity in effecting primary pneumococcal clearance from the nasopharynx
Evidence of the contribution the CD4 T cell response is suggested by the greater incidence of both pneumococcal colonization and disease among HIV-infected compared to uninfected subjects. In addition to Th1 immunity, animal as well as human studies have implicated the Th17 response in pneumococcal clearance.

Although neutrophils are the first cell type to be recruited after colonization, the peak of their presence in the nasopharynx does not correlate with initiation of clearance, and their depletion does not seem to affect bacterial burden during carriage. Recently, a critical role for the sustained presence of macrophages in pneumococcal recognition and clearance, as well as coordination of the adaptive response, has been reported. Mice deficient in pattern recognition receptors show limited ability to generate local chemokine responses to enable recruitment and retention of macrophages in the upper airway and impaired pneumococcal clearance.

Defining the genetic determinants of immunity to pneumococcal disease risk is an area of active investigation. Some familial immune defects are known to confer profound risk for pneumococcal infection. However, the finding of increased susceptibility to pneumococcal disease in certain ethnic groups, despite controlling for disparities in socioeconomic status, suggests that there may be a broader genetic basis for susceptibility. Studies examining host genetics in both pneumococcal colonization and disease cohorts have proposed polymorphisms which may play a role, including a number affecting macrophage function.

Recently, an examination of host genetics in a cohort of 1700 older adults with CAP implicated two commonly-occurring polymorphisms conferring increased expression of
an innate mediator, macrophage migration inhibitory factor (MIF), in protection from disease (362). Microbiologic diagnosis is often challenging in CAP, but older age and community origin of the patients make the pneumococcus a likely pathogen in a majority of cases. The functional significance of these MIF variants, a tetranucleotide repeat in the promoter (-794 CATT) and a linked single nucleotide polymorphism (SNP, -173 G/C), in regulating macrophage response to various infectious and inflammatory stimuli has been demonstrated (82, 83, 273, 313). As pneumococcal carriage is a precursor to disease and macrophage responses are important in clearance, we chose to explore the role of MIF in a murine model of nasopharyngeal colonization.

MIF was the first cytokine discovered and named for its activity of retaining macrophages at the site of inflammation, making it an important factor to examine in pneumococcal colonization (35, 85). The expression of MIF by both immune cells (macrophages and lymphocytes) and epithelial cells (of the lung, gut, and skin) highlight its role in host-pathogen interaction (19, 57). MIF has been demonstrated to mediate recognition of gram-negative bacteria and mycobacteria by regulating macrophage expression of molecular pattern recognition receptors (82, 277, 278). Additionally, MIF promotes production of a variety of inflammatory cytokines by enabling nuclear translocation of NFκB and sustaining the activation of intracellular mitogen-activated protein (MAP) kinases (84, 240, 241). Studies with MIF-deficient (Mif-/-) mice have shown that the role of MIF (detrimental vs. beneficial) in different bacterial infections is dependent on the nature of the pathogen and the type of immunity induced. For instance, Mif-/- mice are protected from overwhelming inflammation in LPS or superantigen-induced shock but are more susceptible to infection with Salmonella typhimurium and Mycobacterium tuberculosis (58, 82, 183).
Early studies attributed the accumulation of alveolar macrophages in rabbit models of pneumococcal pneumonia to the activity of MIF (59). Thereafter, the role of MIF in infections with gram-positive pathogens which do not produce a superantigen, such as the pneumococcus, has received less attention. Additionally, the role of MIF in mucosal immunity or how it may mediate immune responses to the pneumococcus as a commensal as well as an agent of disease remains to be explored.

**Methods**

*Mice.* C57BL/6 (WT) mice were obtained from the Jackson Laboratory at 6–8 weeks of age. Mif<sup>−/−</sup> mice in the C57BL/6 background, backcrossed 10 generations, were obtained as previously described (82). WT and Mif<sup>−/−</sup> mice were age and sex matched in all experiments. All procedures were performed in accordance with the Institutional Animal Care and Use Committee protocols at the University of Pennsylvania.

*Bacterial strains and culture conditions.* The P1121 strain of pneumococcus was utilized for nasal colonization experiments because it is a minimally passaged serotype 23F isolate obtained from the nasopharynx of a subject in a human carriage study (227). Another clinical isolate for serotype 6A also was used (276). 23F pneumococci as well as the previously described pneumolysin deletion (23F<sub>ply−</sub>), point mutant (23F<sub>plyW433F</sub>), and revertant (23F<sub>ply−ply+</sub>) strains were also utilized for macrophage infections where indicated (87). All bacteria were grown in tryptic soy broth (TS, Life Technologies) at 37°C and 5% CO<sub>2</sub> until cultures reached mid-log phase, OD<sub>600</sub> between 0.45 and 0.50.
**Murine model of pneumococcal colonization.** All bacterial strains were animal passaged prior to use in experiments and stored at −80°C in 20% glycerol. Inocula consisted of $10^7$ mid-log-phase PBS-washed bacteria in 10μl PBS and were plated to confirm dose. They were delivered to the nares of unanesthetized mice as previously described (360). At the indicated time points, mice were sacrificed, their trachea cannulated, and 200μl PBS instilled. Lavage fluid was collected from the nares and serially diluted in PBS for plating on TS agar plates supplemented with catalase (Worthington Biochemicals). The lower limit of detection was either 100 CFU/ml or 20 CFU/ml lavage fluid, depending on the experiment. Blood was collected by cardiac puncture and the serum separated. Serum and lavages were assayed for mMIF by specific ELSIA. Serum was additionally assayed for anti-pneumococcal IgG as previously described (87).

Recombinant murine MIF (rMIF) was produced as described previously and ensured to be LPS-free (32). PBS was used to dilute the rMIF to the indicated concentration. As dimethyl sulfoxide (DMSO) was present in the rMIF preparation, an identical amount of DMSO was added to PBS for the vehicle control treatment. Either rMIF or vehicle control were administered in a 10μl volume to the nares of unanesthetized mice for the frequency and duration described.

**RNA Extraction and RT-PCR.** RNA was isolated from the upper respiratory tract following a lavage with 300μl RNA lysis buffer using an RNeasy Mini Kit (QIAGEN) according to manufacturer’s protocol. Complementary DNA was reverse transcribed using a high-capacity reverse transcription kit (Applied Biosystems). Approximately 25ng cDNA was used as a template in reactions with 0.5μM of forward and reverse primers for MIF, GAPDH, and CCL2 and SYBR Green (Applied Biosystems), according
to the manufacturer’s protocol and as previously described (82, 87). Reactions were carried out using the StepOnePlus Real-Time PCR system, and quantitative comparisons were obtained using the ΔΔCT method (Applied Biosystems). Mock-infected WT mice were set as the reference to which relative comparisons were made.

*Flow cytometry.* The nasal lavages of 5 mice from each group were pooled, centrifuged to obtain a cell pellet, and resuspended in PBS with 1% bovine serum albumin. Nonspecific binding was blocked using a rat anti-mouse antibody directed against the FcγIII/II receptor (CD16/CD32) (BD Biosciences), and the cells were stained with the following rat anti-mouse cell surface antibodies: Ly6G, Ly6C, CD11b, CD11c, Siglec-F, and F4/80 (BD Biosciences). All samples were fixed in 4% paraformaldehyde before analysis. Data were acquired using the FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

*Macrophage culture and stimulation.* Human macrophages were prepared from peripheral blood mononuclear cells (PBMCs) isolated by Ficoll-Hypaque gradient centrifugation. The cells were resuspended in RPMI 1640 medium supplemented with 20% human AB serum (Gemini) and plated. After 2 h of culture, the adherent cells were washed extensively with PBS and cultured for 1 week with human serum supplemented media to allow differentiation into monocyte-derived macrophages. THP-1 monocytes (ATCC) were differentiated into macrophages by adding 50ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) to RPMI supplemented with 10% fetal calf serum culture (FCS) medium for 16 hours.
Both human monocyte derived macrophages and THP-1 cells were infected at a multiplicity of infection (MOI) ratio of 10 bacteria per macrophage. The bacteria were spun onto the macrophages by centrifuging at 1500 X g for 10 minutes. Invasion was allowed for 1 hour at 37°C, 5%CO₂, then extracellular bacteria were washed away, and gentamicin-supplemented culture media was added. Conditioned supernatants were collected at the indicated time points. Cell viability was confirmed by light microscopy and lactate dehydrogenase release assay (Roche). Macrophages were treated with the p38-MAP kinase inhibitor, SB203580 (Cell Signaling), at a concentration of 10μM added to the culture media throughout the course of the experiment (or control DMSO supplemented culture medium) where indicated. Supernatants were assayed for huMIF by specific ELISA.

Bone marrow derived macrophages (BMDMs) were prepared by differentiating cells flushed from the femur/tibias of C57BL/6 mice of the appropriate genotype (WT or Mif⁻/⁻) in the presence of supernatant from L929 cells as a source of M-CSF (in DMEM and 10% FCS) for 1 week. The macrophages were harvested and replated for infection experiments. For BMDM experiments, infected macrophages were placed into the incubator immediately after centrifugation and lysed on ice with cold RIPA buffer (Cell Signaling) supplemented with protease inhibitor (Roche) and phosphatase inhibitor (Sigma-Aldrich), at the indicated time points.

*Western Blotting.* Cell lysates were separated on a 10% polyacrylamide gel (Bio-Rad) and proteins were transferred to polyvinylidene difluoride membranes. Membranes were probed first for phospho p38-MAPK using a rabbit monoclonal antibody (Cell
Signaling). The same membrane was then stripped and re-probed for total p38-MAPK (rabbit monoclonal, Cell Signaling).

Statistics. Statistical comparisons were computed using the Mann-Whitney U test (non-parametric, 2-tailed t test) or one way analysis of variance as indicated in the figure legends (Prism 4, GraphPad Software). A P value of less than 0.05 was considered significant.

Results

MIF is required for the clearance of pneumococcal colonization from the nasopharynx.

We found WT and Mif−/− mice to be colonized to a similar degree after inoculation with 23F pneumococci, a clinically relevant serotype which is not associated with pneumonia or invasive disease in mice. By 7 days post-challenge, when bacterial clearance is initiated, a two-fold higher bacterial load was noted in the nasopharynx of the Mif−/− mice compared to their WT counterparts (Figure 1A). In the WT animals, clearance progressed rapidly and was largely complete by 14 days. In contrast, the Mif−/− mice had prolonged colonization through day 28, but were able to clear the infection by day 42 (data not shown). As expected, no symptoms of pneumococcal disease were observed in either group of animals.

To ensure that the clearance defect in Mif−/− mice was not a phenomenon unique to serotype 23F pneumococci, we tested a serotype 6A isolate, which colonizes the murine nasopharynx and also causes bacteremia and sepsis. The Mif−/− mice demonstrated
almost a log higher bacterial load in their nasal washes at 7 days compared to their WT counterparts (Figure 1B). Perhaps as a consequence of the greater bacterial burden, Mif−/− animals also demonstrated greater mortality from sepsis compared to WT (54% vs. 26%, p=0.05).

**Pneumococcal colonization leads to both local and systemic MIF expression.** We next explored whether exposure to pneumococci in the nasopharynx could induce expression of MIF. At 3 days post colonization, MIF expression in the nasopharynx was assayed by qRT PCR performed on RNA extracted from a nasal wash with lysis buffer. We found a basal level of MIF expression in the nasal wash (compared to Mif−/− control, Figure 2A). Upon pneumococcal colonization, the MIF expression was upregulated 2.5-fold. Our attempts to detect MIF protein in the nasopharynx were limited by dilution in the volume required for nasal lavage. Local exposure to pneumococci in the nasal mucosa led to a rise in the circulating level of MIF at 3 days, without any evidence of bacterial dissemination (Figure 2B). Notably, both local upregulation and systemic increase of MIF at 3 days preceded the onset of bacterial clearance, which began at 7 days and accelerated thereafter.

**Cellular recruitment and chemokine expression are impaired in the absence of MIF.** Macrophage recruitment to the nasopharynx has been demonstrated to effect pneumococcal clearance and to be dependent on bacterial recognition and local MCP-1 (CCL2) induction and signaling (87, 366). When we compared the cellular composition of nasal washes from WT and Mif−/− mice over the course of pneumococcal colonization, we observed reduced numbers of macrophages (CD11b+, F4/80+ cells) in the Mif−/−
animals at both 3 and 7 days (Figure 3A). Nasopharyngeal macrophages lacked expression of CD11c, Ly6C, Ly6G, and Siglec-F. The presence of macrophages in the nasopharynx of WT animals at 3 days correlated with transcriptional upregulation of MIF, and preceded the initiation of pneumococcal clearance. The MIF-dependent defect in macrophage numbers was sustained thereafter and could be detected at 14 days post colonization. The defect seemed to be specific to macrophages as neutrophil infiltration in the nasopharynx was unaffected in the absence of MIF (Figure 3B).

We also found that Mif<sup>−/−</sup> animals were impaired in their ability to induce transcription of MCP-1 (CCL2) in the nasopharynx during pneumococcal colonization (Figure 3C). While WT mice upregulated expression of the macrophage chemoattractant almost 10-fold compared to uninfected, this was reduced ~60% in the Mif<sup>−/−</sup> mice. Once again, dilution in the nasopharyngeal lavage precluded our ability to explore the differences in MCP-1 (CCL2) on the protein level.

**Pneumolysin mediates the action of MIF in the nasopharynx**

We next sought to examine the role of bacterial factors in mediating inflammatory macrophage responses in the nasopharynx. We focused on the pneumococcal pore-forming toxin, pneumolysin, which in addition to its role as a cytolysin has been demonstrated to be important for bacterial recognition by the innate immune system, subsequent initiation of the inflammatory cascade, and bacterial clearance (213, 234, 337, 355). Sensing of pneumolysin has been proposed to occur through TLR4, NLRP3, as well as pore-formation and osmotic gradient-dependent mechanisms (271). When we compared the kinetics of pneumococcal clearance using pneumolysin sufficient and deficient pneumococci, we found that carriage was more dense and prolonged in the
setting of pneumolysin deficiency in both the WT and Mif−/− animals (Figure 4A). However, the MIF-dependent defect in pneumococcal clearance was eliminated during colonization with pneumolysin deficient pneumocci, suggesting that the role of MIF in pneumococcal clearance requires pneumolysin. We also found macrophage recruitment to the nasopharynx to be reduced and local upregulation of MIF to be impaired in the setting of colonization with pneumolysin-deficient pneumococci (23Fply−, Figure 4B and C).

**Pneumolysin-stimulated phosphorylation of p38-MAPK is important for cytokine production and impaired in the absence of MIF.**

Bacterial infection is known to induce the production of MIF from a variety of cell types, and MIF has been proposed to modulate host-pathogen interactions by regulating macrophage expression of pattern recognition receptors such as TLR4 and dectin-1 (82, 277, 278). We first confirmed robust MIF production from human macrophages 4 hours after infection with pneumococci (Figure 5A). Next, we examined the requirements of MIF production using a model of pneumococcal infection in human THP-1 monocytes differentiated into macrophages. Since pneumococci have been implicated in the lysis of host cells, we examined MIF production early in the course of infection and confirmed cell viability to exclude death as a source of MIF.

We observed no induction of MIF after infection of the cells with 23Fply− bacteria compared to mock infected controls, consistent with our findings on the role of pneumolysin in the nasopharynx. Genetic correction of the mutation, in the 23Fply−ply+ strain, restored MIF production. We concluded that pneumolysin is required for pneumococcal-induced MIF production from macrophages.
To dissect the basis of the macrophage-pneumolysin interaction, we explored the role of TLR4 in this process. We hypothesized that if TLR4 is crucial for pneumolysin-dependent inflammatory cytokine production, reduced TLR4 in the Mif\(^{-/-}\) mice may underlie their abrogated response to pneumococcal colonization. To test this, we utilized a 23F\(^{ol4}W_{433}F\) mutant, which expresses a pneumolysin that can activate TLR4 but is deficient in its ability to form functional membrane pores. Osmotic stress from pneumolysin pores has been proposed to activate inflammation by an alternative mechanism – inducing phosphorylation of p38-MAPK (213, 271). Phosphorylation of MAPKs is known to be an upstream event in the induction of a number of inflammatory cytokines. We found no MIF production from THP-1 macrophages infected with the 23F\(^{ol4}W_{433}F\) pneumococci, suggesting that MIF’s effects in pneumococci-induced inflammation are TLR4-independent and may be related to an alternate mechanism such as pore formation and p38-MAPK phosphorylation. To evaluate this, we treated macrophages with SB203580, a p38-MAPK inhibitor (MAPKi), and assayed MIF production. As SB203580 inhibits all isoforms of p38MAPKs, there is a potential for off-target effects with its use. We found MIF production to be diminished in the macrophages treated with the MAPKi compared to vehicle control treated cells (Figure 5B).

Phosphorylation of p38-MAPK is involved in the secretion of inflammatory cytokines from immune cells (271). Therefore, we next explored phosphorylation of p38-MAPK in WT and Mif\(^{-/-}\) BMDMs after pneumococcal infection. Phospho p-38 MAPK was demonstrated immediately after infection in the WT cells and sustained over the course of the experiment (Figure 5C). By contrast, although some phospho p-38 MAPK was
noted at early after infection (2 min) in the Mif\(^{-/-}\) cells, it was not observed thereafter (5 or 10 mins). Interestingly, MAPK phosphatase 1 (MKP1), which has been postulated to be involved in the resolution of inflammatory responses, is observed to be downregulated by MIF and found to be constitutively active in the setting of MIF-deficiency (3).

**MIF is involved in the generation of the adaptive immune response to pneumococcal colonization.**

Given the important role of macrophages in coordinating the adaptive immune response, we next examined the role of MIF in generation of antibody responses to pneumococcal colonization and secondary bacterial clearance (87). After six weeks of colonization, when WT and Mif\(^{-/-}\) animals were clear of pneumococci in the nasopharynx, serum was obtained and analyzed for total anti-pneumococcal IgG titers by ELISA. We found Mif\(^{-/-}\) mice to have 10-fold lower antibody titers compared to their WT counterparts (Figure 6A), despite more prolonged bacterial exposure. To evaluate the consequences of an impaired adaptive response, we re-inoculated WT and Mif\(^{-/-}\) mice with the colonizing inocula of 23F pneumococci 6 weeks after primary challenge. Prior studies of secondary challenge in previously colonized WT mice demonstrated rapid pneumococcal clearance (366). We noted that Mif\(^{-/-}\) mice remained colonized with \(~10^4\) pneumococci 5 days after secondary challenge compared to WT mice which had \(<10^3\) bacteria recovered (Figure 6B). These results demonstrate that the MIF-dependent macrophage effects during pneumococcal colonization also impact downstream adaptive immune responses.

**Nasopharyngeal treatment with rMIF restores the MIF-dependent defects in pneumococcal clearance.**
Finally, we sought to rescue MIF effects on pneumococcal clearance by replacing the cytokine in $Mif^{-/-}$ mice. $Mif^{-/-}$ mice were dosed with 100ng of rMIF or vehicle control in the nasopharynx every other day after pneumococcal inoculation for a period of 2 weeks. The rMIF-treated mice had a 1.5 log reduction in their nasopharyngeal bacterial load compared to vehicle control treated animals (Figure 7A). We also evaluated the cellular composition of the nasopharyngeal lavage from the $Mif^{-/-}$ animals with and without MIF treatment. We found MIF administration to be associated with an accumulation of macrophages to a level similar to that observed in WT animals (Figure 7B). Taken together, the results of our MIF replacement experiments confirm that the presence of MIF in the nasopharynx has a direct effect on the recruitment and retention of macrophages, which enables them to promote pneumococcal clearance.

**Discussion**

We show herein that MIF is expressed by macrophages upon pneumococcal infection in a pneumolysin-dependent manner, via a mechanism that requires the phosphorylation of p38-MAPK. Additionally, we demonstrate that MIF promotes the MCP-1 (CCL2)-mediated recruitment and retention of macrophages in the nasopharynx to allow for clearance of primary carriage, and also is required for the generation of adaptive responses - antibody production and clearance after repeat pneumococcal challenge. Taken together with our finding that administration of rMIF to the MIF-deficient animals recovers these defects, our studies indicate that MIF is both necessary and sufficient for the accumulation of macrophages in the nasopharynx and subsequent pneumococcal clearance.
Our work in the mouse model suggests that a setting of relative MIF deficiency may be associated with inability to clear pneumococcal carriage. Although it was not possible to demonstrate a correlation between higher nasal colonization and pneumonia in the animal model using the 23F serotype, clinical data correlating colonization burden to pneumonia suggests that in humans, relative MIF deficiency may confer a propensity for downstream disease. These results are in support of the clinical findings reported by Yende and colleagues reporting older adults with the low-expresser MIF genotype to be more likely to develop CAP as well as suffer adverse outcomes (362). The functionality of human MIF polymorphisms in the response to infectious stimuli has been demonstrated in gram-negative bacterial infection as well as in tuberculous and meningococcal disease (82, 83, 273). One study also has suggested that MIF mRNA upregulation in peripheral blood mononuclear cells upon pneumococcal infection is influenced by MIF genotype (325). Further investigation is necessary to analyze the MIF-genotype effect on nasopharyngeal and systemic pneumococcal responses. Additionally, genetic examinations of dedicated patient cohorts will be required to ascertain the impact of immune factor polymorphisms on the risk for pneumococcal colonization and disease. Reduced ability to clear primary colonization or develop adequate adaptive responses to deal with subsequent challenge, both of which were affected by MIF in our model, are risk factors for pneumococcal disease in children and adults (112).

Genetic examinations thus far suggest a dual role for MIF in infection with gram-negative pathogens – promoting pathogen elimination in some scenarios but causing inflammatory damage in others. In meningococcal disease, the low-expresser MIF
genotype is associated with mortality from disease but protective from its occurrence (273). High-expression of MIF is correlated with morbidity and mortality in sepsis but protects older adults from developing gram-negative bacteremia (83, 195). In lower respiratory tract infection with Pseudomonas aeruginosa, the absence of MIF protected mice from neutrophil-dependent inflammatory pathology, and genetic low-expressers of MIF among individuals with cystic fibrosis were protected from pseudomonas pneumonia (42, 263). Fewer studies have examined the role of MIF in the response to gram-positive pathogens, and work is underway to determine whether MIF is beneficial or detrimental in host responses to pneumococcal infection in the lower respiratory tract. A preliminary genetic examination of patients with pneumococcal disease found meningitis to be associated with high-expression MIF genotypes, suggesting that MIF may play divergent roles depending on the anatomic site of host-pneumococcal interaction and whether the inflammatory response is beneficial or detrimental to the host (95).

MIF appears to be critical for the control of infections where recruitment and retention of macrophages plays a central role in mounting an effective immune response. The source of the macrophages in the nasopharynx is an active area of investigation. Studies to date have implicated embryologically divergent origins for the circulating monocytes and alveolar macrophages in the lung; whether there are analogous populations of cells in the nasopharynx is unknown (137). The absence of CD11b on both alveolar and nasopharyngeal macrophages suggests phenotypic similarities, but the relatively small number of cells in the latter group make their immunologic characterization challenging. MIF may play a role in the migration of circulating monocytes into tissues in an MCP-1 (CCL2) dependent manner, setting up a positive-feedback loop as more cells are
recruited (134). Additionally, some of the seminal studies of MIF described its ability to inhibit migration and promote retention of alveolar macrophages (59). Recruitment of circulating monocytes or retention of tissue macrophages may serve as the mechanism by which MIF promotes nasopharyngeal macrophage accumulation in pneumococcal colonization. We found no MIF-dependent defects in uptake or killing of pneumococci in vitro by BMDMs (data not shown), leading us to the conclusion that it is MIF’s role in the recruitment/retention of macrophages in the nasopharynx which underlie its importance in clearance of colonization.

This study also highlights the immunomodulatory properties of pneumolysin, which serves as a virulence factor for in invasive pneumococcal disease, but has been found to promote bacterial clearance from the nasopharynx (33, 337). We report that TLR4-independent stimulation of macrophages to effect inflammatory cytokine production requires phosphorylation of p38-MAPK, a process which had been previously reported in epithelial cells, both in vitro and in vivo (29, 271). The action of a bacterial cytolsin to promote pattern recognition receptor-independent cytokine production in innate immune cells has been described with the β-hemolysin of another gram positive pathogen – group B streptococcus (28). A potential mechanism for MAPK-dependent secretion of MIF may be through the binding of the transcription factor, specificity protein (Sp) 1, to the MIF promoter (279).

Investigating host and bacterial factors which effect immunity in the nasal mucosa contributes to the understanding of how the pneumococcus is able to exist as a commensal in the upper airways but cause invasive disease in the lower tract (347). Although the role of MIF in pulmonary immune responses has been examined, this work
is the first examination of its function in the upper airways. MIF production has been observed in human gastric and intestinal mucosa upon gram-negative bacterial infection (109, 207). We demonstrate a role for MIF in development of respiratory mucosal immunity – macrophage retention and chemokine production - required for pneumococcal clearance. We focused on MIF production from macrophages in the pneumococcal response, but further studies are necessary to determine whether there are additional cellular sources (e.g. dendritic cells or epithelial cells). Finally, our work adds pneumolysin-mediated phosphorylation of p38-MAPK to pattern recognition receptor activation as a mechanism of macrophage activation in response to pneumococcal challenge. Further investigations of the role of MIF in mucosal immunity will be important to the understanding local protective responses in response to vaccination as well as the contribution of host genetics to the risk of pneumococcal disease.

**Figure Legends**

**Figure 1.** **MIF is important for the clearance of pneumococcal colonization.** Wild type (WT) and MIF-deficient (Mif<sup>−/−</sup>) mice were inoculated intranasally with 10<sup>7</sup> CFU pneumococci. Lavages of the upper respiratory tract were performed thereafter to determine colonization density (CFU/ml). WT mice (black squares) display accelerated clearance of 23F pneumococci compared to Mif<sup>−/−</sup> mice (open squares, A). The MIF-dependent defect in colonization is evident using both 23F and 6A strains of pneumococci at 7 days (B). N≥5 mice per experiment, at least two experiments. The dashed line indicates the limit of detection. Error bars represent S.D. and horizontal
lines indicate mean values. Y-axis units depicting CFU/ml are on a log base 10 scale. *P < 0.05, **P < 0.01, Mann-Whitney U test.

**Figure 2.** Pneumococcal colonization leads to local upregulation and systemic production of MIF in WT mice.

Upper respiratory tract lavages were obtained 3 days after inoculation using RNA lysis buffer. RNA was isolated and reverse transcribed, and MIF expression level was measured by quantitative RT-PCR relative to GAPDH controls. Baseline MIF expression and MIF upregulation after colonization was noted in WT (A). Serum was obtained by cardiac puncture at 3 days post-colonization and analyzed for MIF by specific ELISA. Increased circulating MIF was noted in WT mice (B). Values are relative to mock-colonized WT mice ± SD (n≥10 mice per group). *P < 0.05, **P < 0.01, unpaired t test.

**Figure 3.** MIF is required for the macrophage influx and MCP-1 upregulation in response to pneumococcal colonization.

Upper respiratory tract lavages were obtained 3 and 7 days after inoculation and the composition of the cellular infiltrate was determined by flow cytometry. Numbers of macrophages (F4/80+, CD11b–, A) and neutrophils (Ly6G+, CD11b+, B) are shown in WT (gray bars) and Mif–/– mice (white bars). Each bar represents the average number of events ± SD in 4 experiments, each with 5 mice. MCP-1 upregulation in the WT mice was demonstrated by quantitative RT PCR of RNA from nasal lavages and found to be reduced in the Mif–/– mice (C). RT PCR values are relative to mock-colonized WT mice ± SD (n≥10 mice per group). *P < 0.05, **P < 0.01 Mann-Whitney U test or unpaired t test.
Figure 4. Pneumolysin mediates the effect of MIF on pneumococcal colonization.

Colonization experiments were performed in WT (close squares) and Mif\(^{-/-}\) (open squares) mice using \(10^7\) CFU of strain 23F and strain 23F\(^{ply-}\). Nasal lavage was obtained at 14 days. 23F\(^{ply-}\) (pneumolysin-deficient ) pneumococci colonized both WT and T Mif\(^{-/-}\) mice to a greater degree than 23F. The MIF-dependent defect in pneumococcal clearance was eliminated in the absence of pneumolysin (A). Macrophage influx, quantified by flow cytometry, was greater in WT mice colonized with strain 23F compared to 23F\(^{ply-}\) (B). MIF upregulation, assessed using quantitative RT PCR of nasal lavage was present in strain 23F colonization and absent in and 23F\(^{ply-}\) colonization (C). Flow cytometry, mean ± SD, n=5 mice per experiment, 4 experiments. RT-PCR, relative to mock-colonized WT mice ± SD, n≥10 mice per group. Y-axis units depicting CFU/ml are on a log base 10 scale. *\(P < 0.05\), **\(P < 0.01\), Mann-Whitney U test or unpaired t test.

Figure 5. MIF is produced from macrophages in a process that requires the pore-forming function of pneumolysin.

Human macrophages differentiated from PBMCs were infected with 23F pneumococci at an MOI of 10:1 and their culture supernatant assayed for MIF production by specific ELISA (A). THP-1 macrophages, differentiated by PMA, were infected with the indicated strain of pneumococci. MIF production after infection was diminished in the 23F\(^{ply-}\) and 23F\(^{plyW433F}\) strains compared to 23F, and restored in the revertant 23F\(^{ply-\rightarrow ply+}\) strain (B). MIF production was abrogated by inhibition of p38MAPK phosphorylation by treatment with SB203580 (a specific MAPKi, C). Phosphorylation of p38MAPK was observed by western blotting after infection of cultured bone marrow derived

102
macrophages (BMDMs) from WT mice with 23F pneumococci, and quantified by densitometry. p38 MAPK phosphorylation was diminished in BMDMs of Mif<sup>−/−</sup> mice (D). Mean ± SD values depicted from 4 independent experiments. Representative western blot shown and densitometry performed from 4 independent experiments. *P<0.05, **P < 0.01, ***P<0.001, one-way ANOVA.

Figure 6. MIF promotes the generation of adaptive responses to pneumococcal colonization.

Mice were inoculated intranasally with 10<sup>7</sup> CFU of 23F pneumococci and 21 days after inoculation, were sacrificed, serum was isolated, and levels of anti-pneumococcal serum IgG determined by ELISA. Values are expressed as geometric mean titers. WT mice (black squares) had circulating higher antibody titers to pneumococcus compared to Mif<sup>−/−</sup> mice (open squares, A). Mice were allowed to clear primary colonization for 6 weeks, and then re-challenged with 10<sup>7</sup> CFU 23F pneumococci. Nasal lavage was obtained at 5 days. Mif<sup>−/−</sup> mice had higher levels of colonization compared to WT (B). N≥5 mice per experiment, at least two experiments. Y-axis units depicting CFU/ml are on a log base 10 scale. **P < 0.01, Mann-Whitney U test.

Figure 7. Nasopharyngeal treatment with rMIF recovers the MIF-dependent defect in macrophage recruitment and pneumococcal clearance.

Mif<sup>−/−</sup> mice were colonized with 10<sup>7</sup> 23F pneumococci, and treated every other day with either 100ng of rMIF in PBS or vehicle control. Nasal lavage was obtained at 14 days. MIF treatment let to greater clearance of pneumococcal colonization compared to control (A). Macrophage influx, quantified by flow cytometry, was also greater in the rMIF treated mice compared to vehicle treated controls (B). Horizontal lines indicate
mean values, dashed line is the limit of detection. Flow cytometry, mean ± SD, n=5 mice per experiment, 3 experiments. Y-axis units depicting CFU/ml are on a log base 10 scale.

*P < 0.05, **P < 0.01, Mann-Whitney U test.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
CHAPTER 4:

Brief Report: Macrophage migration inhibitory factor (MIF) is detrimental in pneumococcal pneumonia and a target for therapeutic immunomodulation.

Jeffrey N. Weiser¹, Aoife M. Roche¹, Christopher B. Hergott¹, ², Meredith I. LaRose², Tarah Connolly³, William L. Jorgensen⁴, Lin Leng³, Richard Bucala³, Rituparna Das²

¹Departments Microbiology and ²Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA

³Department of Medicine, Yale School of Medicine, New Haven, CT

⁴Department of Chemistry, Yale University, New Haven, CT

This work was originally published in The Journal of Infectious Diseases, May 2015, Epub ahead of print, pii: jiv262.
Abstract

Mortality from pneumococcal pneumonia remains high despite antibiotic therapy, highlighting the pathogenic potential for host inflammation. We demonstrate that macrophage migration inhibitory factor (MIF), an innate immune mediator, is detrimental for survival as well as lung pathology, inflammatory cellular infiltration, and bacterial burden in a mouse model of pneumococcal pneumonia despite being necessary for clearance from the nasopharynx. Treatment of animals with a small molecule inhibitor of MIF improves survival by reducing inflammation and improving bacterial control. Our work demonstrates that MIF modulates beneficial vs. detrimental inflammatory responses in the host-pneumococcal interaction and is a potential target for therapeutic modulation.

Introduction

Mortality from invasive pneumococcal infection remains at 10% today. Even with appropriate antibiotic administration mortality is still a concern in the treatment of pneumonia, implicating a pathogenic component of the host response (18, 64). Development of novel, antibiotic-independent, therapeutic strategies are needed to address the disease burden associated with pneumococcal lung infections.

The pathogenesis of pneumococcal pneumonia is governed by the interplay of inflammatory responses - some are required to control bacterial growth whereas others are dispensable or detrimental and result in inflammatory pathology. After infection,
sensing of the pneumococcus by epithelial and resident immune cells leads to the production of cytokines and chemokines, which recruit innate effector cells, neutrophils and monocytes, to the lung. Neutrophils and monocytes/macrophages are agents of pneumococcal killing, but their role in the regulation of inflammation has recently come to light. Depletion of neutrophils in pneumonia improves survival and lung pathology without compromising bacterial control (216). Additionally, IL-1 signaling in monocytes and epithelial cells promotes expression of the neutrophil chemoattractants KC/CXCL1 and MIP-2/CXCL2, resulting in increased pulmonary neutrophilia and exacerbated disease (217). The importance of cooperation between monocytes and neutrophils is highlighted by findings that mice lacking either macrophages or components of the macrophage apoptosis machinery demonstrate increased neutrophil recruitment and impaired pneumococcal control (217, 275).

We recently reported that macrophage migration inhibitory factor (MIF), a cytokine expressed abundantly by both immune and epithelial cells at sites of host-pathogen interaction, retains macrophages in the nasopharynx in response to pneumococcal colonization and is required for bacterial clearance (224). This supports the observation that older adults who are low-expressers of MIF, by way of a commonly occurring genetic polymorphism, are at greater risk for community-acquired pneumonia (362). However, given that host-pathogen interactions may vary in different regions of the respiratory tract, we next sought to define the role of MIF in pulmonary immunity during pneumococcal pneumonia.

Methods
Mice. Adult C57BL/6 (wild type, WT) mice were age/sex matched with MIF-deficient (Mif−/−) mice in the C57BL/6 background for all experiments (224). Procedures were performed in accordance with the Institutional Animal Care and Use Committee protocols of the University of Pennsylvania.

Model of pneumococcal colonization and pneumonia. An animal-passaged serotype 3 strain of Streptococcus pneumoniae (ATCC 6303) was used for all experiments. Cultures were grown in tryptic soy broth (TS, Life Technologies) at 37°C to mid-log phase, OD620~1.0. For colonization experiments, 10⁷ mid-log phase bacteria in 10μl of PBS were delivered to the nares of awake mice (224). At the indicated time points, mice were sacrificed, their trachea cannulated, and lavage fluid collected from the nares for bacterial enumeration. Pneumonia was modeled by administering 10⁴ mid-log phase bacteria in 80μl of PBS to the nares of isoflurane-anesthetized mice (Santa Cruz Biotechnology). During survival experiments animals were monitored twice a day for signs of illness such as decreased activity and labored breathing, and euthanized if they were in extremis. Otherwise, they were sacrificed by CO₂ asphyxiation at the indicated times.

For MIF replacement experiments, 100ng of endotoxin-free recombinant MIF (rMIF) dissolved in 80μL of PBS, or PBS alone, was administered to the nares of anesthetized mice (224). For MIF-inhibition, 40mg/kg of 3-(3-hydroxybenzyl)-5-methylbenzooxazol-2-one, designated MIF098, dissolved in PEG 400 plus (2-hydroxypropyl)-β-cyclodextrin vehicle, or vehicle alone, was administered i.p. twice daily beginning on the day of
infection (320). MIF098 did not directly inhibit pneumococcal growth, even at ten-fold the concentration used in vivo.

Bronchoalveolar lavage was performed with 1ml of cold PBS for protein analysis or 3ml for flow cytometry. Fluid was centrifuged to pellet cells prior to protein determination (bicinchoninic acid (BCA) assay, Thermo Scientific) and ELISAs (eBioscience). Blood was obtained by cardiac puncture and diluted 1:10 in PBS before plating. For bacterial enumeration, the right lung lobes were collected in PBS, mechanically disrupted, and serial dilutions plated. Histologic examination was performed on hematoxylin and eosin (H&E) stained sections of the left lung. Pathology was scored as follows: 0-no involvement; 1-localized infiltrates of neutrophils in alveoli, no bacteria; 2-dense infiltrates of neutrophils in airways with involvement of adjacent alveoli, no bacteria; 3-consolidation of neutrophil inflammation in bronchioles and alveoli, lobar pneumonia, with intact visible bacteria; 4, overwhelming infection with bacteria greater than inflammatory cells in alveoli or bronchioles.

Flow cytometry. Single cell suspensions were prepared by incubating minced tissue (left lung) with collagenaseIV (Worthington Biochemicals) and DNaseI (Sigma-Aldrich) for 1 hour at 37°C. The digest was disrupted by passing through a cell-strainer (BD Bioscience). Red blood cells were lysed and cells were washed in DMEM/FBS (Life Technologies). Cells were counted after trypan blue staining using an automated counter (Life Technologies). Cells were stained with CD11b-PerCP, Gr-1/Ly6G-PE, Ly6C-APC-Cy7, Siglec F-BV421, MHCII-AF700, CD11c-PE-Cy7, CD45-650NC (BD Biosciences), Annexin V-APC and Zombie Yellow Fixable Viability Dye (BioLegend) according to the
manufacturers’ protocol. Samples were fixed prior to analysis. Data was acquired using the LSR II flow cytometer (BD Biosciences), and analyzed using FlowJo (Tree Star).

Acknowledgements. This work was supported by the National Institutes of Health (grants R01AI05168 [to J. N. W.], R01AI042310 [to R. B.], and K08AI097223 [to R. D.]).

Results

MIF is detrimental for survival and lung injury in pneumococcal pneumonia.

In our serotype 3 pneumonia model, mice developed severe illness that resulted in death beginning at 2 days post-infection, with 90% of the WT animals succumbing by 7 days (Figure 1A). As is the case in human pneumococcal disease, bacteremia was noted in a majority (60%) of ill-appearing mice. They also had significant pulmonary pathology, with alveolar and bronchial inflammation (overall pathology scored as 3, Figure 1B).

Mif−/− animals had improved survival during pneumococcal pneumonia, with 46% mortality and no bacteremia (Figure 1A). Overall, the Mif−/− animals demonstrated fewer clinical signs of illness and lost less weight than their WT counterparts. The lungs of the Mif−/− mice had reduced parenchymal infiltration and their airways remained free of inflammatory cells (overall pathology scored as 1, Figure 1B).

MIF-related pathology is associated with greater pulmonary bacterial load, inflammatory cell infiltrate, and cytokine/chemokine production.
There was 100-fold bacterial growth over 2 days in WT animals. Mif\(^{-/-}\) animals, in contrast, were better able to control bacterial proliferation, correlating with their increased survival and attenuated lung pathology (Figure 1B and D). Since our observations seemed to indicate opposing roles for MIF in pneumonia vs. colonization, we repeated carriage experiments with the same serotype 3 isolate. This strain colonized the murine nasopharynx effectively and did not produce symptomatic illness. While the WT mice began clearing the bacteria after 7 days, Mif\(^{-/-}\) mice showed no change in burden by 14 days (Figure 1C).

In order to better understand the role of inflammation in pneumonia, we next characterized the pulmonary cellular infiltrate in WT and Mif\(^{-/-}\) mice over the course of disease. WT and Mif\(^{-/-}\) animals demonstrated a progressive infiltration of neutrophils into the lung parenchyma, but Mif\(^{-/-}\) mice had approximately 5-fold fewer neutrophils than WT at 2 days (Figure 1E). Additionally, Mif\(^{-/-}\) animals had approximately 1.5-fold fewer monocytes/macrophages in the lung than WT. While total numbers of alveolar macrophages in BAL remained similar between groups (data not shown), we observed a significant increase in early apoptosis in these cells in Mif\(^{-/-}\) mice, which correlated with a later increase in dead cells (Figure 1F). These differences in apoptosis were not observed among neutrophils or monocytes/macrophages.

Next, we compared the chemoattractant microenvironment of the lungs between WT and Mif\(^{-/-}\) mice. Similar to our observations in nasal colonization, local MIF production in lungs was induced early during pneumococcal infection and sustained over the course of the experiment (Figure 2A) (224). Higher protein levels were present in the bronchoalveolar lavage (BAL) fluid obtained from the WT compared to the Mif\(^{-/-}\) mice.
animals, indicating higher levels of inflammation in the WT group. Finally, levels of the specific chemoattractants of neutrophils and monocytes/macrophages, IL-1β and MCP-1/CCL2 respectively, were significantly higher in BAL obtained from WT compared to Mif−/− mice.

**Modulation of MIF regulates outcome in pneumococcal pneumonia.**

In order to investigate whether the presence of MIF was both necessary and sufficient to induce detrimental inflammation in pneumococcal pneumonia, we delivered rMIF to the lungs of Mif−/− mice. We found that local administration of rMIF decreased survival over the course of pneumonia and increased the pulmonary bacterial burden 48h after infection, compared to PBS administration (Figure 2B and C). Next to assess the role of MIF inhibition in pneumococcal pneumonia along with its therapeutic potential, we treated WT mice with either the small molecule receptor antagonist for MIF, MIF098, or vehicle control, over the course of infection (320). Survival in the WT vehicle treated animals mirrored that seen in the untreated mice (Figures 1A and 2B). Treatment with MIF098 both delayed initiation of mortality and improved overall survival from 10% to >50%, identical to that observed in Mif−/− mice. When we examined pulmonary bacterial loads 2 days after infection, we found a 100-fold reduction in burden in the MIF098 treated animals compared to vehicle control (Figure 2C). The bacterial burden in MIF098 treated mice was similar to Mif−/− animals treated with vehicle control. Finally, we demonstrated a reduction in both the numbers of infiltrating neutrophils and inflammatory monocytes/macrophages with MIF098 treatment (Figure 2D&E).

**Discussion**
We show herein that MIF is detrimental for survival in pneumococcal pneumonia. Mice deficient in MIF have improved survival with reduced lung pathology, more effective bacterial control, and decreased infiltration of the lungs by innate immune cells compared to their WT counterparts, which may be mediated by enhanced apoptosis of alveolar macrophages in the absence of MIF. These findings are in contrast to pneumococcal colonization of the nasopharynx where MIF is required for bacterial clearance. Taken together, our experiments suggest that MIF-mediated inflammation plays a defining role in the outcome of the host-pneumococcal interaction in the upper vs. lower respiratory tract. Additionally, we demonstrate that modulation of inflammation by a small molecule antagonist of MIF improves outcomes in pneumococcal pneumonia.

There are parallels between the innate immune responses elicited by the presence of pneumococci in the nasopharynx and the lung. While neutrophils are recruited early to both sites, their relative contribution to bacterial control has been shown to be limited (216, 222). Mice lacking IL-1 receptor have decreased pulmonary neutrophilia and bacterial dissemination in pneumococcal disease (217). Previous work in a model of Toxoplasma gondii infection confirms our observation of reduced IL-1β production in the setting of MIF-deficiency, but its mechanism remains to be determined (115). Additionally, neutrophil-mediated lung pathology is associated with failure of pneumococcal control; whether the predominance of neutrophils or enhanced inflammation in the WT animals may be a cause of the greater bacterial growth in these animals compared to $Mif^{-/-}$ remains to be investigated (339).
During colonization, MCP-1/CCL2 upregulation in WT mice correlates with the sustained presence of macrophages in the nasopharynx required for pneumococcal clearance, all of which are impaired in MIF-deficiency (87, 224). In pneumonia, CCL2/MCP-1 levels are also higher in the BAL of WT mice compared to Mif−/− mice and are associated with the increased number of monocytes/macrophages in the tissue. MIF may promote the migration and retention of circulating monocytes into tissues in a MCP-1/CCL2 dependent manner, setting up a positive-feedback loop as more cells are recruited (134). Additionally, MIF is known to prolong the survival of inflammatory cells by inhibiting p53 dependent apoptosis (287); the enhanced apoptosis we observed in alveolar macrophages may underlie improved outcomes in Mif−/− mice by promoting the resolution of inflammation.

Few host factors have been examined systematically in both pneumococcal colonization and disease. But studies examining the response to the pneumococcal cytolysin, pneumolysin, in host-bacterial interaction provide another example of differing roles of inflammation in the nasopharynx vs. the lung. Pneumolysin-deficient pneumococci are better able to persist in models of carriage because of an attenuated inflammatory response but are less virulent in pneumonia where they induce decreased recruitment of innate immune cells (224, 339).

Our observations on the detrimental role of MIF implicate it as a target for immunomodulation in pneumococcal pneumonia. While therapeutic potential of MIF inhibition has been evaluated for regulating inflammatory pathology in rheumatologic disease, this is the first examination of the use of a small molecule inhibitor of the cytokine in response to bacterial infection (52). Future work is needed to determine the
timing and duration of MIF inhibition necessary for an optimal impact on the excess morbidity and mortality caused by inflammation in pneumococcal pneumonia.

**Figure Legends**

**Figure 1.** Macrophage migration inhibitory factor (MIF) is detrimental to survival and lung pathology in a murine model of pneumococcal pneumonia.

(A) Pneumonia was induced in WT (closed squares) and Mif\(^{-/}\) (open squares) mice by intranasally inoculating anesthetized mice with \(1 \times 10^4\) CFU of Type 3 pneumococci in 80\(\mu\)l of PBS. WT mice demonstrated reduced survival than their Mif\(^{-/}\) counterparts. (B) When lungs from the mice were examined by H&E staining at 48 hours post infection, WT animals demonstrated greater lung pathology (cellular infiltrates of the parenchyma and airways) compared to Mif\(^{-/}\). While Mif\(^{-/}\) mice have impaired ability to clear Type 3 pneumococcal colonization from the nasopharynx (modeled by inoculating awake mice with \(1 \times 10^7\) CFU pneumococci in 10\(\mu\)l of PBS, C), they have reduced lung bacterial burdens during pneumococcal pneumonia (D). (E) WT mice demonstrate increased numbers of pulmonary neutrophils as well as monocytes compared to their Mif\(^{-/}\) counterparts during pneumonia (48h). Cellular composition assessed by flow cytometry, where Ly6C\(^+\)Ly6G\(^+\) are designated as neutrophils and Ly6C\(^+\)Ly6G\(^-\) cells as monocytes, per \(10^5\) CD45\(^+\) live cells counted. (F) Mif\(^{-/}\) mice have increased ratio of alveolar macrophages undergoing early apoptosis at 12h, which correlates with increased numbers of dead alveolar macrophages at 48h. Cellular composition of BAL assessed by flow cytometry, where Siglec F\(^-\)CD11c\(^+\) cells are designated as alveolar macrophages, with Annexin V and Zombie yellow viability dye used to determine early apoptosis and
Figure 2. Modulation of MIF regulates inflammation, influencing both bacterial load and survival in pneumococcal pneumonia.

(A) MIF was produced locally in the lung during the course of pneumococcal pneumonia. Increased MIF was associated with greater general inflammation in the lungs assessed by total BAL protein in the WT vs. Mif⁻/⁻ animals. Specific cytokine attractants of both monocytes and neutrophils (MCP-1 and IL-1β, respectively) were greater in the WT mice compared to their Mif⁻/⁻ counterparts. MIF, MCP-1, and IL-1 β were assessed by specific ELISAs and total protein determined by BCA assay. (B) Mif⁻/⁻ mice receiving local MIF supplementation (by delivery of 100ng rMIF in 80μL of PBS to the airways beginning from the day prior to infection, and continuing daily thereafter) had decreased survival compared to their counterparts receiving PBS alone. (C) Additionally, they had higher bacterial burdens than their PBS-receiving Mif⁻/⁻ counterparts. Conversely, when MIF was inhibited in the WT mice using a small molecule inhibitor (MIF-098, dosed at 40mg/kg i.p., twice daily, beginning on the day of infection), the mice had greater survival compared to those receiving vehicle control (VC, B). Additionally, greater survival in the MIF098 treated animals was associated with reduced pulmonary bacterial load compared to VC treated WT animals (C). (D&E) Pharmacologic MIF inhibition also reduced both neutrophil and monocyte infiltration compared to VC treatment. All experiments performed at 48h, unless otherwise stated. N≥10 mice per group in survival experiments, and ≥4 mice per group in all others. *P<0.05, **P < 0.01, ***P<0.001, log-rank test for survival curves, Mann-Whitney U test for CFU and cell number comparisons.
rank test for survival curves, Mann-Whitney U test for cytokine, CFU, and cell number comparisons.
Figure 1
Figure 2
CHAPTER 5:

Detection of peptidoglycan from the gut microbiota governs the lifespan of circulating phagocytes at homeostasis.

Christopher B. Hergott\textsuperscript{1}, Aoife M. Roche\textsuperscript{1}, Edwin Tamashiro\textsuperscript{1,2}, Thomas B. Clarke\textsuperscript{3}, Aubrey G. Bailey\textsuperscript{1}, Alice Laughlin\textsuperscript{1}, Frederic D. Bushman\textsuperscript{1}, and Jeffrey N. Weiser\textsuperscript{1,4,5}

\textsuperscript{1}Department of Microbiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

\textsuperscript{2}Department of Ophthalmology, Otorhinolaryngology, and Head and Neck Surgery, Ribeirao Preto School of Medicine, University of Sao Paulo, Sao Paulo, Brazil

\textsuperscript{3}MRC Centre for Molecular Bacteriology and Infection, Imperial College London, London, United Kingdom, SW7 2AZ

\textsuperscript{4}Department of Pediatrics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

\textsuperscript{5}Department of Microbiology, New York University School of Medicine, New York, NY 10016, USA
Abstract

Homeostatic regulation of myeloid cell abundance and function rely upon continuous turnover of circulating phagocytes from the bloodstream. While growing evidence suggests that these cells possess intrinsic mechanisms driving spontaneous senescence and apoptotic cell death, it remains largely unknown whether environmental signals influence myeloid cell turnover in vivo. Here, we show that the gut microbiota regulates the steady-state cellular lifespan of neutrophils and inflammatory monocytes, the two most abundant circulating myeloid cells and key contributors to inflammatory responses. Treatment of mice with broad-spectrum antibiotics increases the expression of surface markers previously linked to phagocyte senescence, accelerates cellular turnover, and increases the rate of spontaneous apoptosis among myeloid cells while leaving lymphoid lifespan unperturbed. The gut-restricted aminoglycoside neomycin can fully recapitulate the impact of broad-spectrum antibiotic treatment on phagocyte viability, corresponding with reduced abundance of bacterial taxa that express the peptidoglycan dipeptide γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP). Administration of iE-DAP is sufficient and its intracellular receptor, Nod1, is necessary to mediate the influence of the gut flora on systemic myeloid cell lifespan. Systemic neutralization of interleukin 17A (IL-17A), a key regulator of myeloid cell longevity stimulated by Nod1 signaling, eliminates the stimulatory effects of the microbiota on myeloid cell persistence. Together, these results uncover a novel microbial influence on a central component of myeloid homeostasis and suggests perturbations of commensal communities can influence systemic, steady-state regulation of cell fate.
Introduction

Each day, approximately $10^{11}$ mature neutrophils emerge from the bone marrow and enter systemic circulation, comprising the most abundant leukocyte subset in the human bloodstream (15). These terminally differentiated phagocytes migrate swiftly into inflamed tissues following infection or injury, where they eliminate invading pathogens, release an array of inflammatory mediators, and help orchestrate subsequent phases of immunity (10, 38, 214). To maintain tissue and immune homeostasis in the absence of inflammation, steady-state production of neutrophils must be counterpoised by continuous elimination in the bone marrow, spleen, and liver (206, 341). The uniquely brief lifespan of these cells, averaging less than 24 hours, is essential for replenishing the pool of circulating phagocytes and averting the vascular and tissue damage risked by their potent cytotoxic capacity (118, 229). Peripheral clearance also regulates neutrophil abundance indirectly by restraining systemic concentrations of IL-17A and IL-23, cytokines that drive development of neutrophils in the bone marrow (315). However, pathologically enhanced neutrophil turnover has been linked to congenital neutropenia and a wide range of immunodeficiency syndromes, illustrating the stringent regulation of cell survival required to maintain immunologic fitness (122, 179, 250). Despite the central role played by cell turnover in regulating phagocyte homeostasis, the factors that influence neutrophil lifespan during health remain incompletely understood.

Steady-state turnover of neutrophils follows a program of spontaneous cell senescence (termed neutrophil “aging”), exit from circulation, and programmed cell death (289). Aged neutrophils that have resided longest in the bloodstream shed the surface selectin
CD62L and enhance display of the chemokine receptor CXCR4, changes which facilitate extravasation and homing to tissue-resident macrophages for uptake (62, 218). Simultaneously, accumulating oxidative damage and metabolic dysregulation cooperate to trigger spontaneous apoptosis upon the exit of neutrophils from the bloodstream (123, 181, 182, 288). Tight coordination of these processes allow efficient and immunologically quiescent clearance of expiring phagocytes. While the mechanisms of cell death in neutrophils have long been subject to intensive study, previous work has focused largely on the diverse cell-intrinsic pathways governing constitutive neutrophil apoptosis (123, 308). Comparatively less attention has been paid to extrinsic factors influencing neutrophil viability in the steady state.

Extension of neutrophil lifespan during inflammation highlights the ability of environmental signals to modulate phagocyte turnover kinetics. Numerous viral and bacterial pathogens prolong neutrophil survival to stabilize a replicative niche or to promote tissue damage that facilitates microbial growth (254, 291, 309). Moreover, enhanced viability can augment neutrophil responses to some bacterial infections, driven by pro-inflammatory cytokines like IL-1β, TNFα, and IL-17A (72, 77). Neutrophils obtained from patients with systemic inflammatory disease, including rheumatoid arthritis, anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis, gout, and cystic fibrosis, also exhibit delayed apoptosis, as do neutrophils exposed to hypoxic injury (229, 327). The stimulatory impact of microbial and cytokine signals are thought to expand the window in which recruited neutrophils can exert optimal inflammatory function, strengthening their contribution to host defense. Whether similar signaling influences neutrophil lifespan in the absence of inflammation remains unclear.
A growing body of evidence suggests signals emanating from the gut microbiota prime systemic innate immunity in health and regulate phagocyte homeostasis (69). Germ-free mice show steady-state reductions in the abundance of both neutrophils and mononuclear phagocytes, changes ascribed previously to defects in hematopoietic development (23, 53, 173). However, mature phagocytes from animals raised germ-free or treated with antibiotics also exhibit a range of functional defects, including impaired trafficking to tissues, production of reactive oxygen species, and bactericidal capacity (70, 169, 255). Given that the commensal flora can influence phagocyte function in the periphery, we hypothesized that steady-state microbial signals may enhance the lifespan of circulating neutrophils and that this effect may extend to other short-lived, circulating phagocytes.

Here, we use in vivo cell-tracking and ex vivo cell viability assays to demonstrate that a distinct, neomycin-sensitive cohort of intestinal microbiota continuously regulates the death and turnover rates of neutrophils and Ly6C+ inflammatory monocytes in healthy mice. We show that signaling through nucleotide-binding oligomerization domain-containing protein 1 (Nod1), a cytoplasmic peptidoglycan sensor which recognizes the dipeptide structure γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), is necessary and sufficient to mediate the microbial influence on phagocyte longevity. Lastly, we find that the inflammatory cytokine IL-17A is required to transmit the Nod1-dependent microbial signal to neutrophils and inflammatory monocytes throughout the body, illuminating a signaling axis through which commensal microbes regulate systemic immune cell fate at homeostasis.
Methods

Mice. Six- to eight-week-old wild type C57BL/6 mice were obtained from Jackson Laboratories and maintained in strict accordance with a protocol approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Congenic \textit{Nod1}^{-/-}, \textit{Tlr4}^{-/-}, and \textit{Nod2Tlr2}^{-/-} mice were described previously (70, 87). Germ-free C57BL/6 mice were obtained from the University of Pennsylvania Gnotobiotic Mouse Facility and maintained in germ-free conditions until sacrifice. All mice were sacrificed by CO$_2$ inhalation followed by cardiac puncture.

\textit{Antibiotic treatment}. All antibiotics were administered in drinking water \textit{ad libitum}. Mice received sterile-filtered tap water supplemented with either a broad-spectrum antibiotic cocktail (1 g/L each of neomycin (Fisher), vancomycin (Santa Cruz), metronidazole (Sigma), ampicillin (Corning), and gentamicin (Life Technologies)), 1 g/L of neomycin or metronidazole alone, or control tap water. Water was changed every 3-4 days and the treatment regimen was maintained for 3-4 weeks.

\textit{Tissue processing and cell isolation}. Bone marrow parenchyma was flushed from the femora and tibia of mice with RPMI 1640 (Corning) supplemented with 5% fetal bovine serum (FBS) (GIBCO). Murine blood was obtained from retro-orbital sinuses following induction of anesthesia by isoflurane (Santa Cruz) or by cardiac puncture for terminal bleeds. Blood samples were anticoagulated with 0.1 M EDTA (Invitrogen) and
erythrocytes were lysed with ACK lysis buffer (Life Technologies) for 5 minutes at room
temperature before flow cytometric analyses. Splenocytes were isolated by
homogenization over 40 µm nylon cell strainers (Falcon) into RPMI 1640 + 5% FBS.

Flow cytometry. After resuspension of cells in PBS with 1% BSA, FcR blocking was
performed with 1:100 dilution of anti-CD16/32 (BD Biosciences, 2.4G2). Cellular
viability/integrity was assessed by Fixable Viability Dye eFluor780 (eBioscience). Surface
marker staining was performed for 30 minutes at 4° C, antibodies diluted 1:150, against:
CD45 (eBioscience, 30-F11); CD11b (M1/70), CD11c (N418), Ly6G (1A8), Ly6C (AL-21),
F4/80 (BM8), CXCR4 (L276F12), CD62L (MEL-14), CD4 (GK1.5), CD8 (53-6.7), and
B220 (RA3-26) (all from BioLegend). All cytometry was performed on a BD LSRII flow
cytometer and analyzed using FlowJo software (Tree Star).

Ex vivo apoptosis assays. To measure the duration of cellular viability and kinetics of
spontaneous apoptosis ex vivo, we applied freshly isolated splenic or bone marrow
parenchyma to 96-well plates, with ~10^5 cells and 200 µL RPMI + 5% FBS per well. No
antibiotics or cytokines were added to ex vivo culture media. Plates were incubated at
37° C and removed at the indicated time points before examination by flow cytometry.
Apoptosis among each cell population gated during analysis was measured by staining
with Annexin V (1:20) in Annexin V Binding Buffer (BioLegend) for 15 minutes at room
temperature. Viable cells were identified as Annexin V-Fixable Viability Stain.
BrdU pulse-chase assays. 5-bromo-2-deoxyuridine (BrdU) incorporation was measured as described previously (364) with the following modifications: Mice were given 3 intraperitoneal (i.p.) injections of 2 mg BrdU (Life Technologies), spaced 3 hours apart, in 100 μL PBS. After circulating leukocytes were obtained by retro-orbital bleeding (~100 μL), BrdU incorporation was measured by flow cytometry (BioLegend, Bu20a, 1:20) at the indicated time points post-injection after fixation and permeabilization per manufacturer’s instructions. Decreases in the proportion of BrdU+ cells were fit to a one-phase exponential decay equation, \( N(t) = N(0)e^{-\gamma t} \), where \( N \) represents the BrdU+ fraction over time \( t \) and the rate constant \( \gamma \) is solved.

16S rDNA collection, quantification, and sequencing. Stool samples were collected from neomycin-treated and control mice on days 7, 14, and 21 after initiation of antibiotic treatment, with ileum contents also collected on day 21. Samples were flash-frozen at -80°C and DNA was extracted using Zymo ZR Fecal Miniprep Kits. 16S rDNA copy number was quantified by qRT-PCR, relative to a standard curve constructed from a TOPO vector containing E. coli 16S rDNA. PCR reactions were performed in triplicate as previously described (147) using the following 16S rDNA-specific primers: Forward, 5’-AGAGTTTGATCCTGGCTCAG-3’; Reverse, 5’-CTGCTGCCTYCCGTA-3’; Probe, 5’-FAM-TAA+CA+CATG+CA+AGT+CGA/3BHQ1-3’ (“+” precedes position of LNA base). 16S rDNA metagenomic sequencing was performed as described previously on the Illumina HiSeq platform, using bar-coded primers aligned to the V1/V2 region (301). Data was analyzed and quality-controlled using the QIIME pipeline v.1.8 and phylogenetic trees were constructed using FastTree v.2.1.3 with default parameters (60, 268).
Ligand treatments and cytokine neutralization. To stimulate Nod1 signaling in vivo, we injected mice i.p. with two doses of 100 µg C12-iE-DAP (InvivoGen), an acylated derivative of the minimal Nod1 dipeptide ligand iE-DAP. Injections were given 12 and 2 hours before sacrifice for ex vivo apoptosis assays and at 48 and 72 hours after BrdU injections for in vivo myeloid cell turnover experiments. Neutralization of IL-17A was achieved through two i.p. injections of 100 µg anti-IL-17A monoclonal antibody (BioXCell, 17F3) or IgG1 isotype control antibody (MOPC-21), performed at 12 and 2 hours before sacrifice for ex vivo apoptosis assays and at 48 and 72 hours after BrdU injections for in vivo myeloid cell turnover experiments.

Statistics. Data are displayed as mean ± SEM. Except where indicated otherwise, statistical significance was assessed by 2-tailed Student’s t test for pairwise comparisons and ANOVA with Newman-Keuls post-test for comparisons of >2 groups. For all analyses, p < 0.05 was considered statistically significant.

Acknowledgements. We thank Kristin Blouch and Dr. Susan Ross for TLR4−/− mice and Drs. Jamie Lemon and Cierra Casson for helpful discussions. This work was supported by NIH grants AI038446, AI105168, and AI060516 to J.N.W.

Authorship Contributions. C.B.H. and J.N.W. designed the research. C.B.H. performed the majority of the experiments, with E.T.W. and A.M.R. contributing to collection and analysis of microbial samples. T.B.C. provided essential reagents. A.L., A.G.B., and F.D.B. performed and analyzed metagenomic sequencing experiments. C.B.H., F.D.B.,
and J.N.W. analyzed and interpreted experimental findings. C.B.H. and J.N.W. wrote the paper with important contributions from A.G.B. and F.D.B. All authors reviewed the manuscript before submission.

**Results**

**Antibiotic treatment accelerates turnover of circulating phagocytes from the bloodstream.**

Turnover of phagocytes from circulation begins with a coordinated program of cell senescence, defined recently in neutrophils to include shedding of CD62L and up-regulated CXCR4 expression (62). To determine whether the microbiota influences the display of these aging markers, we collected blood from mice treated for 4 weeks with broad-spectrum antibiotics (ABX) and evaluated neutrophils (CD45^+CD11b^+Ly6C^+Ly6G^+ cells, see Figure S1) by flow cytometry compared to conventionally treated mice (CNV). To control for diurnal oscillations in receptor expression, we collected all blood for phagocyte aging analyses at zeitgeber time 5 (ZT5), 5 hours after initiation of ambient light (62). Neutrophils from antibiotic-treated mice showed significant elevation of CXCR4 expression and decreased CD62L levels, suggesting a signature of accelerated cell turnover in mice depleted of intestinal flora (Figure 1A). Neutrophils from mice treated only with neomycin (NEO) resembled those from mice treated with broad-spectrum antibiotics, indicating that a neomycin-sensitive cohort of intestinal microbes may accelerate the intravascular aging of bloodstream phagocytes.
We next performed BrdU pulse-chase assays to assess directly whether the observed changes in surface markers corresponded with accelerated turnover of phagocytes from circulation. After pulsing mice intraperitoneally (i.p.) with BrdU, we measured the fraction of BrdU⁺ neutrophils (PMNs) and inflammatory monocytes (IMs, CD45⁺CD11b⁺Ly6C⁺Ly6G⁻, Figure S1) in the bloodstream over time (Figure 1B). Previous studies have shown that BrdU is not mitogenic at these doses, is incorporated most readily into the numerous late precursors giving rise to mature phagocytes in the bone marrow, and that circulating phagocytes reach saturation with BrdU approximately 2 days after its administration (73, 322, 364). Accordingly, we found that nearly 100% of bloodstream PMNs (Figure 1C) and IMs (Figure 1D) were BrdU⁺ at 2 days post-injection. This saturation occurred irrespective of antibiotic treatment. To assess whether the microbiota instead influences the circulating longevity of labeled phagocytes, we tracked the contraction of BrdU⁺ cells from the blood of antibiotic-treated and control mice over the next 3 days. In contrast to the equivalent frequencies of labeled cells at Day 2, treatment with broad-spectrum antibiotics significantly accelerated the elimination of BrdU⁺ PMNs and IMs from circulation (Figure 1C&D), portrayed quantitatively by enhanced decay kinetics when set to one-phase exponential decay equations (Figure 1E).

Neomycin was again sufficient to reproduce the impact of the broad-spectrum antibiotic cocktail on phagocyte longevity. This corresponded with modest but significant decreases in the frequencies of total PMNs and IMs in the bone marrow (Figure 1F) and spleen (Figure 1G) of mice treated with neomycin alone, comparable to differences observed previously with broad-spectrum antibiotics and germ-free mice (91, 173). Taken together, these data provide evidence that the neomycin-sensitive gut microbiota augments the circulating lifespan of bloodstream phagocytes in vivo.
Antibiotic treatment corresponds with impaired phagocyte survival ex vivo.

Following exit from the bloodstream, senescent phagocytes undergo spontaneous apoptosis before clearance by tissue-resident macrophages (4, 206). We asked whether the microbiota influences the duration of phagocyte viability and resistance to spontaneous cell death. Since apoptotic cells are cleared immediately in vivo and do not accumulate at steady state (266), we determined the lifespan of neutrophils and inflammatory monocytes ex vivo in cultures lacking growth factors or cytokines. At 6 or 24 hours after harvest of bone marrow or splenic parenchyma, we measured the proportion of phagocytes that remained viable and excluded Annexin V, a stain that detects early apoptosis by binding surface-exposed phosphatidylserine (Figure 2A, representative gating for neutrophils). In concordance with their accelerated turnover from the bloodstream, PMNs and IMs from mice treated with broad-spectrum antibiotics or neomycin entered apoptosis more rapidly than controls (Figure 2B). Accelerated cell death occurred in cells from both the bone marrow and spleen, was apparent at 6 hours, and expanded at 24 hours post-harvest. In contrast, neomycin treatment had no measurable impact on the apoptosis of splenic lymphocytes, fibroblasts, or endothelial cells (Figure S2A). Splenic macrophages exhibited apoptosis kinetics similar to that of IMs, likely reflecting the monocytic origin of some of these cells (Figure S2B) (1).

To verify that the depletion of microbes was responsible for accelerated phagocyte apoptosis rather than a direct effect of antibiotics, we repeated the ex vivo apoptosis assays using germ-free mice. Similar to the effects observed upon treatment with
antibiotics, germ-free mice trended toward lower frequencies of PMNs and IMs in the bone marrow (Figure 2C) and spleen (Figure 2D) and exhibited accelerated apoptosis when incubated \textit{ex vivo} (Figure 2E&F). In contrast, treating mice solely with metronidazole, which preferentially targets anaerobes had no impact on phagocyte viability (data not shown) (203). Thus, a distinct, neomycin-sensitive cohort of microbes regulates the cellular lifespan of mature phagocytes in addition to their turnover from the bloodstream.

**Treatment with neomycin stably alters the community structure of the gut microbiota.**

Since neomycin was sufficient to recapitulate the impact of broad-spectrum antibiotics on phagocyte turnover, we sought next to characterize the impact of this gut-restricted aminoglycoside on the murine intestinal microbiota (13, 192). After collecting weekly fecal samples and harvesting ileal contents 21 days after initiation of treatment, we extracted genomic material and performed 16S rDNA qPCR to quantify changes in intestinal microbial abundance. In contrast to reductions described previously with broad-spectrum antibiotics (352), we observed no significant differences in fecal or ileal 16S rDNA copy number between neomycin-treated and conventional mice (Figure 3A). However, metagenomic sequencing of intestinal contents revealed stable and reproducible shifts in microbial community architecture at both sample sites, beginning within 1 week after the onset of treatment (Figure 3B&C). Most notably, \textit{Lactobacillaceae}-family bacteria dropped from approximately 25\% of fecal microbes among conventional mice to less than 10\% with neomycin, paired with a \textasciitilde40\% increase in anaerobic S24-7-family \textit{Bacteroidetes} species (Figure 3B). This redistribution concurs
with previous reports showing sensitivity among lactobacilli and resistance among anaerobes to aminoglycosides \textit{in vitro} \cite{51, 78}. \textit{Clostridiaceae} and \textit{Turicibacter} species, while comprising only \(~4\%\) and \(~10\\%\) of microbes in control mice respectively, were effectively absent in neomycin-treated mouse feces.

The ileal microbiota exhibited changes broadly similar to those observed in fecal samples (Figure 3C). However, given the previously described immunoregulatory role of segmented filamentous bacteria (SFB) residing within the small intestine \cite{155}, we queried directly whether neomycin induced changes in the abundance of SFB species (\textit{e.g.} \textit{Candidatus} Arthromitus). We detected no SFBs in control or treated mice, a finding consistent with previous analyses of mice acquired from Jackson Laboratories \cite{156}. While the effects of antibiotics on the intestinal microbial ecosystem are complex \cite{242}, these results suggest that the impact of neomycin on phagocyte lifespan corresponds with a discrete and stable signature of altered commensal flora. However, the nature of the microbial signal connecting gut commensals to phagocyte homeostasis remained unclear.

\textbf{Nod1 mediates the microbial influence on phagocyte lifespan at homeostasis.}

We previously described a role for the intracellular peptidoglycan sensor Nod1 in driving microbiota-mediated stimulation of neutrophil phagocytic capacity \textit{ex vivo} \cite{70}. As the peptidoglycan of members of the \textit{Lactobacillaceae} family depleted by neomycin treatment bear iE-DAP and stimulate Nod1 \textit{in vitro} \cite{21, 31}, we hypothesized that the
microbiota stimulates this cytoplasmic sensor to regulate phagocyte homeostasis in the absence of infection. To determine whether Nod1 signaling stimulates the viability of neutrophils and inflammatory monocytes, we repeated ex vivo phagocyte apoptosis assays using Nod1−/− mice. PMNs and IMs from both the bone marrow (Figure 4A) and spleen (Figure 4B) of Nod1−/− mice entered apoptosis significantly more rapidly than those of wild type controls, instead resembling phagocytes from neomycin-treated mice. Treating Nod1−/− mice with neomycin produced no additional increase in the kinetics of phagocyte apoptosis, reinforcing that Nod1 is responsible for relaying the stimulatory signal from the flora into changes in cellular viability. In contrast, the lifespan of phagocytes from Nod2Tlr2−/− and Tlr4−/− mice resembled those of wild type controls despite well-described roles for these sensors in regulating granulopoiesis and phagocyte activation (53, 163, 191). To assess whether signaling through Nod1 was sufficient to recapitulate the impact of the microbiota on cellular lifespan, we repeated the apoptosis assays after injecting mice i.p. with the acylated Nod1 ligand C12-iE-DAP (332). Ligand treatment rescued the viability of phagocytes from neomycin-treated mice such that their lifespan was indistinguishable from conventional mice while conferring only a modest survival benefit to the conventional cells.

To ascertain whether Nod1 signaling plays a similarly central role in regulating phagocyte turnover in vivo, we subjected Nod1−/− mice to BrdU pulse-chase assays (Figure 4C). Congruent with their impaired survival ex vivo, PMNs and IMs disappeared from circulation more quickly in knockout mice than in controls and knockout phagocyte turnover kinetics were indistinguishable from that of neomycin-treated animals (Figure 4D). Further, administration of C12-iE-DAP markedly delayed turnover of both PMNs
and IMs from the blood and equalized the longevity of phagocytes from conventional and neomycin-treated mice (Figure 4C&D). Together, these data reveal that recognition of iE-DAP by Nod1 is both necessary and sufficient to mediate the stimulatory impact of the flora on phagocyte lifespan.

**Neutralization of IL-17A abrogates the role of the microbiota and Nod1 on phagocyte lifespan.**

While Nod1-mediated sensing of peptidoglycan from the microbiota is essential for the maintenance of gut mucosal immunity (41), how these signals are transmitted to instruct systemic immune homeostasis remains poorly understood. Nod1 signaling drives systemic responses to infection primarily through the liberation of cytokines that recruit and activate phagocytes (219). We predicted that similar signaling regulate phagocyte lifespan in the steady state. We focused our studies on IL-17A, a key cytokine orchestrating myeloid cell development, function, and survival (91, 199, 235), because its production has been shown to be stimulated by both the gut microbiota and Nod1 signaling (14, 119, 156). Neutralization of IL-17A markedly impaired the survival ex vivo of PMNs and IMs from the bone marrow (Figure 5A) and spleens (Figure 5B) of conventionally treated mice. However, the blockade had limited impact on cells from neomycin-treated or *Nod1/−* animals, resulting in equal cellular viability across all groups in the absence of IL-17A signaling. BrdU pulse-chase assays revealed similar results in vivo, with neutralization of IL-17A phenocopying neomycin treatment or lack of Nod1 in accelerating the turnover of PMNs and IMs from the bloodstream (Figure 5C&D). Collectively, these results demonstrate that neomycin-sensitive microbes, Nod1, and IL-
17A comprise requisite components of a common signaling axis that governs systemic phagocyte lifespan at homeostasis.

**Discussion**

Cell turnover is a key component of immune homeostasis, and accumulating evidence suggests that failure to properly regulate the lifespan of circulating phagocytes can impair host defense. Here, we showed that the gut microbiota governs the viability and circulating lifespan of neutrophils and inflammatory monocytes at steady state. Our observations highlight how broad-spectrum antibiotic therapy may negatively impact the availability of circulating myeloid cell populations. As disruptions of commensal microbial communities are associated with a growing set of inflammatory, infectious, and neoplastic diseases, mechanistic understanding of the microbial influence on innate immunity during health may reveal actionable therapeutic targets when this relationship is disturbed (281).

We found that treating mice with broad-spectrum antibiotics accelerated each of the fundamental steps in neutrophil turnover: senescence, extravasion, and apoptotic cell death. Driven by our previous findings linking the microbiota to stimulation of neutrophil bactericidal capacity *ex vivo* (70), we hypothesized that microbial signals may regulate a broader set of fundamental properties among mature neutrophils at homeostasis. We focused on cell turnover because, in addition to regulating cell abundance directly, mounting evidence suggests neutrophil elimination drives
regulatory circuits that feed back on other components of myeloid homeostasis in vivo, including neutrophil development in the bone marrow. Phagocytosis of apoptotic neutrophils by macrophages has been shown to suppress IL-23 production in these cells, thereby inhibiting IL-17A liberation by tissue-resident lymphocytes and suppressing granulopoiesis and neutrophilia (315). More recently, Casanova-Acebes et al. have shown that rhythmic migrations of senescent neutrophils to the bone marrow modulate the hematopoietic niche to more broadly disfavor progenitor cell retention and maturation (62). Therefore, while alterations of the microbiota have been linked to defects in myeloid cell development previously (23, 173), whether this is a primary phenomenon or secondary to enhanced turnover remains an area of active investigation.

In addition to neutrophils, the stimulatory effect of the flora on cell longevity extended to Ly6C+ inflammatory monocytes, the second-most abundant and short-lived circulating phagocyte (364). The potential fates of inflammatory monocytes are more diverse than that of neutrophils and considerably less is known about the mechanisms guiding their turnover. Ly6C+ inflammatory monocytes have been shown to differentiate into long-lived Ly6C-CX₃CR₁+ monocytes that patrol vascular endothelia and resident macrophages and dendritic cells populating some tissues (22, 125, 162, 364). However, recent evidence suggests that, in addition to inhabiting the first stage of a developmental sequence of mononuclear phagocytes, inflammatory monocytes emerge from the bone marrow and turn over in tissues at homeostasis while maintaining Ly6C+ monocyte identity (158). In contrast, tissue-resident macrophages and dendritic cells arise from a number of cellular origins, including non-hematopoietic cells seeded during embryonic development (364). We focused our investigations on circulating neutrophils and inflammatory monocytes.
because their short lifespans contrast with the more persistent nature of tissue-resident phagocytes and were therefore more likely to be regulated dynamically by tonic microbial signals.

Our finding that neomycin recapitulates the impact of broad-spectrum antibiotics on phagocyte lifespan has precedence in other investigations on the immunomodulatory effects of the microbiota, namely in regulating antiviral responses to influenza infection in the lungs (152). Despite its ongoing clinical utility (44, 117), the precise nature and kinetics of neomycin-induced shifts in commensal community structure have not been explored previously. We found that neomycin treatment suppressed the abundance of lactobacilli in the murine intestine, a family of microbes previously shown to express Nod1 ligands and activate Nod1 in vitro (21, 31). This decrement was matched with a compensatory increase in the abundance of Bacteroidetes species, many of which also express Nod1 ligands. However, detailed analyses of the radial distribution of microbes in the gut suggest that obligate anaerobic bacteria, including Bacteroidetes species, reside predominantly in the luminal center of the intestinal tract (5). In contrast, lactobacilli and other oxygen-tolerant bacteria often reside in closer contact with the mucosal surfaces, where oxygen tension is greater and microbial products are sensed more readily by the epithelium (251, 344). This redistribution may explain the decrease in Nod1 signaling observed upon neomycin treatment.

Our results also indicate that the pro-inflammatory cytokine IL-17A connects microbial detection by Nod1 to systemic changes in phagocyte lifespan. IL-17A has been shown to delay the apoptosis of neutrophils and mononuclear phagocytes largely through
stimulation of downstream cytokines, including G-CSF and GM-CSF (43, 235). Since the signaling pathways downstream of IL-17A diverge by target cell type and may have overlapping or redundant functions, we chose to focus on IL-17A as the most proximal cytokine driving phagocyte survival and therefore the most likely to be amenable to therapeutic intervention. The division of function for downstream cytokines to survival of neutrophils and inflammatory monocytes remain an area of ongoing investigation.

As disruptions of the intestinal microbiota are associated with a broad range of inflammatory and infectious disease, efforts to manipulate the flora directly and attain a healthful commensal community have developed rapidly over the past decade. These include transplantation of exogenous microbial communities into the gut environment, which has been shown to improve disease outcomes in some severe intestinal infections (197). Our findings imply these interventions may improve the persistence and function of phagocytes that monitor for foreign threats throughout the body, providing systemic benefits from local manipulations of microbial communities. Furthermore, growing understanding of the central importance of apoptosis and cell turnover to immune homeostasis has spurred an increase in the development of therapeutics designed to target this component of phagocyte physiology directly. For example, inhibitors of cyclin-dependent kinases have been shown to improve resolution of pathogenic pulmonary inflammation by hastening the apoptosis of infiltrating neutrophils (280). Our results suggest that the makeup of intestinal commensal communities may modulate the efficacy of this therapy and others like it, potentially providing an opportunity for adjuvantive therapies targeting the gut microbiota directly to improve the outcome of patients with diverse inflammatory diseases.
Figure Legends

Figure 1. Antibiotic treatment accelerates turnover of circulating phagocytes from the bloodstream.

(A) Quantification of mean fluorescence intensity (MFI) for CD62L and CXCR4 on neutrophils from the blood of mice treated with tap water (CNV, black), broad-spectrum antibiotics (ABX, open), or neomycin (NEO, grey). (B) Representative flow cytometry plots from BrdU pulse-chase assays. Events represent CD45<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>+</sup> cells stained for intracellular BrdU content and Ly6G (neutrophils: Ly6G<sup>+</sup>; inflammatory monocytes: Ly6G<sup>−</sup>) on days 2 and 4 after BrdU injection, compared to no-BrdU controls. (C) Frequency of BrdU<sup>+</sup> events among blood neutrophils (PMNs) and inflammatory monocytes (D, IMs) assessed on days 2-5 after BrdU administration. (E) Rate constants (k, day<sup>−1</sup>) for one-phase exponential decay quantified from BrdU<sup>+</sup> frequencies depicted in (C) and (D). (F) Steady-state cell frequencies (% of CD45<sup>+</sup> events) of PMNs and IMs measured from the bone marrow and spleens (G) of CNV- and NEO-treated mice. All data presented as mean ± SEM with ≥ 5 mice per group. Statistical significance was assessed by Student’s t test for pairwise comparisons and one-way ANOVA with Newman-Keuls post-test for comparisons of >2 conditions. NS = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

Figure 2. Antibiotic treatment corresponds with impaired phagocyte survival ex vivo.
(A) Representative flow cytometry plots of neutrophils (PMNs, gated on CD45+CD11b+Ly6C+) obtained from bone marrow (BM) and spleens and allowed to incubate *ex vivo* for 0, 6 or 24 hours before staining with Annexin V to detect apoptotic cells. Non-apoptotic, viable cells were quantified by gating on Annexin V- events (SSC = side scatter). (B) Assessment of Annexin V- event frequency (% Viable) among PMNs and inflammatory monocytes (IMs) obtained from the BM and spleens of conventional (CNV), broad-spectrum antibiotic-treated (ABX, open), and neomycin-treated (NEO, grey) mice following 6 or 24 hours incubation *ex vivo*. (C) Steady-state cell frequencies (% of CD45+ events) of PMNs and IMs obtained from the bone marrow and spleens (D) of CNV (black circles) and germ-free mice (GF, open circles). (E) Survival *ex vivo* of neutrophils and inflammatory monocytes from the bone marrow (24 hours) and spleens (F, 6 hours) of CNV and GF mice. All data presented as mean ± SEM with ≥ 3-5 mice per group. Statistical significance was assessed by Student’s t test for pairwise comparisons and one-way ANOVA with Newman-Keuls post-test for comparisons of >2 conditions. NS = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

**Figure 3. Metagenomic analysis of the murine intestinal microbiota upon treatment with neomycin.**

(A) Quantitative real-time PCR analysis of 16S rDNA copy number from the intestinal contents of mice treated with conventional water (CNV, black) or neomycin (NEO, grey). Fecal samples were collected and analyzed on days 0, 7, 14, and 21 after initiation of neomycin treatment. Ileal contents were collected on day 21. (B) Family-level phylogenetic analyses of fecal bacterial sequences from conventional and neomycin-treated mice. Frequencies of significantly altered bacterial taxa in feces were compared
individually at day 21. (C) Family-level phylogenetic analysis of ileal contents taken at day 21, with individual frequency comparisons of significantly altered taxa. All data represent averages from 8 mice in each treatment condition, housed as pairs in 4 separate cages. Box-and-whisker plots depict medians ± inter-quartile range. Statistical significance for all comparisons was analyzed by Kruskal-Wallis tests with correction for false discovery rate (FDR). * = p < 0.05, ** = p < 0.01.

**Figure 4. The intracellular peptidoglycan sensor Nod1 is necessary and sufficient to mediate the microbial influence on phagocyte lifespan.**

(A) Quantification of cell survival *ex vivo* (Annexin V, % Viable) among neutrophils (PMNs) and inflammatory monocytes (IMs) from the bone marrow (24 hours) and spleens (B, 6 hours) of conventional WT, Nod1+/−, neomycin (NEO)-treated Nod1+/−, Nod2Tlr2−/−, and Tlr4−/− mice. Cellular lifespan was measured similarly for phagocytes obtained from CNV and NEO-treated mice after intraperitoneal injection of the Nod1 ligand C12-iE-DAP (Ligand), with 100 μg doses given 12 and 2 hours before sacrifice. (C) BrdU pulse-chase assays. BrdU+ frequency among PMNs and IMs from the bloodstream of CNV (black), NEO (grey), and Nod1−/− mice (open), measured on days 2-5 after systemic BrdU administration. Mice received injections of vehicle (1% DMSO in PBS, solid curves) or 100 μg C12-iE-DAP (Ligand, hashed curves) on days 2 and 3 after injection of BrdU. (D) Rate constants (*k*, day−1) for one-phase exponential decay quantified from BrdU+ frequencies depicted in (C). All data presented as mean ± SEM with ≥ 5 mice per group. Statistical significance was assessed by Student’s t test for pairwise comparisons and one-way ANOVA with Newman-Keuls post-test for
comparisons of >2 conditions. NS = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

**Figure 5.** Neutralization of IL-17A abrogates the impact of the microbiota and Nod1 on phagocyte lifespan.

(A) Assessment of cellular lifespan *ex vivo* (Annexin V, % Viable) among neutrophils (PMNs) and inflammatory monocytes (IMs). Survival of cells from the bone marrow (24 hours) and spleens (B, 6 hours) was quantified for WT, neomycin-treated WT, and Nod1−/− mice after 100 µg injections of IgG1 isotype control (Isotype) or neutralizing anti-IL-17A antibody (α-IL-17A), given 12 and 2 hours before sacrifice. (C) BrdU pulse-chase assays. Quantification of BrdU+ frequency among PMNs and IMs from the bloodstream of CNV (black), NEO (grey), and Nod1−/− mice (open). Mice received injections of 100 μg of IgG1 isotype control (solid curves) or IL-17A-neutralizing antibody (hashed curves) on days 2 and 3 after administration of BrdU. BrdU+ frequency was assessed on days 2, 4, and 5 after BrdU injection. (D) Rate constants (k, day−1) for one-phase exponential decay quantified from BrdU+ frequencies depicted in (C). All data presented as mean ± SEM with ≥ 4 mice per group. Statistical significance was assessed by Student’s t test for pairwise comparisons and one-way ANOVA with Newman-Keuls post-test for comparisons of >2 conditions. NS = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

**Figure S1.** Gating strategies for circulating neutrophils and inflammatory monocytes.
Neutrophils (PMNs) and inflammatory monocytes (IMs) were gated by the following heuristic: (I) Leukocytes gated from erythrocytes by forward- and side-scatter; (II) CD45$^+$CD11b$^+$ to gate mature myeloid cells; (III) PMNs gated by Ly6C$^+$Ly6G$^+$ and IMs gated by Ly6C$^+$Ly6G$^-$.

Figure S2. Effects of neomycin treatment on apoptosis among splenic lymphocytes, endothelial cells, fibroblasts, and macrophages.

Assessment of Annexin V- event frequency (% viable) among: (A) Lymphocytes (CD45$^+$ events that are CD4$^+$, CD8$^+$, or B220$^+$), endothelial cells (CD45$^-$CD90.2$^+$), fibroblasts (CD45$^-$CD31$^+$); or (B) macrophages (CD45$^+$F4/80$^{hi}$) from the splenic parenchyma of conventional (black) or neomycin-treated mice (grey) after 6 hours’ incubation ex vivo. All data presented as mean ± SEM with 4 mice per group. Statistical significance was assessed by Student’s t test. NS = not significant, * = p < 0.05.
Figure 1

A

B

C

D

E

F

G

152
Figure 2
Figure 3

A

Feces

16S rDNA Copies (per mg)

Duration of Treatment (Days)

Illeal Contents

10^2
10^3
10^4
10^5
10^6
10^7
10^8
10^9
10^10

0 7 14 21

CNV
NEO

21 21

10^0
10^2
10^4
10^6
10^8
10^10
Figure 3, continued.

B

Feces

CNV

NEO

Proportion of 16S rDNA

Study Day

Selected Fecal Taxa

Day 21

Proportion of 16S rDNA

CNV

NEO

NA; NA
p._Actinobacteria; f._Coriobacteriaceae
p._Bacteroidetes; f._S24-7
p._Firmicutes; f._Christensenellaceae
p._Firmicutes; f._Clostridiales
p._Firmicutes; f._Erysipelotrichaceae
p._Firmicutes; f._Lachnospiraceae
p._Firmicutes; f._Lactobacillaceae
p._Firmicutes; f._Mogibacteriaceae
p._Firmicutes; f._Peptococcaceae
p._Firmicutes; f._Ruminococcaceae
p._Firmicutes; f._Streptococcaceae
p._Firmicutes; f._Turicibacteraceae
p._Firmicutes; NA
p._Tenericutes; f._Anaeroplasmataceae
p._Tenericutes; NA
p._Verrucomicrobia; f._Verrucomicrobiaceae
Figure 3, continued.

C

Ileal Contents
Day 21

Proportion of 16S rDNA

CNV NEO

Selected Ileal Taxa
Day 21

Proportion of 16S rDNA

CNV NEO

p. Fimbriimonas
p. Bacteroides; f. S24-7
p. Firmicutes; f. Christensenellaceae
p. Firmicutes; f. Clostridiaceae
p. Firmicutes; f. Erysipelotrichaceae
p. Firmicutes; f. Lachnospiraceae
p. Firmicutes; f. Lactobacillaceae
p. Firmicutes; f. Mogibacteriaceae
p. Firmicutes; f. Peptococcaceae
p. Firmicutes; f. Ruminococcaceae
p. Firmicutes; f. Streptococcaceae
p. Firmicutes; f. Turicibacteraceae
p. Firmicutes; NA
p. Tenericutes; f. Anaeroplasmataceae
p. Tenericutes; NA
p. Verrucomicrobia; f. Verrucomicrobiaceae

NA; NA
Figure 4

A  Bone Marrow

PMNs

IMS

B  Spleen

PMNs

IMS

C  Blood

PMNs

IMS

D  PMNs

IMS

Decay Constant (k)

Decay Constant (k)

Vehicle  Ligand

Vehicle  Ligand

WT  NEO  Nod1−/−

WT  NEO  Nod1−/−

WT  NEO  Nod1−/−
Figure 5

A. Bone Marrow

PMNs
- Isotype
- α-IL-17A

% Visible

IMs
- Isotype
- α-IL-17A

% Visible

B. Spleen

PMNs
- Isotype
- α-IL-17A

% Visible

IMs
- Isotype
- α-IL-17A

% Visible

C. Blood

PMNs

% BrdU

Time (Days)

Blood

% BrdU

Time (Days)

D. Decay Constant (k)

PMNs
- Isotype
- α-IL-17A

Decay Constant (k)

IMs
- Isotype
- α-IL-17A
Figure S1
Figure S2

A

- Lymphocytes
- Endothelial Cells
- Fibroblasts

B

- Macrophages

% Viable

<table>
<thead>
<tr>
<th></th>
<th>CNV</th>
<th>NEO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelial Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NS
CHAPTER 6:

Discussion

Christopher B. Hergott

Department of Microbiology, University of Pennsylvania,

Philadelphia, PA, 19104, USA
**Bacterial Exploitation of Phosphorylcholine Mimicry Suppresses Inflammation to Promote Airway Infection.**

While prolonged pneumococcal infection of the upper airway was known to require efficient evasion of acute, neutrophil-mediated phagocytosis, the mechanism underlying this phenomenon remained unknown. We found that the pneumococcus disarms the functional capacity of responding neutrophils through hydrolysis of platelet-activating factor (PAF), a secreted inflammatory phospholipid essential for neutrophil activation, viability, and phagocytic capability in the airway. PAF hydrolysis relies upon molecular mimicry between phosphorylcholine (ChoP) residues present on the pneumococcal surface and within PAF, allowing the microbe to leverage a surface ChoP hydrolase, Pce, to remove PAF from the airway.

We found that pneumococci deficient in Pce were cleared rapidly from the upper airway over the first week of infection, corresponding with the period of maximal neutrophil influx into the airway (366). This survival defect was in agreement with previous findings in infant rats over the first two days of infection, though no mechanistic explanation was explored in that study (129). Further evidence linking Pce to neutrophil evasion was provided by a previously completed *in vitro* genomic screen for anti-phagocytic factors in which the gene encoding Pce was detected (81). Purified Pce enzyme had also been shown to mediate ChoP hydrolysis from artificial substrates *in vitro*, suggesting this catalytic activity was likely responsible for its immune evasion properties (340). Whether ChoP hydrolysis from the cell wall or another substrate remained less clear.
Using bacterial flow cytometry, we assessed whether ChoP abundance on the exterior of the pneumococcus was modified in the presence or absence of Pce and found no difference. Furthermore, we noted that the neutrophils recruited to pneumococci lacking Pce were both more abundant and more activated despite the mutant’s enhanced clearance from the airway, suggesting that Pce may modulate the physiology of responding neutrophils directly. Given previous in vitro evidence that Pce is capable of hydrolyzing ChoP from PAF, along with the well-known stimulatory activity of PAF on neutrophils, we hypothesized that this exploitation of phosphorylcholine mimicry governed pneumococcal neutrophil evasion in the upper airway (144, 178).

Systemic neutrophil depletion with monoclonal antibodies demonstrated that neutrophils were responsible the survival defect observed among Pce-deficient pneumococci. Pce was also rendered unnecessary for pneumococcal survival in mice lacking PAFR or treated with PAFR inhibitors, suggesting that the enzyme targets PAF signaling to disable responding neutrophils in the upper airway. These findings stand in contrast to previous investigations of PAFR in pneumococcal infections, carried out primarily in pneumonia, sepsis, and meningitis models. Previous conceptions of PAFR-pneumococcus interactions focused on the receptor’s role as a bacterial adhesin, binding cell wall ChoP to enhance bacterial persistence at the mucosa and invasion across mucosal barriers (76). Accordingly, PAFR-deficient mice showed impaired pneumococcal dissemination in these models, though PAFR exerted little effect on intravascular bacterial survival (47, 48). In contrast, we found that PAFR exerts no role in governing wild type pneumococcal persistence in the upper airway and exerts a protective effect against Pce-deficient pneumococci. Furthermore, we determined that
Pce inhibits bacterial invasion into the blood from the upper airway, suggesting that enhanced PAF signaling protects the host from systemic pneumococcal dissemination. These results indicate that the primary function for PAFR in the upper airway is responding to cognate ligand binding and stimulating neutrophil-mediated clearance, rather than serving as a receptor for direct binding by microbial ChoP. Given that nearly all invasive pneumococci arise from localized infections of the upper airway, we propose that the cognate ligand “signaling” paradigm reflects a more physiologically relevant function of PAFR than the microbial “binding” paradigm in the natural course of infection (154).

The wide range of extracellular bacteria that display surface ChoP, including *H. influenzae* and *N. meningitidis*, led us to hypothesize that similar PAF mimicry may be utilized by other microbes (350). Despite the lack of a direct homolog for Pce, we uncovered a functional analog on the surface of *H. influenzae* named GlpQ. Like Pce, GlpQ hydrolyzes phosphorylcholine from conjugated substrates and inhibits acute, neutrophil-mediated clearance of the microbe during upper airway infection. Future work will focus on expanding the repertoire of undiscovered PAF-cleaving enzymes expressed on the exterior of ChoP-expressing microbes. This can proceed without need for genetic similarity to Pce and in a high-throughput manner, as our *in vitro* chromogenic assay for ChoP hydrolysis provides a functional, quantifiable readout for this catalytic activity. Transposon libraries can be generated in suspected microbes, similar to that performed previously for the pneumococcus in anti-phagocytosis screens (81). Insertion mutants showing diminished ChoP hydrolysis activity can be explored mechanistically and in mouse infection models. A potential complication of this method
is that other microbes may possess multiple enzymes functioning in parallel to hydrolyze ChoP efficiently. Indeed, while no residual activity remained in the pneumococcus after deletion of Pce, ~20% of ChoP hydrolysis remained for \textit{H. influenzae} after deletion of GlpQ. Thus, multi-gene deletions or bioinformatics queries for the active site structure in question may be required to elucidate the full complement of unknown PAF-hydrolyzing enzymes in other bacterial species.

The main clinical relevance of this work arises from the finding that a critical threshold of PAF concentration exists for clearance of both \textit{S. pneumoniae} and \textit{H. influenzae}, above which neither pathogen can evade acute neutrophil responses. This may illuminate a potentially actionable target for intervening in upper airway infection before the onset of invasive disease, especially among individuals at elevated risk of dissemination (e.g. children, the elderly, the immunocompromised). If PAF levels can be safely elevated in the upper airway, clearance of a wide spectrum respiratory pathogens may accelerate. One potentially promising avenue for elevating PAF levels is the inhibition of a host enzyme, called PAF acetylhydrolase, charged with hydrolyzing PAF as a negative feedback mechanism to promote resolution of inflammation. PAF acetylhydrolase is secreted into the airway lumen during infection and inhibits respiratory tract neutrophil responses (188, 331). Preliminary experiments suggest mice lacking PAF acetylhydrolase clear colonizing pneumococci entirely within a few days, accompanied by an abundant influx of activated neutrophils (data not shown).

Importantly, the safety of a PAF acetylhydrolase inhibitor (named darapladib) has already been demonstrated in human clinical trials and no systemic inflammatory symptoms were observed (153). If proven to be efficacious in inhibiting airway bacterial
infection, darapladib could represent a broadly-acting, host-directed antibacterial therapeutic less susceptible to bacterial escape than drugs targeting microbial structures.

**Macrophage Migration Inhibitory Factor Promotes Clearance of Pneumococcal Colonization**

A sustained influx of macrophages is required for definitive elimination of pneumococci from the upper airway (366). However, which factors mediate macrophage persistence in the respiratory tract and how such factors are elicited by the microbe have remained less clear. We demonstrated that macrophage persistence in the upper airway lumen requires the cytokine macrophage migration inhibitory factor (MIF). Colonizing pneumococci stimulate MIF secretion through the action of its pore-forming toxin pneumolysin, which triggers pro-inflammatory p38-MAPK signaling in tissue-resident macrophages. MIF liberated into airway stimulates the durability and phagocytic capacity of recruited macrophages. MIF also facilitates secretion of CCL2, the key chemokine governing macrophage recruitment. Collectively, these functions markedly accelerate pneumococcal clearance from the respiratory tract.

Stable colonization of the upper respiratory tract is a prerequisite for the development of pneumococcal pneumonia and disseminated disease (348). We found that mice lacking MIF exhibit higher bacterial burdens in the upper airway and reduced abundance of airway luminal macrophages. These experiments are in agreement with population-based studies showing decreased incidence of pneumococcal pneumonia among adults with activating polymorphisms in the gene encoding MIF (362). Increasingly, genetic
screens for genetic polymorphisms provide an empiric basis upon which to design mechanistic investigations. Traditionally restricted to monogenic disorders with readily apparent clinical phenotypes, modern sequencing technologies have allowed detailed analysis of patient genotype information as individuals are monitored for susceptibility to infectious disease. In the past 15 years, functional polymorphisms have been discovered within a range of factors governing microbial detection and response, including TLR2, TLR4, NOD1, TNFα, and IL-1β (174, 290, 335). In addition to providing a platform for scientific study, these genetic variants may delineate patients that are particularly susceptible to infectious diseases and may therefore require more careful clinical monitoring and/or prophylaxis.

We found that MIF is released into the respiratory tract by pneumococci through the action of its pore-forming toxin, pneumolysin. Originally thought to function chiefly through lysis of host cells, this toxin is also capable of modulating intracellular signaling pathways, inhibiting bactericidal mechanisms, and impairing phagocyte trafficking to disable phagocytic responses (234). However, its role in pneumococcal colonization is less clear. As shown in Figure 4A, pneumolysin-deficient bacteria persist at higher loads than wild type bacteria at two weeks post-inoculation. Thus, pneumolysin seems to confer detrimental effects to the microbe in the upper airway, accompanied by the more robust macrophage response it initiates. Whether the poor survival of pneumolysin-positive pneumococci belies some other evolutionary benefit remains unclear. One possible interpretation is that pneumolysin functions primarily in the lower airway tract, where it is essential for maintaining bacterial persistence (33). Expectoration of persistent organisms from the lung may continually re-seed the upper respiratory tract,
providing an ongoing platform from which the robust immunity of the lower airway can be evaded. Alternatively, pneumolysin may provide some yet-unknown advantage to pneumococci during transmission from host to host. Disabling and destroying leukocyte aggregates by pneumolysin may free the pneumococcus to be shed in respiratory droplets and more readily acquired by a new host. Finally, recent evidence suggests the elicitation of inflammation can provide direct nutritional benefits to colonizing pneumococci, potentially yielding a smaller population of bacteria that exhibit enhanced fitness within the nutrient-limiting conditions inherent to respiratory droplet transmission (305).

Our findings also illuminate a potentially cooperative relationship between luminal cytokines and systemic chemokines in generating enduring macrophage responses to pneumococcal colonization. Previous findings have demonstrated that detection of the pneumococcus in the upper airway by TLR2 and NOD2 stimulated release of CCL2, the key chemokine governing initial macrophage recruitment into the tissues (87). The present study expands this paradigm by showing that MIF governs the sustained presence of these cells in the lumen of the airway, where they persist and clear bacteria more readily. Additionally, we found that MIF binding to resident macrophages stimulates additional secretion of CCL2 from the airway, generating a positive feedback loop of macrophage recruitment and retention. Accordingly, mice lacking either CCR2 (the receptor for CCL2) or MIF exhibit macrophage responses with lower cell abundance and qualitative phagocytic defects.
Macrophage Migration Inhibitory Factor is Detrimental in Pneumococcal Pneumonia and a Target for Therapeutic Immunomodulation

While MIF-stimulated macrophage persistence is essential for pneumococcal clearance from the upper airway, it remained unclear whether this host-protective effect also functioned during fulminant pneumonia. In contrast its impact in the upper airway, we found that MIF signaling precipitated more rapid host mortality, impaired bacterial clearance, and substantial pulmonary tissue damage during pneumococcal pneumonia. These findings corresponded with enhanced viability of resident alveolar macrophages and increased recruitment of neutrophils and inflammatory monocytes to the lungs of wild type mice when compared to MIF-deficient mice.

We discovered that the detrimental consequences of MIF signaling in pneumococcal pneumonia were incongruent with the beneficial impact observed during upper respiratory tract clearance. However, our findings suggested that MIF stimulates similar functional changes in macrophages residing at both anatomic sites. In the upper respiratory tract, MIF augments bacterial clearance by enhancing the duration of macrophage responses in the airway lumen. Similarly, alveolar macrophages from wild type mice exhibit significantly enhanced cellular viability during pneumococcal pneumonia compared to similarly infected MIF-deficient animals. These results are consistent with well-described mechanisms through which MIF signaling delays apoptotic cell death by suppressing nitric oxide production and p53 signaling (240).
In our study, MIF-driven delays in alveolar macrophage apoptosis were accompanied by substantially increased pulmonary recruitment of neutrophils and inflammatory monocytes, along with elevated pneumococcal burden and accelerated host mortality. Multiple previous studies have demonstrated a host-protective role for apoptotic cell death among alveolar macrophages during pneumococcal pneumonia (93, 94). Inversely, inhibition of alveolar macrophage apoptosis (e.g. in iNOS-deficient mice) corresponds with drastically enhanced pulmonary inflammation, bacteremia, and host demise. Activated neutrophils recruited to the lungs confer the bulk of tissue damage in these infection models, responsible for liberating a plethora of proteases, reactive oxygen species, and other cytotoxic mediators that spur further inflammation and disrupt vascular integrity (136).

We also found that treating mice systemically with a small-molecule inhibitor of MIF called MIF098 reduced inflammatory leukocyte infiltration and augmented pneumococcal clearance from the lungs. Like the inhibitors of PAF acetylhydrolase, MIF antagonists present an opportunity for broadly efficacious, host-directed immunomodulatory therapy potentially more resistant to microbial evolutionary escape. While MIF-directed small molecules have shown some efficacy in combating animal models of inflammatory diseases, they remain largely undeveloped in the context of bacterial infections (198). Our results in pneumococcal pneumonia may be particularly promising because the drug is efficacious against fulminant, ongoing disease. This may help preclude the need for the difficult patient monitoring inherent in detecting nascent infections before they develop into readily detectable clinical entities.
Detection of peptidoglycan from the gut microbiota governs the lifespan of circulating phagocytes at homeostasis.

It is now widely appreciated that the gut commensal flora regulates systemic innate immunity, yet the mechanisms driving many of these stimulatory influences remain poorly understood. We showed that the gut microbiota regulates the viability and circulating lifespan of neutrophils and inflammatory monocytes at homeostasis. Microbial effects were dependent upon a distinct community of neomycin-sensitive microbes in the intestine, required host signaling through the cytoplasmic peptidoglycan sensor Nod1, and necessitated IL-17A liberation to relay commensal signals to circulating phagocytes.

Most investigations into the impact of the commensal flora on phagocytes have focused on the influence of these signals on their hematopoietic development or bactericidal capacity (91, 173). However, the rate at which phagocytes turn over in the tissues continuously instruct these other parameters. Persistence in the vasculature and in the tissues dictates the window in which phagocytes can combat pathogenic threats (206). Phagocyte turnover also restrains the rate of new phagocyte development during homeostasis through suppression of IL-17A and IL-23 release (315). Tight regulation of phagocyte lifespan becomes particularly important in inflammatory environments, in which the cells must exert their functions efficiently but undergo apoptosis with sufficient rapidity to facilitate resolution (103). Dysregulated persistence of phagocytes at inflammatory sites leads to tissue damage and, if the threat is infectious, suboptimal pathogen control (104). Phagocyte turnover kinetics remain important even at rest, as
continuous disposal and degradation of aged cells regulates the size of peripherhal organs and inhibits spontaneous auto-inflammation (206).

Our results reveal that neutrophil and inflammatory monocyte lifespans are dictated at homeostasis by tonic and systemically disseminated signals from the gut microbiota. Broad-spectrum antibiotic treatment accelerated the exit of circulating phagocytes from the bloodstream and accelerated their spontaneous cell death *ex vivo*. The turnover and apoptosis kinetics among cells from control mice resembled those seen in previous studies, reinforcing that our assays accurately reflect physiologic phagocyte lifespan ranges (364). We also sought to identify a particular microbial community responsible these stimulatory signals by narrowing the coverage of our antibiotic treatment. Neomycin recapitulated the impact of the broad antibiotic cocktail, a finding congruent with previous analyses of the microbiota and antiviral pulmonary responses (152).

Neomycin is an aminoglycoside antibiotic that absorbs poorly into systemic circulation when given orally. It exhibits strong activity against gram-negative bacteria with less consistent coverage among gram-positive bacteria and little impact on anaerobes (78). We characterized the community architecture of the intestinal microbiota before and after neomycin treatment. We found that while facultative gram-negative taxa were relatively rare in all conditions, gram-positive lactobacilli were depleted particularly well upon drug treatment. This finding was consistent with previous studies reporting high sensitivity of lactobacilli to neomycin (251). Furthermore, the peptidoglycan of multiple lactobacilli express iE-DAP, the cardinal ligand for Nod1 and the first signal connecting the microbes to systemic phagocyte responses (31). Accordingly, we found that Nod1
signaling is both necessary and sufficient to mediate microbial extension of phagocyte lifespan.

The path by which iE-DAP transits from the bacterial cell wall in the gut lumen to Nod1 within the cytoplasm of host cells remains incompletely understood. Intact bacteria or cell wall components shed during microbial fission are taken into sensing cells through endosomal compartments, from which they traffic into the cytosol through the peptide transporters SLC15A3 and SLC15A1, meeting and binding to Nod1 on the cytoplasmic leaflet of the endosomal membrane (61). Binding of ligand to Nod1 triggers oligomerization of the sensor, which facilitates downstream signaling through downstream kinases to activate NF-κB (318).

In addition to stimulating the release of cytokines and neutrophil chemokines, Nod1 signaling also promotes the differentiation of IL-17A-producing T cells in the intestinal lamina propria (119). Our results demonstrate that IL-17A is essential for mediating microbial and Nod1-dependent signals to stimulate phagocyte lifespan. Systemic neutralization of IL-17A renders cells from control, knockout, and antibiotic-treated mice nearly equivalent in persistence and turnover kinetics. How IL-17A instructs phagocyte lifespan is likely complex and multifactorial. While it remains possible that IL-17A acts on neutrophils and inflammatory monocytes directly, the majority of evidence suggests that IL-17A instead stimulates the lifespan of these cells through additional cytokine intermediates. The best-studied terminal cytokine for neutrophil lifespan extension is G-CSF (201). G-CSF binding to its receptor (G-CSFR1) on neutrophils triggers diverse intracellular signaling pathways that converge to block the activation of caspase 3 (212).
Monocytes also receive signals from GM-CSF, though the two cytokines are by no means mutually exclusive (43). Collectively, these signals integrate to inhibit pro-apoptotic machinery with phagocytes and promote their persistence in the periphery.

Future directions for this project will focus on determining whether microbiota-mediated stimulation of phagocyte lifespan manifests as a differential outcome in some clinically significant disease scenario. Preliminary data suggests that mice treated with neomycin or lacking Nod1 exhibit impaired bacterial clearance of *S. pneumoniae* and *S. aureus* (data not shown). This is consistent with previous reports showing accelerated sepsis from these pathogens among Nod1-deficient mice, though the present results suggest this is directly attributable to the neomycin-sensitive cohort of microbes. However, host defense phenotypes among Nod1-deficient mice are not necessarily caused by differential phagocyte lifespan, especially since Nod1-mediated stimulation also impacts neutrophil phagocytic capacity (70).

Dissecting whether a causal relationship exists between phagocyte lifespan and infection outcome *in vivo* would require isolated manipulation of lifespan determinants without confounding effects on phagocytic capacity. One potentially promising strategy for investigating this in neutrophils is using myeloid-specific Mcl-1 knockout mice. Mcl-1 has been termed the “master regulator” of intracellular neutrophil apoptotic signaling is essential for maintaining normal neutrophil (but not macrophage) lifespan (98, 239). Myeloid-specific depletion of Mcl-1 (by Cre recombinase under the LysM promoter) accelerates the turnover of neutrophils so sharply as to render mice nearly devoid of neutrophils successfully recruited to gastrointestinal infections (167). This result
supports the overarching hypothesis that neutrophil lifespan is central to its role in host defense, but does not dissect the role of the flora in governing these changes. Because these mice are functionally neutropenic, there may be a very narrow window in which to analyze the effects of neomycin treatment or Nod1 deletion on a background of myeloid Mcl-1 deficiency.
References


66. Clark PO, Hanahan DJ, and Pinckard RN. Physical and chemical properties of platelet-activating factor obtained from human neutrophils and monocytes and


144. Hermoso JA, Lagartera L, González A, Stelter M, García P, Martínez-Ripoll M, García JL, and Menéndez M. Insights into pneumococcal pathogenesis from the


as they survey steady-state tissues and transport antigen to lymph nodes.


181. Kobayashi SD, Voyich JM, Buhl CL, Stahl RM, and DeLeo FR. Global changes in gene expression by human polymorphonuclear leukocytes during receptor-mediated phagocytosis: cell fate is regulated at the level of gene expression.


