

Multi-faceted PMN and epithelial responses triggered by *S. pneumoniae* PLY promote bacterial translocation across the pulmonary barrier

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Abstract

Streptococcus pneumoniae (*Sp*; also known as pneumococcus) is the most common agent of community-acquired pneumonia, can spread systemically to cause invasive pneumococcal disease (IPD), and is an important source of morbidity and mortality particularly in the elderly population. Pneumolysin (PLY), a pore-forming toxin and major *Sp* virulence factor, is not only directly cytotoxic but also capable of triggering tissue-damaging host responses. Limitations of pneumococcal vaccine efficacy, an increase in *Sp* antimicrobial resistance, and the heightened susceptibility of the aged to serious *Sp* infection, indicate a need for adjunctive host-directed therapies (HDTs) that target pathological immune responses to diminish IPD.

A key step in the progression of localized *Sp* pulmonary infection to bacteremia and IPD is *Sp* breach of the respiratory epithelial barrier. Neutrophils, also known as polymorphonuclear leukocytes (PMNs), are essential for infection control but can also promote tissue damage and pathogen spread. 12-lipoxygenase (12-LOX) eicosanoid synthesis triggers robust PMN infiltration into the airways and is required for *Sp* systemic spread in a mouse pneumonia model. *In vitro* modeling using immortalized H292 cells indicated that PLY stimulates 12-LOX. Here, to reveal PMN-driven pathologies to the epithelial barrier that promote *Sp* trans-epithelial dissemination, we infected stem cell-derived air-liquid interphase (ALI) epithelial organoids, which compared to immortalized cell lines more accurately reflect the complexity of the airway epithelium. We found that hepxilin A3 (HXA₃), a 12-LOX-secreted lipid chemoattractant, was sufficient to induce PMN transmigration and epithelial barrier disruption upon *Sp* infection. Moreover, HXA₃, in contrast to a well-studied control chemoattractant, f-Met-Leu-Phe, promoted pro-inflammatory conditioning of PMNs, reflected by enhanced neutrophil elastase (NE) release, that compromised airway integrity.

A key element in epithelial barrier function is the apical junction complex (AJC), which limits paracellular leakage. We found that PLY, in addition to its ability to activate 12-LOX, was sufficient and necessary to trigger efficient cleavage and mis-localization of the epithelial AJC protein E-cadherin of polarized H292 monolayers. By examining a time course of pulmonary *Sp* challenge in mice, we found that disruption of alveolar E-cadherin by PLY preceded PLY-promoted PMN infiltration and bacteremia. Infection of 12-LOX-deficient ALI monolayers, which are incapable of producing the confounding dissemination-promoting factor HXA₃, showed that early AJC perturbation was in fact required for maximal PMN transmigration and subsequent bacterial movement across the compromised epithelial barrier.

To test potential HDTs that target pathways facilitating *Sp* spread upon lung infection, particularly in the aged host, we intratracheally challenged young (2-month-old) or aged (>20-month-old) mice, which exhibit greater lung burden and more severe bacteremia upon *Sp* infection. We discovered that aging is correlated with poorer alveolar AJC organization, elevated NE in bronchial alveolar lavage fluid, and increased bloodstream-lung permeability, and confirmed these findings during *Sp* infection of ALI cultures generated from young or aged mice. Treatment with Nexinhib20, an inhibitor of NE release, Sivelestat, an inhibitor of NE activity, or bardoxolone methyl (CDDO), an agonist of Nrf2-mediated AJC fortification, each effectively limited development of IPD following pulmonary challenge in young mice, while a combination of Sivelestat and CDDO provided additional barrier integrity protection in aged mice.

These studies elucidate the multiple means by which PLY compromises airway epithelial barrier function and promotes IPD. PLY (i) disrupts epithelial AJCs to prime the airway epithelium for transepithelial movement of PMNs, and (ii) triggers epithelial secretion of HXA₃, which enhances NE production by PMNs while driving migration of these tissue-destructive cells into the airways. Pharmacologic interventions that preserve

barrier function, by preventing AJC dissolution or inhibition of NE release or activity, have potential as HDT candidates to mitigate epithelial barrier destruction by PMNs and the ensuing lethal IPD.

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List of Abbreviations

12-LOX/Alox15	12-lipoxygenase
ABTS	Diammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)
AJ	Adherens junction
AJC	Apical junction complex
ALI	Air-liquid interphase
ARDS	Acute respiratory distress syndrome
B6	C57Bl/6
BALF	Bronchoalveolar lavage fluid
BBB	Blood-brain barrier
BMDM	Bone marrow-derived macrophage
BSC	Basal stem cell
CBS	Cholesterol binding site mutant
CDC	Cholesterol binding cytolysin
CDDO	Bardoxolone methyl
CF	Cystic fibrosis
CFU	Colony forming units
Ci-Di-Cy/CDC	Cinnamyl-3,4-dihydroxy- α -cyanocinnamate
COPD	Chronic obstructive pulmonary disease
CrEL	Cremophor EL
DCF	2',7'-Dichlorofluorescein
DMSO	Dimethyl sulfoxide
EAD	Extracellular adenosine
E-cad/ <i>CDH1</i>	E-cadherin
EpCAM	Epithelial cell adhesion molecule
FITC	Fluorescein Isothiocyanate
fMLP	f-Met-Leu-Phe
h.p.i	Hour post infection
HBSS	Hanks buffered salt solution
HDT	Host directed therapy
HRP	Horseshoe radish peroxidase
HXA3	Hepoxilin A3
HXA3-ME	Hepoxilin methyl ester
<i>i.p</i>	Intraperitoneal
<i>i.t</i>	Intratracheal
<i>i.v</i>	Intravenous
IF	Immunofluorescence
IJOQ	Intercellular junction organization quotient
IL	Interleukin
ILY	Intermedilysin
IPD	Invasive pneumococcal disease
IPTG	Isopropyl β -D-1-thiogalactopyranoside
JAM	Junctional adhesive molecule

LC3	Microtubule-associated protein 1A/1B-light chain 3
LRT	Lower respiratory track
MDR	Multi-drug resistant
MMP	Matrix metalloprotease
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MRC-1	Mannose receptor C type 1
NE	Neutrophil Elastase
NET	Neutrophil extracellular trap
Nex	Nexinhib20
OPH	Opsonophagocytic
PACE	Pneumonia-associated adverse cardiac events
PBS	Phosphate buffered saline
PFO	Perfringolysin O
PFT	Pore forming toxin
PI	Propidium iodide
PLY	Pneumolysin
PMN	Polymorphonuclear cells, or neutrophils
ROS	Reactive oxygen species
SAGM	Small airway epithelium growth media
Siv	Sivelestat
SLO	Streptolysin O
Sp/SPN	<i>Streptococcus pneumoniae</i> , or pneumococcus
TCEP	Tris(2-carboxyethyl)phosphine
TJ	Tight junction
TMB	3,3',5,5'-Tetramethylbenzidine
TNF- α	Tumor necrosis factor alpha
WT	Wild type
ZO	Zonular occludin

Chapter 1: Introduction¹

¹Pereira, J. M.*; Xu, S.*; Leong, J. M.; Sousa, S. The Yin and Yang of Pneumolysin During Pneumococcal Infection. *Front Immunol* 2022, 13, 878244. DOI: 10.3389/fimmu.2022.878244, Published.

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1. Introduction

1.1. *Sp* pathogenesis leading to invasive disease

Streptococcus pneumoniae (*Sp* or pneumococcus) is an extracellular gram-positive bacterium that asymptomatically colonizes the upper respiratory tract of 5-10% of healthy adults [1]. Children under 5 and adults over 65 are particularly susceptible, and approximately 2.5 million individuals succumb to *Sp* infections globally each year, making this bacterium a critical and deadly pathogen [2, 3].

Sp transmits predominantly via aerosol as the bacteria can be harbored in the nasopharynx asymptomatically. To colonize the nasopharynx, *Sp* forms biofilms attaching to the upper respiratory tract mucosa [4] (Figure 1.1A), where shed bacteria are transmitted to other hosts via nasal secretions [5]. *Sp* carriage can progress to localized infection of tissues contiguous with the nasopharynx, such as the sinusoidal cavities to cause sinusitis, the middle ear to cause otitis media, or the eye to cause keratitis [1] (Figure 1.1B). Nasopharyngeal carriage also predisposes the host to develop pneumonia (Figure 1.1C), as aspiration into the lower respiratory tract (LRT) allows *Sp* to come into contact with small bronchioles and the alveoli [6]. Finally, in approximately 20% of pneumococcal pneumonia patients, the bacterium is capable of entering the bloodstream (Figure 1.1D) to cause invasive pneumococcal disease (IPD), resulting in serious conditions such as septicemia, cardiac dysfunction, and meningitis [1] (Figure 1.1E).

The versatility of this bacterium is reflected in its remarkable lifestyle adaptations to the host environment depending on the site of infection and degree of disease, aided by pneumolysin (PLY), a multi-faceted virulence factor integral to all stages of *Sp* pathogenesis [7] and produced by almost all clinical isolates and currently known *Sp* serotypes [8]. In the nasopharynx, PLY triggers only a mild pro-inflammatory response enough to promote bacterial shedding in secretions [5]. This is in stark contrast to PLY

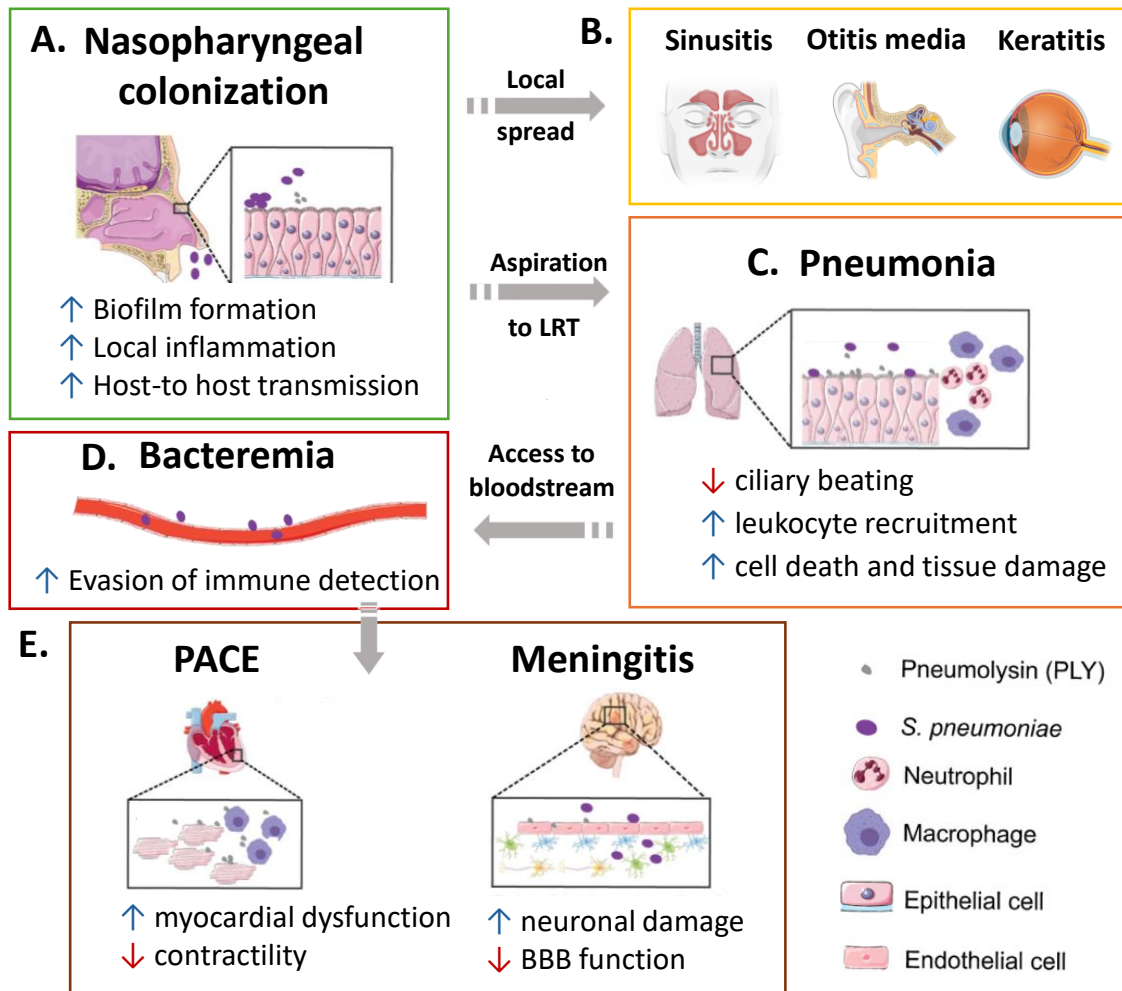


Figure 1.1. *Sp* pathogenesis facilitated by PLY. *Sp* is an opportunistic bacterium capable of transitioning from being a harmless commensal to causing lethal systemic diseases. At each step of *Sp* pathogenesis, PLY is critical for promoting virulence and its key activities are listed. **(A)** *Sp* asymptomatically colonizes the nasopharynx where it is shed in nasal secretions for transmission. **(B)** Prolonged colonization may lead to local *Sp* spread to neighboring tissues such as the sinus, the middle ear, or the eyes. **(C)** Upon aspiration to the lower respiratory tract (LRT), *Sp* can establish infection in the lungs and cause pneumonia. **(D)** Excessive inflammation-associated damage to the pulmonary barrier during pneumonia may permit bacterial access to the bloodstream, leading to bacteremia and onset of invasive pneumococcal disease (IPD). **(E)** Systemically disseminated bacteria are able to invade distal organs such as the heart, causing pneumonia-associated adverse cardiac events (PACE), and the meninges, upon crossing of the blood-brain barrier (BBB).

behavior in the LRT during pneumonia, where the toxin triggers an exuberant pro-inflammatory response that recruits large numbers of inflammatory leukocytes, damages alveolar epithelial and endothelial cells, and increases pulmonary barrier permeability [9-

11] (Figure 1.1C). During this phase of the infection where the lung is sustaining substantial damage as a result of inflammation, if the bacteria is not cleared in a timely fashion, *Sp* may gain access to the bloodstream and disseminate to cause IPD [12, 13] (Figure 1.1D). While *PLY* is downregulated in the bloodstream and promote immune evasion [14], upon reaching distal organs such as the heart and the spleen, *PLY* promotes tissue colonization [13, 15-17], and/or crossing of the blood-brain barrier to access the meninges [18]. Transition from pneumococcal pneumonia to IPD increases disease lethality by over 3-fold and may leave long-term and irreversible damage in the survivors, such as pneumonia-associated adverse cardiac events (PACE) [14] and permanent neurological damage [19] (Figure 1.1E).

1.2. Aging increases susceptibility to IPD

Infection-related deaths make up one-third of all elderly deaths aged over 65, and pneumococcal pneumonia ranks in the top 10 causes of infection in this population [20]. Elderly patients are at heightened risk of severe morbidity and mortality due to IPD and represent over 80% of all cases and fatalities associated with pneumococcal infections [21]. One reason that the elderly succumb to infection at higher rates is age-related change to the host tissue environment [22] and the immune system [23]. Age-related changes promoting infection are generally categorized into two categories: inflammaging, a chronic elevation of basal and induced inflammation, and immunosenescence, a decline in cell-mediated immunity (Figure 1.2).

1.2.1. Inflammaging and barrier tissue dysfunction

Inflammaging is characterized by a chronic state of elevated baseline inflammation that occurs with aging. This sustained low-grade inflammation is thought to partly originate from barrier tissue defects associated with old age [24]. As an example,

upon aging the intestinal barrier suffers from a loss of apical junction complex (AJC) proteins and increased intestinal permeability to macromolecules [25]. While the barrier remains impermeable to pathogens, increased bacterial endotoxin leakage and altered microbiota composition are sufficient to promote low levels of systemic inflammation [25]. Similarly, during aging of the skin, keratin-forming stem cells become defective in differentiation and form less resilient skin barriers, leading to elevated skin cell pro-inflammatory cytokine production [24].

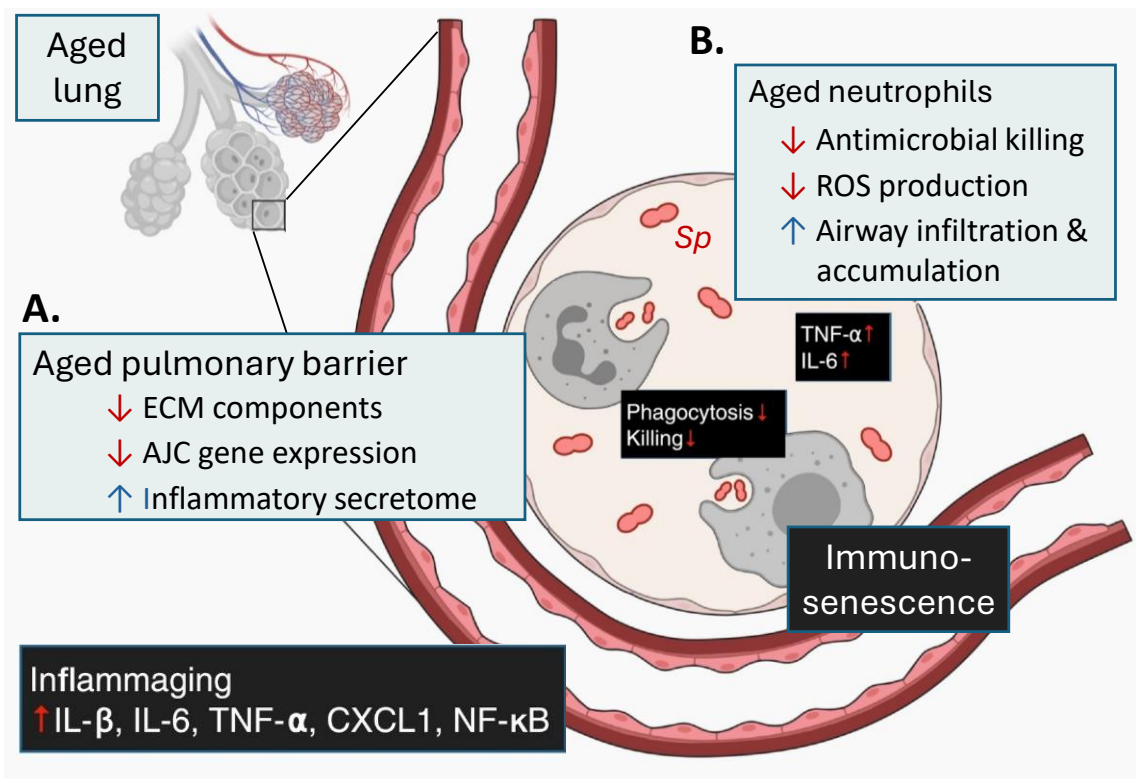


Figure 1.2. Inflammaging and immunosenescence impair immune defense against *Sp* in the elderly host. Inflammatory cytokine levels in the lung are elevated with age both at the steady state and during infection. Phagocytic cell functions also decline with age, resulting in reduced phagocytic killing of bacteria and elevated proinflammatory cytokine accumulation. As a result, the pulmonary barrier (**A**) and lung-infiltrating neutrophils (**B**) show age-related defects that hinder effective bacterial clearance and promote excessive and prolonged inflammation upon *Sp* infection.

Age-related changes also occur in the lungs (Figure 1.2A), including alterations in extracellular matrix components, the epithelial cell secretome, and a shift towards increased inflammation upon encountering microbial pathogens [26-30]. Many genes

associated with the assembly of the AJCs are downregulated in the lungs with increasing age [31], with loss of *CDH1* (gene for E-cadherin) identified as a putative key regulator of age-related defects in epithelial barrier function [32]. Inflammation during *Sp* lung infection is enhanced with age, as characterized by increased airway levels of tumor necrosis factor alpha (TNF- α), interleukin (IL) 6, and decreased anti-inflammatory cytokine IL-10 [22, 33]. Chronic exposure to TNF- α drives premature myeloid cells that are defective in bacterial burden control to exit the bone marrow and extravasate to the site of infection during *Sp* challenge [34]. Heightened inflammation is a major driver for severity of *Sp* infection in the elderly host, as use of alpha-tocopherol, the most bioavailable form of Vitamin E with antioxidant with anti-inflammatory functions [35], downregulates proinflammatory chemokines, limits excessive inflammatory PMN recruitment to the lung [36, 37], and restores effective immune clearance of *Sp* burden in aged mice [37].

1.2.2. PMN immunosenescence

Neutrophils, or polymorphonuclear leukocytes (PMNs), are known to decline in function with age [38-40], exhibiting defects in phagocytosis, reactive oxygen production, NET formation, and chemotactic accuracy (Figure 1.2B). During pneumococcal pneumonia, elderly patients experience exacerbated PMN pulmonary influx [41, 42], which correlates with bacterial burden. PMNs from both elderly human subjects and aged mice are defective in opsonophagocytic (OPH) killing of *Sp* [39, 43]. Upon intratracheal (*i.t*) *Sp* challenge of young or aged C57Bl/6 (B6) mice, aged mice suffered 100-fold higher lung burdens and 1000-fold higher blood burdens [36], correlating with higher levels inflammatory cytokines in the airways, increased PMN infiltration, greater lung tissue damage, and mortality [36]. Adoptive transfer of PMNs from young, but not aged mice, boosted the resistance of aged mice to *Sp* pneumonia [43], rescuing age-

related defects in *Sp* killing [44], diminishing IL-10 production [45], and limiting *Sp* invasion into airway tissue [46]. This protective activity depended upon PMN antimicrobial activity because transfer of PMNs defective in extracellular adenosine (EAD) signaling, a critical pathway required for *Sp* killing by PMNs, failed to rescue aged hosts [43], suggesting that PMNs are a major driver of age-related susceptibility to *Sp*.

1.3. Pneumolysin is a key virulence factor for *Sp* systemic spread

As a member of the cholesterol-dependent cytolysin (CDC) family, PLY is a pore-forming toxin (PFT) that at lytic concentrations disrupts host cell plasma membrane and promotes the uncontrolled influx and efflux of ions, small molecules, and proteins, leading to cell death [47]. To generate pores, soluble PLY anchors to cholesterol on the mammalian plasma membrane, multimerizes into “pre-pore” complexes, and then inserts into the membrane to generate a large 400 Å pore. Studies with neutralizing monoclonal antibodies and binding-defective point mutants indicate that PLY domain 4 is required for membrane docking [47-52]. In particular, two residues (T459 and L460) in a conserved Trp-rich loop in domain 4 are essential for cholesterol recognition, with mutations in these residues resulting in a non-pore forming toxoid [47, 53-55]. PLY shares major structural and functional properties with other bacterial CDCs, such as Streptolysin O (SLO), perfringolysin O (PFO), and intermedilysin (ILY) [53], all of which contain similar cholesterol-binding Trp-rich loops [54].

PLY has functional roles in promoting asymptomatic carriage, colonization, transmission, immune evasion, and systemic dissemination (Figure 1.1), highlighting the multi-faceted functions of this bacterial toxin. However, interestingly, the degree and outcome of PLY action is highly dependent on factors such as infection dose, target cell type, and the immune state of the host. This is exemplified by the paradoxical role of PLY during experimental challenge of different mouse strain backgrounds [56-59].

During *Sp* lung infection of CD1 mice [60], PLY is essential for host immune activation leading to effective pathogen control. In contrast, PLY elicits overwhelming cytotoxicity and inflammation during *Sp* challenge in B6 mice or BALB/c mice, resulting in hosts succumbing to infection [12, 56].

Host-protective responses triggered by PLY are often a result of immune recognition of PLY, which activates macrophages, dendritic cells, PMNs, and the epithelium [7, 61-63]. In mouse models, early inflammation and rapid immune cell recruitment into the lungs are essential for limiting pulmonary pneumococcal burden [64, 65]. Internalization of PLY by alveolar macrophages and dendritic cells via the mannose receptor MRC-1 [58], or, via LC3-associated phagocytosis in bone marrow derived macrophages (BMDMs) [66], results in effective *Sp* killing and the production of anti-inflammatory cytokines to promote disease resolution.

On the other hand, PLY-induced pores can overwhelm cellular homeostatic mechanisms [67], triggering both proinflammatory signaling pathways and irreversible cellular injury that results in the release of molecules heightening inflammation. PLY pore formation triggers inflammasome activation, IL-1 β release, and pyroptotic and necroptotic cell death [57, 68]. PLY activates epithelia cell production of pro-inflammatory cytokines and chemokines [10], while PLY-stimulated PMNs engage in a wide range of tissue-damaging effector functions, many of which are associated with severe pathology in the lung and subsequent *Sp* systemic spread [69, 70].

The balance between host-protective and detrimental activities of PLY is thus a major determinant of *Sp* infection outcome. Indeed, mice that survived *Sp* pulmonary infection largely resolved inflammation early, while those with sustained inflammation and large numbers of pulmonary PMNs beyond 12 hours post infection (h.p.i) exhibit greater bacterial burden, more severe systemic infection, and increased mortality [44, 59]. The state of the immune system, especially in its ability to mount effective yet

controlled inflammation not overwhelmed by actions of PLY, is crucial for optimal host defense against *Sp* infection [56].

1.3.1. PLY interaction with the epithelium

Epithelial cells are a first line of defense against *Sp*, functioning as a physical barrier against invasion and maintaining the mucociliary elevator that removes microbes from the lung. PLY was reported to bind to and promote actin polymerization in epithelial cells *in vitro* [71], inducing cytoskeleton instability [19] and formation of actin stress fibers, filopodia, and lamellipodia [71-73]. By destabilization of the cytoskeleton, PLY interferes with ciliary beating in human and mouse airway epithelium cultures [74-76]. PLY also disrupts organization and function of multiple epithelial and endothelial AJC proteins [12, 77], increasing permeability of the pulmonary barrier and promoting edema [77, 78].

In addition, the mucosal epithelium is a versatile surveillance system capable of secreting a variety of pro-inflammatory signaling molecules to alert and recruit immune cells to respond to invading pathogens. PLY is a potent activator of epithelial cytokine and chemoattractant secretion [9, 10, 79], triggering release of IL-1 β , TNF- α [10], and chemoattractants IL-8 [79] and the 12-lipoxygenase (12-LOX)-dependent lipid mediator hepoxilin A₃ (HXA₃) [9]. Accumulation of inflammatory cytokines and chemokines facilitate the recruitment of PMNs responding to and clearing *Sp*. This robust, and largely 12-LOX-dependent, PMN infiltration is however associated with increased epithelial damage and bacterial dissemination into the bloodstream [59, 80], suggesting that PLY-triggered epithelial chemoattractant secretion is eliciting a type of tissue-destructive neutrophilic inflammation harmful to the host [81].

1.3.2. PLY manipulating PMN functions

In mice, while early PMN influx is essential for clearing *Sp*, the prolonged accumulation of PMNs in the airways results in increased burden and high tissue damage [44]. Upon extravasation and encountering *Sp* in the airways, PMNs are activated by PLY to release IL-8, phospholipase A2, prostaglandin E₂, and leukotriene B₄ [82, 83], reinforcing leukocyte recruitment and inflammation. PLY-exposed PMNs produce reactive oxygen species (ROS) [61, 84, 85], form neutrophil extracellular traps (NETs) and engage in degranulation. During the latter, PMNs secrete a myriad of proteolytic enzymes capable of propagating tissue damage, including metalloproteinases (MMPs) MMP-8 and MMP-9 [70], and serine proteases cathepsin G, proteinase-3, and neutrophil elastase (NE) [69]. PLY can trigger NE release either by degranulation of primary granules or by direct cell lysis [61, 84]. Although NE degrades pneumococcal cell wall-localized aminopeptidase N and can facilitate OPH killing in the presence of an antiphagocytic capsule [84, 86-88], excessive NE activity is detrimental to the host [89]. NE has potent catalytic activity against a range of extracellular matrix proteins, including elastin, proteoglycan, fibronectin, collagen [90], surfactants [89], and epithelial AJC protein such as E-cadherin [91]. In addition, NE further stimulates the lung epithelium to release proinflammatory cytokines and induces epithelial apoptosis [92-94]. Severe pneumonia in human patients and experimental animals is associated with increased NE levels in lung bronchoalveolar lavage fluid (BALF) [95, 96] and in plasma [97], suggesting that NE may be a critical inducer of epithelial permeability during pneumococcal pneumonia [98]. Interestingly, PMN sensitivity to PLY depends on activation state of the PMN, with many PLY-induced effector functions further altered upon co-stimulation with the pneumococcal capsule [89] or with chemoattractants like f-Met-Leu-Phe (fMLP) [70, 85]. PLY is thus a potent activator of multiple PMN functions, and along with other bacterial or tissue environmental factors, can influence PMN functional profile to contribute to inflammation and pathogenesis of *Sp* infection.

1.4. Advantage of host-directed therapies for *Sp* infection

Since 1977, vaccination has been the cornerstone of pneumococcal disease prevention. Currently, the two classes of pneumococcal vaccines, the 23-valent pneumococcal polysaccharide vaccine (PPSV23) and the pneumococcal vaccines based on protein-conjugated polysaccharides (PCV13/ PCV23), protect against only a subset of over 90 different pneumococcal capsular serotypes [99]. Through both the expansion of preexisting non-vaccine pneumococcal serotypes and serotype 'switching', an exchange of capsular polysaccharide genes through transformation, infectious strains not covered by standard vaccination are on the rise [100].

In addition, a worldwide post-vaccination increase in antibiotic resistance frequencies of non-vaccine-type *S. pneumoniae* strains has been reported [101, 102]. *Sp* is naturally transformable and displays high genome plasticity, contributing to the rapid emergence of antibiotic resistance and evasion of vaccine-mediated immunity [1, 103]. Pneumococci resistant to penicillin (minimum inhibitory concentration of ≥ 0.1 $\mu\text{g/ml}$), erythromycin, and trimethoprim-sulfamethoxazole has spread across the world, with isolates identified in Australia, Israel, Spain, Poland, South Africa, and the United States [104-107]. Multi-drug resistant (MDR) pneumococci, classified as resistant to three or more classes of antimicrobials, were first identified in children in 1978 via nosocomial transmission, and has since been reported with increasing frequency [108]. In the United States today, MDR *Sp* is much less common after over 20 years of conjugate vaccine usage; however, the risk for acquiring resistant infections remains in particular populations, such as the elderly [109]. In 2017, *S. pneumoniae* infections led to over 30,000 outpatient visits in the United States alone, and 30% of cases involved isolates resistant to one or more antibiotics [110].

Rather than relying on therapeutics solely targeting the bacterial pathogen, host-directed therapies (HDT) focusing on host responses that may alter infection outcome are an attractive alternative. The host inflammatory response is a critical element for *Sp* pulmonary clearance, but if dysregulated, can damage pulmonary tissue and promote bacterial dissemination into the bloodstream [111]. As a result, a balance between host defense and timely return to homeostasis is critical for resolution of *Sp* infections, and HDT strategies capable of diminishing inflammatory damage to bystander cells may help direct inflammation in an appropriate and timely fashion to restore effective defense against *Sp* [112]. HDTs also presents a customizable strategy for the more susceptible elderly patients. With a better understanding of host deficiencies with age, HDTs can be tailored to the specific immunological mechanism promoting disease. Importantly, HDTs often target common infection immunoregulatory pathways and can be generalizable to diseases beyond *Sp* infections of the lung.

1.5. Research questions and thesis overview

PMN recruitment and response to *Sp* at the mucosal surface is a major determinant of whether *Sp* infection progresses to lethal systemic spread [59, 80]. While PMNs transmigrate across the airway epithelial barrier, they undergo a dynamic activation process orchestrated by microbial products like *PLY*, various chemotactic signals, and the pulmonary tissue environment [113, 114]. These factors individually and when combined have profound influence on the myriad of PMN antimicrobial and tissue-damaging activities, shaping the outcome of infection [115]. In addition, both PMNs and the airway mucosal environment are altered upon aging [23, 116], which results in increased susceptibility to severe *Sp* infection. This dynamic complexity presents a challenge to understanding the cellular mechanisms involved in PMN-assisted *Sp*

translocation across the airway epithelial barrier, and/or any age-related decline that may promote this process.

In this thesis, we utilized pulmonary challenge of mice *in vivo* and airway stem cell-derived 2D epithelial organoid infection *in vitro* to mimic the cellular complexity of PMN-epithelial interactions during *Sp* pulmonary infection. We ask the following research questions:

- (i) What is the mechanism by which 12-Lipoxygenase-dependent PMN transepithelial migration promotes epithelial barrier damage (Chapter 2);
- (ii) What PLY-triggered processes compromise integrity of the epithelial barrier and what degree of disruption is required to permit *Sp* cross-epithelial movement (Chapter 3); and
- (iii) Does host aging lead to defective epithelial barrier function or dysregulated PMN responses that promote susceptibility to systemic disease following pneumococcal pneumonia (Chapter 4).

For each chapter, we performed experimental therapeutic intervention targeted at the host processes identified to promote *Sp* cross-epithelial movement, with the goal of identifying potential HDTs capable of diminishing systemic spread of *Sp* following pneumococcal pneumonia.

Chapter 2: Blocking HXA₃-mediated neutrophil elastase release during *S. pneumoniae* lung infection limits pulmonary epithelial barrier disruption and bacteremia²

² Xu, S.; Tan, S.; Romanos, P.; Reedy, J. L.; Zhang, Y.; Mansour, M. K.; Vyas, J. M.; Mecsas, J.; Mou, H.; Leong, J. M. Blocking HXA₃-mediated neutrophil elastase release during *S. pneumoniae* lung infection limits pulmonary epithelial barrier disruption and bacteremia. *mBio*, 2024: p. e0185624.
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2. Blocking HXA₃-mediated neutrophil elastase release during *S. pneumoniae* lung infection limits pulmonary epithelial barrier disruption and bacteremia

2.1. Introduction

Streptococcus pneumoniae (*Sp*; also known as the pneumococcus) is a Gram-positive bacterium that asymptomatically colonizes the nasopharynx of 5-10% of healthy adults, but can spread to the lower respiratory tract and is the most frequent cause of community-acquired pneumonia [117]. Subsequent bacterial translocation from the airway into the bloodstream can lead to invasive disease, such as septicemia and meningitis, events associated with a three-fold increase in mortality [118]. Invasive pneumococcal infections result in approximately 14 million cases and one million deaths annually worldwide [119]. Vaccination and antimicrobials are first-line strategies in combating pneumococcal diseases. However, the rapid rise of antibiotic resistance and the limited antigenic breadth of effective vaccines have fueled interest in treatment strategies that focus on diminishing tissue-destructive host immune responses [100, 102, 120, 121].

Pneumococcal infection of lung mucosa drives robust recruitment of polymorphonuclear leukocytes (PMNs, or neutrophils), leading to the acute inflammation that is a hallmark of this infection [117]. PMNs confront invading *Sp* with multiple antibacterial mechanisms, including release of reactive oxygen species (ROS) [122], neutrophil extracellular traps (NET) [123], and/or proteases such as cathepsin G (CG) and neutrophil elastase (NE) [87]. Indeed, neutropenic individuals or neutrophil-depleted mice are highly susceptible to systemic *Sp* infection [124, 125]. Nevertheless, sustained pulmonary accumulation of PMNs increases airway permeability with a concomitant risk of disseminated infection [126, 127]. Protease inhibitors that diminish PMN infiltration also reduce bacteremia and lethality after *Sp* pulmonary challenge of mice [128, 129]. Finally, mice that retain high numbers of pulmonary PMNs suffer higher levels of

bacteremia and mortality [59, 80, 130, 131], and depletion of PMNs 18 hours post-infection (h.p.i.) mitigates disease and pathogen spread [44].

Chemotactic cues not only recruit PMNs but also influence their tissue-destructive character [113, 132, 133]. Hence, in addition to their recruitment, PMN-directed pathologies may result from enhanced tissue-damaging PMN activities [113]. The major *Sp* virulence factor pneumolysin (PLY), a cytolysin that drives tissue damage and promotes early bacteremia [13, 67, 134], stimulates the 12-lipoxygenase (12-LOX) pathway in epithelial cells and results in the synthesis and apical secretion of eicosanoid PMN chemoattractants [59, 135, 136]. Among 12-LOX-generated bioactive lipid mediators [137], the hydroxy epoxide heptoxilin A₃ (HXA₃) is a potent chemoattractant [138] that orchestrates mucosal inflammation during both intestinal [139, 140] and pulmonary infections [141]. Like other chemoattractants [133, 142], HXA₃ has both chemotactic and non-chemotactic effects on PMNs [143], triggering intracellular calcium release [143], promoting PMN survival [144], inducing NET formation [145], and stimulating the release of additional arachidonic acid metabolites [146]. Notably, genetic ablation or chemical inhibition of 12-LOX drastically reduces PMN infiltration, bacteremia, and mortality following *Sp* lung challenge of mice [59, 136], suggesting that barrier disruption and systemic *Sp* disease could be mitigated by modulation of PMN effector functions that are enhanced by one or more products of the 12-LOX pathway.

The tissue-destructive functions of PMNs are dramatically altered upon exposure to bacterial factors [113, 147, 148], but the effect of HXA₃ on PMNs in the context of *Sp* infection has not been examined. In addition, 12-LOX promotes the production of numerous bioactive lipids [137], and although HXA₃ has been hypothesized to be the essential driver in PLY-promoted *Sp* dissemination from the lung, this eicosanoid has not been directly implicated in the *Sp*- or PLY-driven PMN chemotaxis. These limitations are in part a reflection of the instability of HXA₃ in aqueous environments [139], as well as

the lack of an easily manipulated *in vitro* experimental model that faithfully reflects *Sp*-mediated inflammation and bacterial translocation across an epithelial barrier. Indeed, the respiratory epithelial culture models previously applied to *Sp* infection are typically based on immortalized cell lines that lack the cellular diversity and *bona fide* barrier function integral to airway epithelium [149]. Here, we characterized the role of PLY in promoting PMN transmigration and epithelial compromise using air-liquid interface (ALI) monolayers derived from bronchial stem cells that recapitulate key features of the airway epithelium. Moreover, ALI monolayers genetically ablated for 12-LOX deficient permitted the demonstration that HXA₃ methyl ester (HXA₃-ME), a stable and active version of HXA₃, is sufficient to promote PMN transmigration and *Sp* barrier breach. Corresponding studies of the signaling capacities of HXA₃-ME on PMN in the context of *Sp* infection showed that HXA₃ is not only a central driver of PMN transmigration across infected epithelium but also enhances the tissue-damaging proteolytic activity of PMNs. Targeting this HXA₃-promoted activity mitigated systemic disease following *Sp* pulmonary challenge of mice, illustrating its therapeutic potential as a host-directed therapy for *Sp* infection.

2.2. Results

2.2.1. The 12-LOX pathway, stimulated by PLY-producing *Sp*, promotes PMN

infiltration, lung permeability, and bacteremia following *Sp* lung infection in mice

Activation of the airway epithelial cell 12-LOX pathway is triggered by *Sp* pneumolysin [136]. We intratracheally (*i.t.*) inoculated BALB/c mice with 1×10^7 CFU of WT *Sp* TIGR4 or the isogenic PLY-deficient mutant *Sp* TIGR4 Δply . At 18 hours post infection (h.p.i.), the two strains reached similar lung burdens (Figure 2.1a, "WT" vs. " Δply "), consistent with previous reports [12, 150]. Both strains also induced pulmonary inflammation, but consistent with the ability of PLY to stimulate the 12-LOX pathway and

increase inflammation [13, 59], PMN pulmonary infiltration was 1.5-fold higher in mice infected with WT *Sp* compared to *Sp* Δply ($p < 0.01$; Figure 2.1b, "WT" vs. " Δply "). To assess damage to the lung barrier, at 18 h.p.i. we delivered 1 mg of 70 kDa FITC-dextran intravenously into infected mice and, after 30 minutes, measured the fluorescence signal in lung homogenates relative to that of serum. Infection by WT *Sp* increased lung permeability more than two-fold relative to uninfected mice ($p < 0.01$), whereas infection with *Sp* Δply had no effect (Figure 2.1c, "WT" vs. " Δply "). Mirroring the

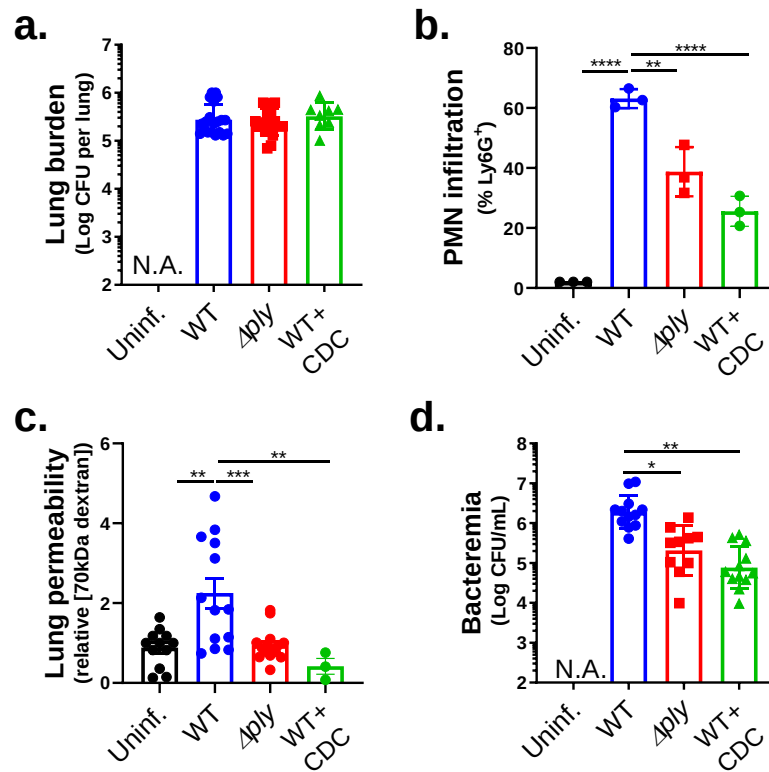


Figure 2.1. The 12-LOX pathway, stimulated by PLY-producing *Sp*, promotes PMN infiltration, lung permeability and bacteremia following *Sp* lung infection in mice. BALB/c mice were infected *i.t.* with 1×10^7 CFU wild type (WT) or PLY-deficient mutant (Δply) TIGR4 *Sp* for 18 h, with or without *i.v.* injection of 8 mg/kg of the 12-LOX inhibitor Ci-Di-Cy. (a) Bacterial lung burden determined by measuring CFU in lung homogenates. (b) PMN infiltration determined by flow cytometric enumeration of Ly6G⁺. (c) Lung permeability quantitated by measuring the concentration of 70 kDa FITC-dextran in the lung relative to serum after *i.v.* administration. (d) Bacteremia measured by enumerating CFU in serum. Each panel is representative of three independent experiments, or pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: * p -value < 0.05 , ** p -value < 0.01 , *** p -value < 0.001 , **** p -value < 0.0001 .

increased lung permeability to FITC-dextran, WT *Sp* infection resulted in a ten-fold higher level of bacteremia compared to *Sp* Δply infection ($p < 0.05$; Figure 2.1d, "WT" vs. " Δply ").

We previously found that inhibition of 12-LOX activity by *i.p* injection of cinnamyl-3,4-dihydroxy- α -cyanocinnamate (Ci-Di-Cy) did not affect *Sp* lung burden but curtailed PMN lung infiltration in C57BL/6 (B6) mice [59]. Here, after infection of BALB/c mice with WT *Sp*, Ci-Di-Cy treatment similarly diminished lung PMN infiltration ($p < 0.0001$) without altering lung burden (Figure 2.1a-b, "WT + CDC"). Lung barrier disruption and *Sp* dissemination also depended on 12-LOX activity because Ci-Di-Cy treatment of *Sp*-infected mice resulted in lower FITC-dextran leakage ($p < 0.01$; Figure 2.1c) and bacteremia at 18 h.p.i. ($p < 0.01$; Figure 2.1d). Therefore, 12-LOX activation by PLY promoted PMN infiltration to the lungs, an event that correlated with increased lung permeability and *Sp* spread to the bloodstream.

2.2.2. The 12-LOX pathway promotes PMN transmigration and epithelial barrier breach upon apical infection of ALI monolayers by PLY-producing *Sp*

Recent advances in airway stem cell biology have allowed for the generation of genetically tractable *in vitro* stem cell-derived epithelial cultures with organized architecture and functional attributes of the airway mucosa, including beating cilia, apical mucus production, and a robust junctional barrier [151]. To identify key steps underlying the promotion of bacteremia by PLY and 12-LOX activation, we modeled interactions between *Sp* and PMNs at the airway epithelium by culturing human airway basal stem cells (BSCs) on 3 μ m pore size Transwell filters. After growth to confluency, media was removed from the apical side of the monolayers, a step that triggers the differentiation of the stem cells to form a monolayer containing the diverse airway epithelial cell types [149], including ciliated cells, mucus-producing goblet cells, and secretory club cells,

found in *bona fide* airway epithelium. We then added 1×10^6 PMNs isolated from human peripheral blood to the basolateral surface of these air-liquid interface (ALI) cultures and assessed their movement to the apical side upon *Sp* infection.

Two hours of apical infection with 1×10^7 *Sp*/Transwell induced robust *PLY*-dependent PMN transmigration across human ALI monolayers, with WT *Sp* triggering two-fold greater migration compared to *Sp* Δply ($p < 0.0001$; Figure 2.2a, "Human ALI"). WT *Sp* infection of monolayers pre-treated with Ci-Di-Cy failed to trigger PMN transmigration (Figure 2.2a, "WT+CDC"), suggesting that PMN transmigration across *Sp*-infected ALI monolayers was dependent on eicosanoid lipid mediators produced by 12-LOX, recapitulating our findings during pulmonary *Sp* challenge in mice.

Given the correlation between PMN infiltration and barrier disruption *in vivo*, we visualized monolayers by fluorescence confocal microscopy. PMNs were distinguished from ALI cells by staining cell nuclei with DAPI and visualizing their F-actin with fluorescent phalloidin. Upon infection with WT *Sp*, PMNs, identified by their multi-lobed nuclei, were found to infiltrate the epithelial monolayers in great numbers. Infection with *Sp* Δply resulted in reduced but detectable PMN infiltration (Figure 2.2b, "Human ALI", yellow arrows). On the other hand, epithelial cells were lost from the Transwell filters post-PMN transmigration. To quantitate epithelial cell loss, we optimized a CellProfiler pipeline to distinguish epithelial cells from PMNs based on the size and shape of their nuclei (see Methods). Quantitation of each cell type indicated that infection with WT *Sp* and concomitant PMN migration triggered a 64% loss in epithelial cells from the monolayer (Figure 2.2b, "WT *Sp*"). This loss was entirely dependent on the presence of PMNs (Supplemental Figure 1.1a-b). It was also partially dependent on *PLY*, because infection with *Sp* Δply resulted in a 41% (and significantly lower) loss of epithelial cells ($p < 0.01$; Figure 2.2b, "*Sp* Δply ").

To quantitate epithelial barrier function, we measured leakage of the basally loaded tracer protein HRP into the apical chamber. A 17-fold increase in HRP flux was observed after PMN transmigration induced by apical infection of ALI monolayers by WT *Sp* ($p < 0.0001$; Figure 2.2c, "Human ALI"). This level of leakage was 1.5-fold higher compared to monolayers that had been pre-treated with Ci-Di-Cy or monolayers that were infected with *Sp Δply* ($p < 0.01$; Figure 2.2c, "Human ALI"). The diminished HRP leakage observed in the latter conditions correlated with a 25- or 9-fold decrease in cross-monolayer bacterial movement ($p < 0.0001$; Figure 2.2d, "Human ALI"). As predicted, disruption to barrier integrity depended entirely on the presence of PMNs (Supplemental Figure 1.1c-d).

We then tested the effect of genetic ablation of 12-LOX by generating ALI monolayers from WT or 12-LOX-deficient *Alox15^{-/-}* mice (Figure 2.2a, "Mouse ALI"). Infection of ALI monolayers from B6 mice with WT *Sp* induced PMN transmigration 7-fold higher than basal (uninfected) levels ($p < 0.0001$) and 2-fold higher ($p < 0.0001$) than that induced by *Sp Δply* (Figure 2.2a, "B6"). In contrast, *Alox15^{-/-}* ALI monolayers failed to trigger significant PMN transmigration during infection by either WT or *PLY*-deficient *Sp* (Figure 2.2a, "*Alox15^{-/-}*").

Confocal microscopy analysis of monolayers after PMN migration revealed that WT infection was associated with a 68% loss of B6 ALI monolayer compared to a 12% loss of *Alox15^{-/-}* ALI monolayers ($p < 0.0001$; Figure 2.2b, "Mouse ALI"). Correspondingly, a 30-fold increase in HRP flux was detected across monolayers infected with WT *Sp* compared to uninfected monolayers ($p < 0.0001$; Figure 2.2c, "Mouse ALI"). This increase in HRP flux was promoted by both *PLY* and 12-LOX, because (a) *Δply Sp* infection of WT B6 monolayers resulted in 2-fold lower flux ($p < 0.05$); and (b) WT *Sp* infection of *Alox15^{-/-}* ALI monolayers resulted in 4-fold lower flux ($p < 0.0001$; Figure 2.2c, "Mouse ALI").

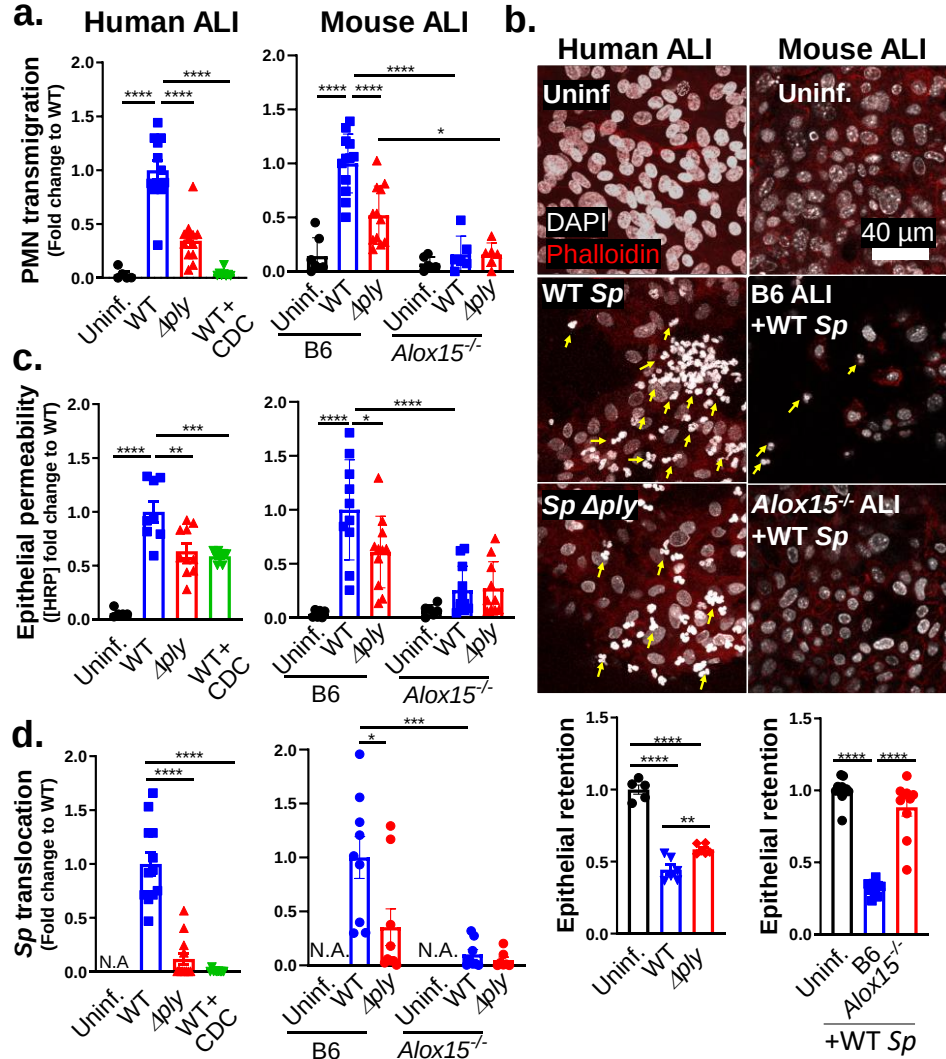


Figure 2.2. The 12-LOX pathway promotes PMN transmigration and epithelial barrier breach upon apical infection of ALI monolayers by PLY-producing *Sp*. Human BSC-derived ALI monolayers (left column) or WT B6 and 12-LOX-deficient *Alox15*^{-/-} mouse BSC-derived ALI monolayers (right column) were apically infected with 1×10^7 WT or Δ ply *Sp* in the presence of basolateral PMNs. (a) After 2 hours of PMN migration, the degree of transmigration as determined by MPO activity in the apical chamber. (b) PMN infiltration and monolayer integrity assessed by fluorescence confocal microscopy after staining nuclei with DAPI and F-actin with fluorescent phalloidin. For clarity, images shown are of extended projections (all z-sections collapsed into 1 plane). Arrows indicate examples of PMN nuclei. Scale bar = 40 μ m for all images. Quantitation of epithelial retention is shown in the graph below the images, performed by enumerating epithelial cell nuclei relative to uninfected ALI in five images per experiment. (c) Epithelial permeability measured by HRP flux relative to monolayers infected with WT *Sp*. (d) *Sp* translocation quantitated by measuring basolateral CFU. Each panel is representative of three independent experiments, or pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001, ****p-value < 0.0001.

The *PLY*- and 12-LOX-dependent barrier disruption correlated with enhanced *Sp* translocation across ALI monolayers, as WT *Sp* translocation across B6 monolayers was 3-fold higher than that of *Sp Δply* ($p < 0.05$) and 10-fold higher than that of WT *Sp* across *Alox15^{-/-}* monolayers ($p < 0.001$; Figure 2.2d, "Mouse ALI"). Notably, although *PLY* has diverse effects on mammalian cells [7, 152], upon infection of 12-LOX-deficient ALI, the presence or absence of *PLY* had no effect on barrier disruption and bacterial translocation. Thus, not only is 12-LOX-dependent PMN transmigration required for barrier breach during *Sp* infection of ALI monolayers, but the critical role of *PLY* in this process is the induction of the 12-LOX pathway.

2.2.3. A soluble factor produced by ALI monolayers via the 12-LOX pathway upon apical *Sp* infection promotes both PMN migration and barrier disruption

Infection of WT but not 12-LOX-deficient ALI monolayers by *Sp* triggered PMN migration and barrier breach (Figure 2.2; "Mouse ALI"; "*Alox15^{-/-}*"). To detect putative soluble factor(s) produced by infected epithelium via the 12-LOX pathway, we first collected apical supernatants from B6 ALI monolayers that had been infected with WT *Sp* (herein referred to as "WT supernatant"), or as controls, infected with *Δply Sp* ("*Δply* supernatant") or left uninfected ("uninfected supernatant"). (We did not include these supernatants of *Alox15^{-/-}* ALI cultures because these monolayers did not support PMN migration under any conditions; Figure 2.2a). Detecting factors that are capable of drawing PMNs across *Sp*-infected ALI monolayers and facilitating bacterial translocation is confounded by the further production of 12-LOX-derived products by infected cells. Hence, we added these supernatants to *Alox15^{-/-}* (not WT B6) ALI monolayers that had been apically infected with WT *Sp*. The addition of WT supernatant triggered PMN transmigration across infected *Alox15^{-/-}* ALI monolayers at a 25- and 2-fold higher level

than that triggered by uninfected supernatant and Δply supernatant, respectively (Figure 2.3a).

To determine if PMN migration in response to a 12-LOX-dependent soluble factor (or factors) disrupted the infected monolayer, we measured cross-epithelial horseradish peroxidase (HRP) leakage. WT supernatant induced 4- and 2-fold more leakage than uninfected supernatant and Δply supernatant, respectively (Figure 2.3b). In turn, HRP leakage correlated with bacterial movement because WT supernatant was associated with 10- and 5-fold higher *Sp* translocation than uninfected and Δply supernatant, respectively (Figure 2.3c). That supernatant of epithelium infected with WT *Sp* was sufficient to rescue PMN migration across *Alox15*^{-/-} ALI monolayers, as well as concomitant barrier disruption and *Sp* translocation, affirmed the presence of a soluble mediator (or mediators) in the epithelial apical supernatant that acts as a PMN chemoattractant and drives barrier breach during *Sp* infection.

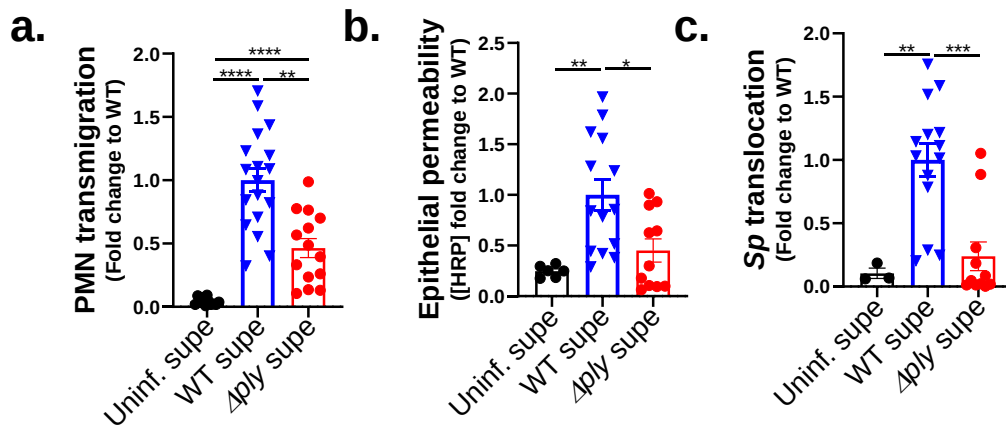


Figure 2.3. A soluble factor produced by ALI monolayers via the 12-LOX pathway upon apical *Sp* infection promotes both PMN migration and barrier disruption. *Alox15*^{-/-} mouse BSC-derived ALI monolayers were apically infected with 1×10^7 WT *Sp* and transferred into apical chambers containing supernatant generated from WT *Sp* infection (WT supe) or Δply infection (Δply supe) of B6 mouse BSC-derived ALI monolayers. (a) After two hours of PMN migration, the degree of transmigration as determined by MPO activity in the apical chamber. (b) Epithelial permeability measured by HRP flux relative to monolayers infected with WT *Sp*. (c) *Sp* translocation quantitated by measuring basolateral CFU. Each panel is representative of three independent experiments, or pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001.

2.2.4. Upon *Sp* infection of ALI monolayers, PMN transmigration induced by HXA₃ but not fMLP promotes barrier breach

The 12-LOX pathway generates a number of bioactive lipids, but based on mucosal infection by several bacterial pathogens [139, 141, 153], hepxilin A3 (HXA₃) is a prime candidate for the 12-LOX-dependent chemoattractant secreted into the apical supernatant by infected B6 ALI monolayers. To test whether HXA₃ is sufficient to trigger PMN transmigration, barrier disruption, and bacterial translocation *in vitro*, we added HXA₃ methyl ester (HXA₃-ME), a stable synthetic form of HXA₃, to the apical chamber of *Alox15*^{-/-} ALI monolayers infected with WT *Sp*, and monitored transmigration of basolateral PMNs. As controls, the well-characterized non-eicosanoid PMN chemoattractant N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP) induced PMN transmigration, whereas the HBSS buffer control did not (Figure 2.4a, "HBSS", "fMLP"). We found that the apical addition of HXA₃-ME induced PMN transmigration equivalent to that triggered by fMLP (Figure 2.4a, "HXA₃"), indicating that HXA₃ is sufficient to induce PMN migration across *Sp*-infected ALI monolayers.

In addition to inducing PMN migration, chemoattractants can alter other PMN functional responses [113], and HXA₃ influences a variety of PMN behaviors [143], such as intracellular calcium release [143], apoptosis inhibition [144], and NETosis [145]. Indeed, despite similar levels of PMN transmigration in response to HXA₃ and fMLP, PMN transmigration induced by fMLP was associated with retention of epithelial monolayer integrity (Figure 2.4b), minimal HRP flux (Figure 2.4c), and the absence of *Sp* transepithelial movement (Figure 2.4d, "fMLP"), whereas that mediated by HXA₃-ME induced loss of 64% of the monolayer, a 4-fold increase in HRP leakage, and a 10-fold increase in *Sp* translocation (Figure 2.4b-d, "HXA₃"). These data indicate that HXA₃ induces a mode of PMN transmigration capable of promoting barrier disruption and bacterial translocation.

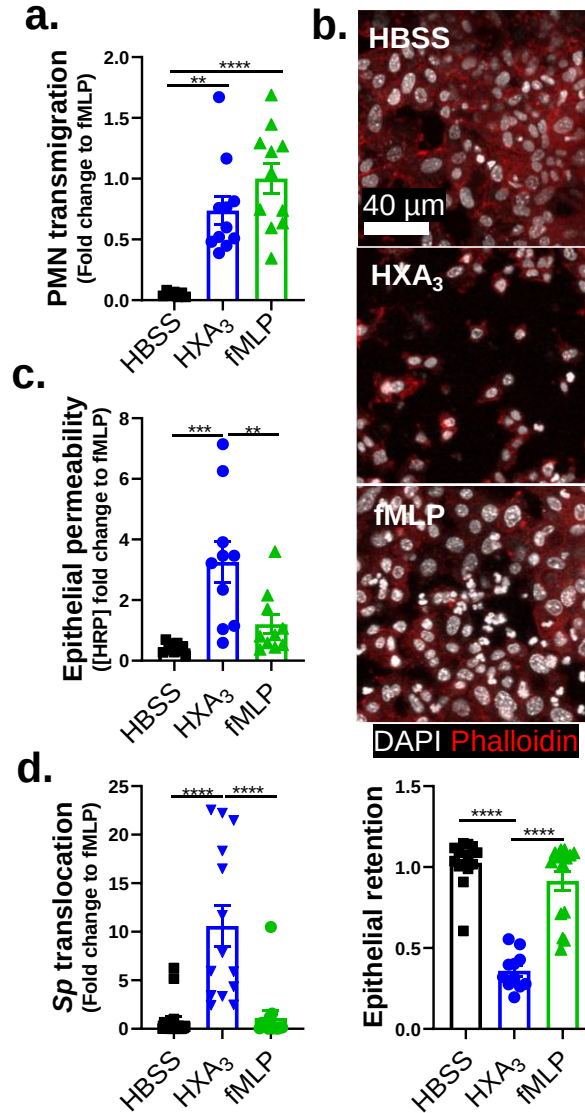


Figure 2.4. Upon *Sp* infection of ALI monolayers, PMN transmigration induced by HXA₃ but not fMLP promotes barrier breach. Alox15^{-/-} mouse BSC-derived ALI monolayers were apically infected with 1×10^7 WT *Sp* and transferred into apical chambers containing 10 nM HXA₃ methyl ester ("HXA₃"), or 10 μM fMLP, in the presence of basolateral PMNs. (a) After two hours of PMN migration, the degree of transmigration as determined by MPO activity in the apical chamber. (b) Monolayer integrity assessed by fluorescence confocal microscopy after staining nuclei with DAPI and F-actin with fluorescent phalloidin. For clarity, images shown are of extended projections (all z-sections collapsed into 1 plane). Scale bar = 40 μm for all images. Shown below the images is epithelial retention quantitated by enumerating epithelial cell nuclei relative to uninfected monolayers in five images per experiment. (c) Epithelial permeability measured by HRP flux relative to monolayers infected with WT *Sp*. (d) *Sp* translocation quantitated by measuring basolateral CFU. Each panel is representative of three independent experiments, or pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001, ****p-value < 0.0001.

2.2.5. HXA₃-stimulated PMNs generate robust NE in response to *Sp* infection

Previous studies show that PMNs respond to purified HXA₃ by resisting apoptosis [144] and generating NETs [145], events that may reinforce PMN inflammatory potential. However, the potentially tissue-damaging state of PMNs is greatly influenced by exposure to microbial pathogens [113]. Hence, to identify key features of PMNs that may lead to monolayer disruption and *Sp* translocation, we compared the effect of HXA₃ and fMLP on various PMN responses in the context of *Sp* infection. To begin this analysis, we first characterized PMN activities in response to *Sp* in the absence of chemoattractant. After 30 min of infection with *Sp*, 94% of PMNs remained viable, i.e., membrane impermeable to propidium iodide (PI; Supplemental Figure 1.2a), and PMNs killed 70% of opsonized *Sp* (Supplemental Figure 1.2b). Infection with *Sp* triggered >7-fold increases in NETosis, PMN apoptosis, and ROS production (Supplemental Figure 1.2c, e-f), and >2-fold increases in MMP and NE release (Supplemental Figure 1.2d, g). (The relative log-fold changes in various activities of infected PMN parameters induced by *Sp* infection are provided in a radar plot; Supplemental Figure 1.2h).

We next profiled the effect of HXA₃ and fMLP on *Sp*-induced responses of infected PMNs. fMLP treatment resulted in a slight increase in membrane-permeant PMNs compared to HBSS or HXA₃ treatment (10% versus 6%; Figure 2.5a). Nevertheless, fMLP- and HXA₃-treated PMNs were equally proficient as HBSS-treated PMNs at opsonophagocytic killing (Figure 2.5b). The presence or absence of fMLP or HXA₃ also did not affect NETosis or MMP secretion by infected PMNs (Figure 2.5c, d). fMLP treatment resulted in slightly higher levels of apoptosis, reflected by surface levels of Annexin V compared to untreated or HXA₃-treated PMNs (Figure 2.5e), a finding consistent with the observation that HXA₃ diminishes PMN apoptosis [144]. Finally,

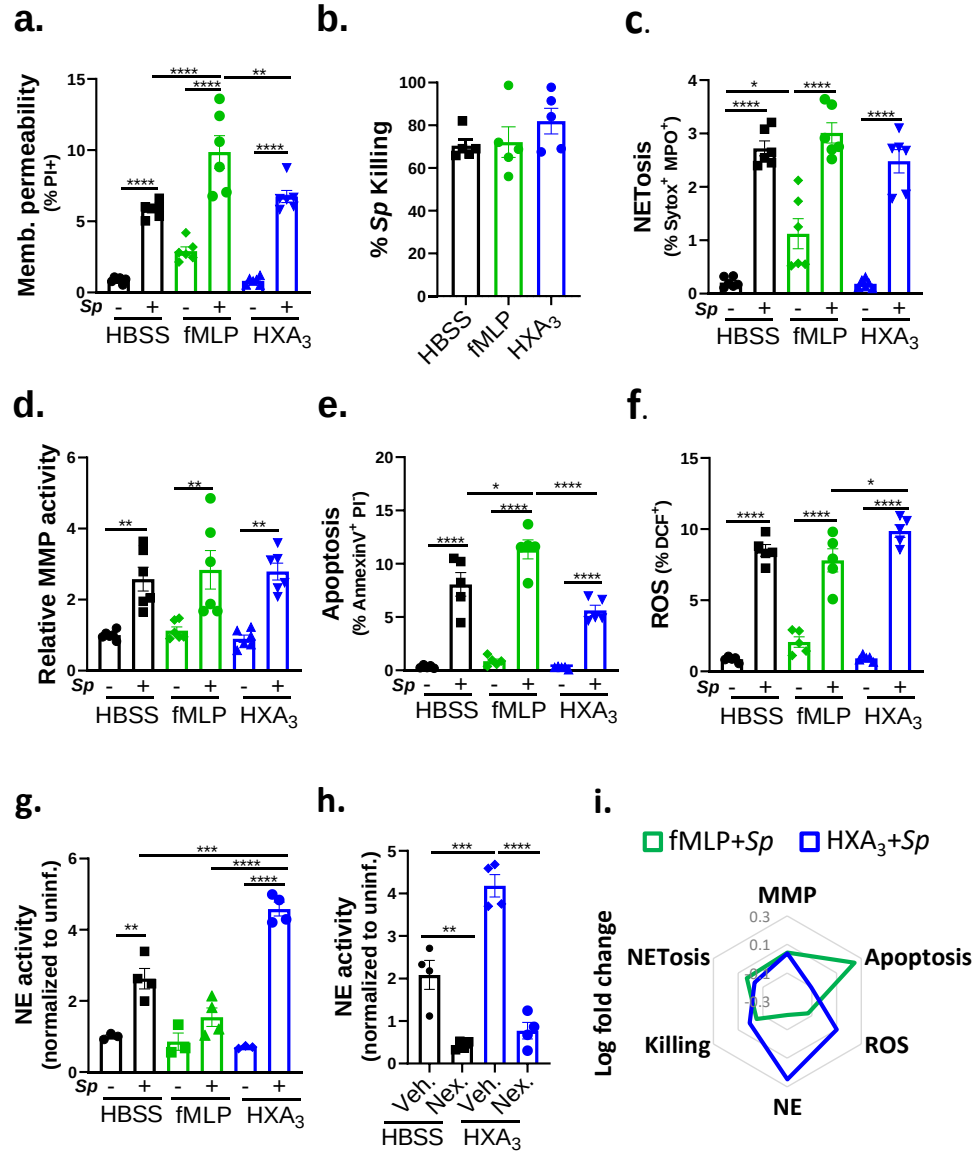


Figure 2.5. HXA₃ enhances NE secretion by *Sp*-infected PMNs. 1×10^6 PMNs were infected with 1×10^7 *Sp* after treatment with control HBSS, 10 μ M fMLP, or 10 nM HXA₃ methyl ester ("HXA₃"), and evaluated for functional performance via (a) PMN membrane permeability determined by propidium iodide staining (PI⁺), (b) opsonophagocytic killing quantitated by plating for CFU, (c) NETosis determined by Sytox and anti-MPO staining (Sytox⁺ MPO⁺), (d) released MMP activity by substrate conversion and expressed relative to uninfected PMNs, (e) apoptosis determined by lack of straining by propidium iodide and positive staining of Annexin V (PI⁺ Annexin V⁺), (f) ROS production by intracellular oxidation of substrate (DCF⁺), and (g) released NE activity by substrate conversion and expressed relative to uninfected PMNs. (h) *Sp*-infected PMNs were treated with HXA₃ methyl ester in the presence or absence of 50 μ M Nexinhib20 (Nex.) and relative NE activity in supernatant quantitated by substrate conversion as in panel g. (i) Radar plot summary of log fold change in PMN activities in (a-g). Each panel shown is representative of three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001, ****p-value < 0.0001.

HXA₃ resulted in slightly higher ROS production than fMLP (10% versus 8%, Figure 2.5f).

The greatest chemoattractant-dependent difference detected in *Sp*-infected PMNs was NE activity. fMLP stimulation appeared to diminish NE activity compared to PBS, although this difference did not reach statistical significance (Figure 2.5g). In contrast, HXA₃ resulted in an almost 2-fold increase relative to the control ($P < 0.001$). NE has been implicated in severe lung injury during *Sp* infection [69, 89] and is delivered by PMNs largely through the release of exosomes and primary granules [154]. The increase in HXA₃-triggered NE activity was eliminated by Nexinhib20, which blocks NE release by both exosomes and primary granules [155] (Figure 2.5h, "Nex"). The relative changes in various activities of infected PMN parameters induced by fMLP or HXA₃ are summarized in Figure 2.5i.

2.2.6. PLY-producing *Sp* promotes release of NE and primary granules in a 12-LOX-dependent manner during experimental lung infection

Given the enhanced NE release by HXA₃-stimulated, *Sp*-infected PMNs *in vitro*, we assessed *in vivo* degranulation of primary granules, a major mechanism of NE release [154] of PMNs. 18 hours after i.t. infection of BALB/c mice with *Sp*, we measured the relative level of the primary granule marker CD63 on the surface of pulmonary PMNs. CD63 surface expression was 3.5-fold higher on PMNs from lungs of mice infected with WT *Sp* compared to uninfected mice (Figure 2.6a, "Uninf." vs. "WT"). This elevated level was reduced by 25% during infection with *Sp* Δply ($P < 0.05$; Figure 2.6a, " Δply "), suggesting that PLY-induced HXA₃ production contributed significantly to degranulation. Consistent with this, inhibition of 12-LOX with Ci-Di-Cy after infection with WT *Sp* infection resulted in a similar decrease in PMN CD63 surface expression (Figure 2.6a, "CDC").

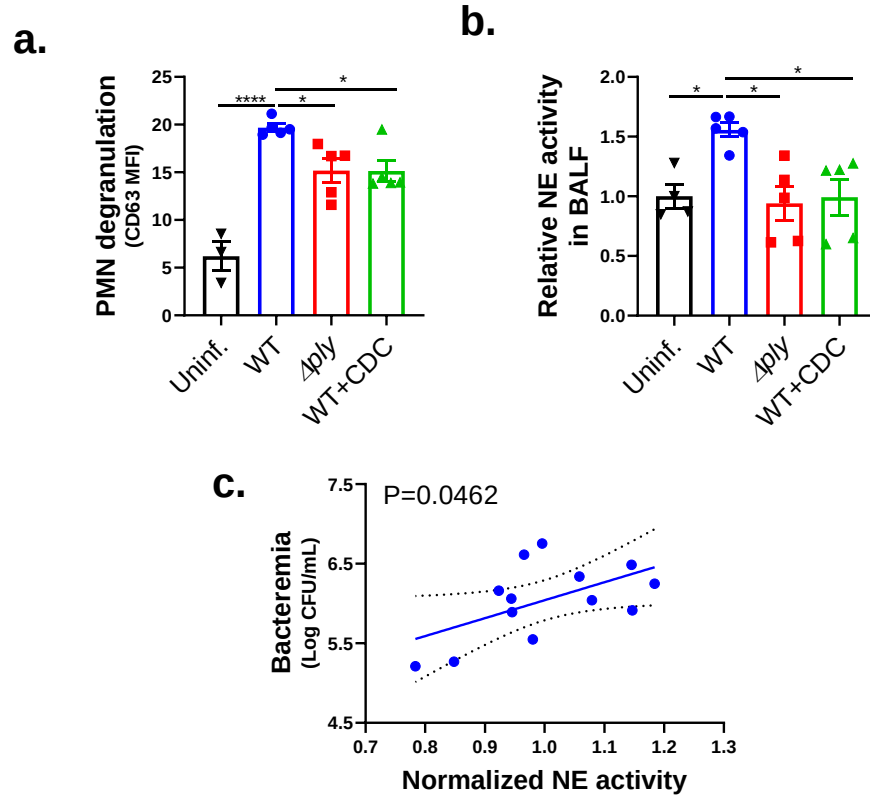


Figure 2.6. PLY-producing *Sp* promotes release of NE and primary granules in a 12-LOX-dependent manner during experimental lung infection. BALB/c mice were infected *i.t.* with 1×10^7 CFU WT or Δply *Sp* for 18 h, with or without *i.p* injection of 8 mg/kg of the 12-LOX inhibitor Ci-Di-Cy. (a) FACS analysis of degranulation determined by CD63 expression on Ly6G⁺ lung infiltrating PMNs. (b) NE activity in cell-free BALF determined by substrate conversion, expressed relative to the NE activity in cell-free BALF from uninfected mice. (c) Correlation between normalized NE activity in (a) and bacteremia determined by enumerating CFU in serum. Each panel shown is representative of three independent experiments, or pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value < 0.05, ***p-value < 0.001, ****p-value < 0.0001.

To determine if PMN degranulation corresponded to increased pulmonary NE levels, bronchial alveolar lavage fluid (BALF) of BALB/c mice at 18 h.p.i. was centrifuged to remove PMNs and other cells, and then assessed for NE activity. Activity was 50% higher in WT *Sp*-infected mice compared to uninfected mice or mice infected with *Sp* Δply (Figure 2.6b, " Δply "), a finding consistent with previous reports (7). Ci-Di-Cy treatment of infected mice, which dramatically decreases PMN lung infiltration [136], reduced BALF NE to levels indistinguishable from that of uninfected mice (Figure 2.6b,

"CDC"). In mice infected with WT *Sp*, BALF NE activity significantly correlated with bacteremia (Figure 2.6c). These findings suggest that PLY-triggered HXA₃ promotes lung-infiltrating PMNs to release NE during pulmonary *Sp* challenge, thus enhancing bacteremia.

2.2.7. Inhibition of NE release mitigates disruption of the lung epithelial barrier and bacteremia following *Sp* lung infection

NE degrades epithelial cell junctions and extracellular matrices *in vitro* [98, 156] and has been implicated in the pathogenesis of several human disorders that involve inflammatory damage [154]. To determine if inhibition of PMN degranulation or NE activity protects lung barrier function during *Sp* infection in mice, we delivered the degranulation inhibitor Nexinhib20 or the NE inhibitor Sivelestat [129] by intraperitoneal (*i.p.*) injection to BALB/c mice (see Methods), followed by *Sp* lung challenge. Neither inhibitor altered bacterial lung burden or PMN infiltration at 18 h.p.i (Figure 2.7a-b). Nexinhib20 significantly diminished PMN degranulation, measured by surface CD63, compared to vehicle-treated mice (Figure 2.7c); Sivelestat did not achieve significant effect. Notably, both Nexinhib20 and Sivelestat prevented an increase in the NE activity of BALF (Figure 2.7d).

The decrease in PMN degranulation associated with Nexinhib20 significantly protected the lung epithelial barrier, reducing epithelial barrier permeability to intravenous 70 kDa FITC dextran by 55% (Figure 2.7e, "Nex."); Sivelestat treatment exhibited a similar trend, reducing permeability by 25% (Figure 2.7e, "Siv."). Importantly, both inhibitors diminished bacteremia significantly by >10-fold (Figure 2.7f). These data suggest that NE release by HXA₃-activated lung infiltrating PMNs contributes to barrier disruption.

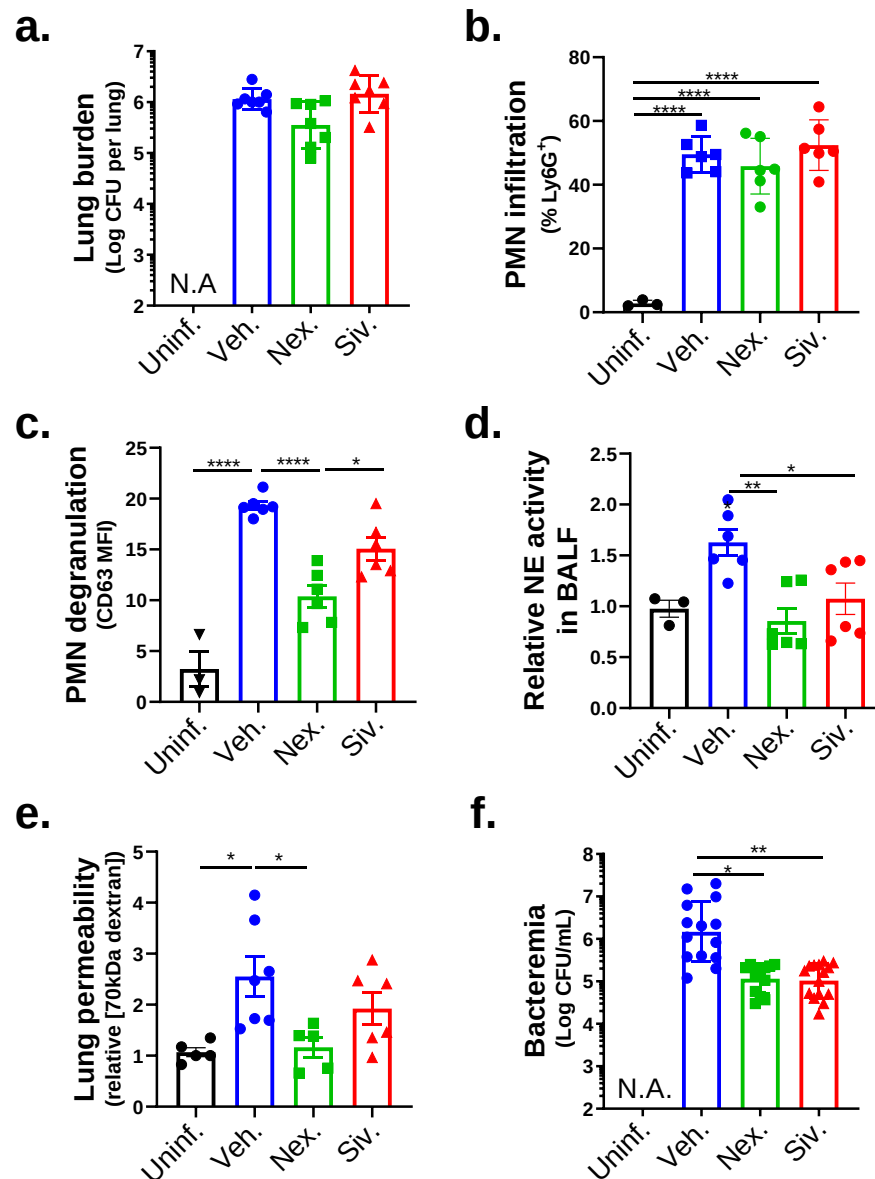


Figure 2.7. Inhibition of NE release mitigates disruption of the lung epithelial barrier and bacteremia following *Sp* lung infection. BALB/c mice were infected *i.t.* with 1×10^7 CFU WT *Sp* for 18 h, with or without i.p injection of 30 mg/kg Nexinhib20 (Nex) or 30 mg/kg Sivelestat (Siv) one hour prior to infection. (a) Bacterial lung burden determined by measuring CFU in lung homogenates; (b) PMN infiltration determined by flow cytometric enumeration of Ly6G⁺; (c) Degranulation determined by CD63 expression on Ly6G⁺ lung infiltrating PMNs by FACS; (d) Relative NE activity in BALF determined by substrate conversion; (e) Lung permeability determined by measuring the concentration of 70 kD FITC-dextran in lung relative to serum after *i.v.* administration; and (f) Bacteremia determined by enumerating CFU in serum. Each panel is representative of three independent experiments, or pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value < 0.05, **p-value < 0.01, ****p-value < 0.0001.

2.3. Discussion

Lung infections by viral and bacterial pathogens, especially multi-drug resistant bacteria, remain a major cause of death and require searches for therapies that target infection-associated pathogenic host processes [120, 121]. Pulmonary infiltration by PMNs can drive lung damage and concomitant transepithelial movement of pathogens, including *Sp* [81, 157, 158], leading to life-threatening systemic infection. Indeed, transepithelial migration of PMNs in response to activation of the 12-LOX pathway disrupts cultured epithelial monolayers [136] and promotes lethal bacteremia in a mouse *Sp* lung challenge model [59]. However, PMNs are also key immune cells critical for early defense against *Sp* infection [159]. Hence, efficacious host-directed therapies to combat *Sp* spread must selectively target PMN effector mechanisms that promote barrier disruption while leaving intact activities required for pathogen control. Identification of the critical pathologic activities of PMNs during *Sp* infection of the lung requires model systems that faithfully reflect key features of PMN-*Sp* interactions at the respiratory mucosa.

The bronchial BSC-derived ALI epithelial model recapitulates important aspects of the architecture of *bona fide* airway epithelium, including the diversity of cell types and the formation of mature apical junction complexes that facilitate a functional mucosal barrier [151, 160]. Here, we show that *Sp* infection of human and murine BSC-derived ALI monolayers mirror essential features of epithelial barrier breach following pulmonary *Sp* challenge in mice [11, 59], including the requirement for PMN transmigration that is entirely dependent on 12-LOX and partially dependent on PLY [136]. PLY does not trigger detectable PMN transmigration or concomitant bacterial translocation after genetic ablation of 12-LOX pathway, suggesting that PLY-triggered pro-inflammatory and barrier disrupting activity in the lung is entirely due its ability to stimulate this

pathway. That a *PLY*-deficient *Sp* was still capable of triggering 12-LOX-dependent PMN migration across ALI monolayers, albeit at lower than wild-type levels (Figure 2.2), is consistent with previous work indicating that *Sp* is also capable of stimulating PMN transmigration via *PLY*-independent means [136].

Chemotactic cues can have remarkably diverse effects on PMNs, including altering effector functions, antimicrobial activity, and inflammatory potential [133, 161]. For example, in models of sterile lung injury, infiltrating PMNs are apoptotic and produce tissue-repair molecules such as TGF- β , VEGF, and resolvins [162-164]. Conversely, in cystic fibrosis (CF) models, PMNs undergo transcriptional changes that reduce bactericidal activity and enhance tissue-damaging degranulation [147, 165]. Similarly, PMNs that migrate into COVID-19-infected airways display a hyperinflammatory phenotype that drives lung pathology [148]. Here, we show that chemotactic cues ultimately lead to divergent infection outcomes in *Sp* infection of ALI monolayers. Based on analogy to mucosal infection by other pathogens [139-141], HXA₃ was previously deemed likely to be the 12-LOX-dependent PMN chemoattractant driving acute inflammation during *Sp* infection [59, 80]. Here, the experimental utility of 12-LOX-deficient ALI monolayers permitted the definitive identification of HXA₃ as indeed being sufficient to induce PMN transmigration and mucosal barrier disruption triggered by *Sp* infection. In turn, this finding was essential to permit a direct comparison of *Sp*-driven chemotaxis with that triggered by a well-studied control chemoattractant, fMLP [133, 166], revealing that HXA₃-promoted specific pro-inflammatory conditioning of PMNs is critical for epithelial monolayer destruction.

The identification of HXA₃ as sufficient for PMN-mediated mucosal barrier breach during infection by *Sp* prompted an exploration of pathologically important HXA₃ responses. Changes in PMN physiology upon stimulation by purified HXA₃ include increased calcium flux, NETosis, and anti-apoptotic programs [139, 144, 145], but here

we investigated HXA₃ response in the context of *Sp* infection. By far the largest difference upon *ex vivo* treatment of *Sp*-infected PMNs with HXA₃ compared to fMLP was a 4-fold higher level of NE activity (Figure 2.5). HXA₃ alone does not enhance PMN NE activity (Figure 2.5), indicating that this response requires co-stimulation by both bacteria and chemoattractant and emphasizing the importance of including microbial agents in studies of PMN responses to infection-triggered chemotactic agents. Moreover, pulmonary PMNs from mice challenged *i.t.* with *Sp* exhibited PLV- and 12-LOX-promoted elevation of degranulation, a means to release NE, as well as elevated NE levels in BALF, indicating that HXA₃ triggered NE release during mouse lung infection as well (Figure 2.6).

Disease manifestation in response to pathogens can be mitigated either by effective actions of the host immune system to clear the microbe or by control of infection-triggered immune responses that are detrimental to the host [167]. NE, along with other serine proteases, contributes to *Sp* killing by PMNs *ex vivo* [87]. However, we found that inhibition of NE activity during mouse lung infection by pretreatment with the NE inhibitor Sivelestat did not affect bacterial lung burden (Figure 2.7), nor did it alter PMN lung infiltration. Rather, inhibition of NE, which degrades extracellular matrix components [168] and alveolar epithelial cell junction proteins [91] that maintain epithelial integrity [169], decreased bacteremia by more than 90%. These findings indicate that, in the mouse model, the pathological activities of NE outweigh any beneficial role in direct pathogen killing [87, 125].

Nexinhib20 blocks formation of exosomes and degranulation of primary granules [155], the two means by which NE is released from PMNs. During mouse infection, this inhibitor diminished degranulation of lung PMNs as well as NE activity in BALF. Although Nexinhib20 diminishes surface localization of adhesion molecules and can limit PMN recruitment to sites of tissue damage [170], we found that this inhibitor did not alter PMN

infiltration into the lung post-*Sp* challenge. Nexinhib20 has been shown to ameliorate PMN-directed tissue damage in models of myocardial ischemia-reperfusion [170] and pulmonary LPS-induced injury [171]. Here we demonstrated the ability of the drug to mitigate injury during microbial infection. Treatment with Nexinhib20, like treatment with Sivelestat, did not alter pulmonary bacterial load (Figure 2.7), yet bacteremia was decreased >10-fold, corresponding to protection of pulmonary barrier function measured by leakage of 70 kDa dextran (Figure 2.7). Primary granules contain numerous proteases that may have diverse physiologically activities [172, 173], such as the activation or inactivation of cytokines and other biologically active host factors [174, 175], that may impact the course of *Sp* infection, so further characterization of the effects of Sivelestat and Nexinhib20 *in vivo* is required to garner a full understanding of how they diminish bacteremia.

NE has been implicated in the pathogenesis of several human disorders that involve inflammatory damage, including CF, chronic obstructive pulmonary disease, bronchopulmonary dysplasia, and acute respiratory distress syndrome (ARDS) [154]. The pathogenic role of NE activity during *Sp* infection of the mouse lung revealed here is likely reflected in human infection because higher NE levels in BALF and serum of patients with bacterial pneumonia is associated with worse clinical outcomes [96, 97, 176]. Sivelestat is clinically approved for the treatment of ARDS in Korea and Japan [177] and for COVID-19 induced ARDS in China [178]. Although studies of efficacy in patients have yielded inconsistent results [154, 179-181], ongoing efforts to improve delivery, e.g., by nebulizer, have yielded favorable results in improving efficacy and limiting adverse effects [182]. Similarly, intrapulmonary delivery of Nexinhib20-loaded nanoparticles in experimental animals increases drug availability and decreases LPS-induced acute lung injury [171]. Future studies are required to determine the efficacy of NE inhibition in limiting *Sp* systemic disease.

Finally, HXA₃ production is a conserved mucosal inflammatory response in a multitude of bacterial infections, and possibly in acute lung injury, asthma, and inflammatory bowel syndrome [132, 138, 139, 141, 183]. Given the prominent role of PMNs in mediating tissue damage, targeted mitigation of HXA₃-triggered changes in PMNs that promote damage but do not compromise host defense has potential efficacy for a broad range of disorders. The identification of such changes, such as excessive NE release, is an important step in developing such strategies.

2.4. Materials and Methods

2.4.1. Bacterial strains and growth conditions

Mid-exponential growth phase aliquots of *S. pneumoniae* TIGR4 (serotype 4) were grown in Todd-Hewitt broth (BD Biosciences) supplemented with 0.5% yeast extract in 5% CO₂ and Oxyrase (Oxyrase, Mansfield, OH), and frozen in growth media with 20% (v/v) glycerol. Bacterial titers in aliquots were confirmed by plating serial dilutions on Tryptic Soy Agar plates supplemented with 5% sheep blood (blood agar) (Northeast Laboratory Services, Winslow, ME). The TIGR4 PLY-deficient mutant (Δply) was a gift from Dr. Andrew Camilli (Tufts University School of Medicine, MA). For experiments, *S. pneumoniae* strains were grown in Todd-Hewitt broth, supplemented with 0.5% yeast extract and Oxyrase, in 5% CO₂ at 37°C and used at mid-log to late log phase.

2.4.2. Murine infections

BALB/c mice, C57BL/6J mice, and *Alox15* knockout (*Alox15*^{-/-}) mice (B6.129S2-*Alox15*^{tm1Fun/J}) were obtained from Jackson Laboratories. All animal experiments were performed in accordance with Tufts University Animal Care and Use Committee approved protocols. BALB/c mice were intratracheally challenged with 1×10⁷ colony

forming units (CFU) of *S. pneumoniae* in 50 µl phosphate-buffered saline (PBS) to induce pneumococcal pneumonia. Control mice received PBS. The role of 12-LOX on *S. pneumoniae*-induced inflammation in BALB/c mice was investigated by inhibiting this enzyme with cinnamyl-3,4-dihydroxy- α -cyanocinnamate (Ci-Di-Cy) at 8 mg/kg, in 3% DMSO, 3% cremaphor EL (CrEL) in PBS as the vehicle. Ci-Di-Cy was injected intraperitoneally (*i.p.*) twice daily, starting one day before infection. The role of NE on *S. pneumoniae*-induced inflammation was studied in BALB/c mice by treatment with Nexinhib20, which blocks release of primary granules [155] or Sivelestate, which inhibits this enzyme [129, 184]. A single dose of Nexinhib20 at 30 mg/kg, in 3% DMSO, 3% CrEL in PBS, or Sivelestat at 30 mg/kg in PBS was injected *i.p.* 1 hour prior to infection. Mice were euthanized at 18 h.p.i.. Blood was obtained by cardiac puncture. Bronchoalveolar lavage fluid (BALF) was collected by washing the lungs twice with 1 ml PBS via a cannula, then whole lungs were removed and bacterial burden enumerated by plating lung homogenate on blood agar plates.

2.4.3. Assessing lung barrier function

For assessment of lung permeability, mice were intravenously injected with 70 kDa MW FITC-Dextran at 5 mg/kg 30 minutes prior to euthanasia. Whole lungs were isolated and homogenized in 1 ml PBS, which was then subjected to fluorescence quantitation using a Synergy H1 plate reader (BioTek). Readouts were normalized to fluorescence in the serum of the same animal, diluted 1:10 in PBS.

2.4.4. Measuring PMN infiltration and degranulation

For flow cytometric quantitation of lung PMNs, mice were euthanized at 18 h.p.i. and lung tissues were digested with 1 mg/ml Type II collagenase (Worthington) and 50 U/ml Dnase (Worthington) to obtain a single-cell suspension. Cells present in the

suspension were stained on ice for 30 minutes with APC-conjugated anti-Ly-6G (clone 1A8) or PE-conjugated anti-CD63 (Biolegend) and then washed two times in FACS buffer (Biolegend). Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and the fluorescence intensities of the stained cells were determined. Collected data were analyzed using FlowJo software (v10.7, BD) to determine the numbers of infiltrating (Ly6G⁺) PMNs, and their level of degranulation (mean fluorescence intensity of CD63).

2.4.5. Establishment of epithelial air-liquid interface monolayers

Human bronchial basal cells were isolated and expanded from lung tissue harvested from donors without lung disease through the New England Organ Bank under an IRB-approved protocol (MGH #2010P001354). In brief, using a previously published basal cell isolation protocol [68, 151], EpCAM⁺ epithelial basal cells were isolated from human trachea and mainstem bronchi tissue. Mouse airway basal cells were obtained from C57BL/6J (B6) or *Alox15*^{-/-} mouse trachea.

Harvested basal cells were cultured in complete small airway epithelial growth media (SAGM) (Lonza, Cat. CC-3118), with propagation for up to 10 passages, using the dual SMAD inhibition protocol [151]. To generate monolayers permissive to modeling PMN transmigration [160], Transwells with permeable (3 μ m pore size) polycarbonate membrane inserts and a culture area of 0.33 cm² (Corning product #3415) were collagen coated and seeded with 80 μ l of the airway basal cells suspension (containing > 200,000 cells) in SAGM, resulting in a density of >6000 cells/mm², and submerged in complete SAGM for airway basal cell recovery and expansion for 1–2 days to ensure monolayer confluence. The media in both chambers was then replaced with complete Pneumacult-ALI medium (StemCell Technology, Cat. 05001) for an additional day. To initiate air-liquid interface, ALI medium in the chamber contacting the cell apical surface was

removed (designated as day 0). Media was changed every 1-2 days during differentiation.

ALI monolayers used in experiments were cultured for at least 21 days to allow for full maturation of both cilia and goblet cells, but no more than 34 days to avoid overgrowth or loss of epithelial barrier [149]. Transepithelial electrical resistance was assessed using a voltmeter (EVOM2, Epithelial Voltohmmeter, World Precision Instruments, Inc.) prior to migration assays to ensure the establishment of a polarized epithelial barrier.

2.4.6. Infection of ALI monolayers

Epithelial cell supernatants were generated from B6 ALI monolayers infected with 1×10^7 WT or Δply *S. pneumoniae* for 1 hour at 37°C with 5% CO₂, and then placed in 24-well receiving plates containing HBSS with Ca²⁺ and Mg²⁺ in the apical chamber for an additional 2 hours to allow for HXA₃ generation. The apical chamber supernatants were then collected and transferred to new Transwells with ALI monolayers for PMN transmigration assays.

2.4.7. Production of cell supernatants containing HXA₃

Epithelial cell supernatants were generated from B6 ALI monolayers infected with 1×10^7 WT or Δply *S. pneumoniae* for 1 hour at 37°C with 5% CO₂, and then placed in 24-well receiving plates containing HBSS with Ca²⁺ and Mg²⁺ in the apical chamber for an additional 2 hours to allow for HXA₃ generation. The apical chamber supernatants were then collected and transferred to new Transwells with ALI monolayers for PMN transmigration assays.

2.4.8. PMN transepithelial migration assay

Whole blood obtained from healthy human volunteers under an IRB-approved protocol (Tufts University protocol #10489) was used to isolate neutrophils using the Easysep direct human neutrophil isolation kit (Stemcell), and 1×10^6 PMNs were added to the basolateral chamber after two hours of apical infection of the ALI monolayers with *S. pneumoniae*. Purified HXA₃ methyl ester (Cayman) at 10 nM and fMLP (Sigma) at 10 μ M were supplemented apically as indicated. To test the effect of HXA₃-containing cell supernatants, the apical media was replaced with cell supernatants prepared as described above.

After two hours of transmigration, PMNs in the apical chamber were quantified by MPO activity assay, as described [136]. Briefly, 50 μ l of 10% Triton X-100 and 50 μ l of 1 M citrate buffer were added to lyse transmigrated PMN, and 100 μ l of lysed PMNs from each well was transferred to a 96-well plate. 100 μ l of freshly prepared 2,2'-azinobis-3-ethylbenzotiazoline-6-sulfonic acid (ABTS) with hydrogen peroxide solution was added to each well and incubated in the dark at room temperature for 5-10 minutes. Absorbance at a wavelength of 405 nm was read on a microplate reader and measurement was converted to neutrophil number using a standard curve.

2.4.9. Fluorescence microscopy assessment of ALI monolayer integrity

At the end of the two hours of infection, followed by two hours of PMN transmigration across ALI monolayers, the degree of cell confluency of ALI monolayers on Transwell filters was assessed by fluorescence microscopy. To prepare samples for fluorescence microscopy, 4% paraformaldehyde fixed ALI monolayers were permeabilized with 0.1% Triton-X 100 in PBS with 3% BSA. ALI monolayers were then stained with DAPI (for nuclei) and Alexa Fluor 594 phalloidin (for F-actin), and visualized on excised filters with a Leica SP8 spectral confocal microscope (Leica). Epithelial cell retention on filters was quantitated by counting of DAPI-stained epithelial cell nuclei per

field of view, carried out with CellProfiler pipeline optimized with size and roundness exclusion criteria for epithelial cell nuclei identification. Counts were normalized to uninfected controls

2.4.10. Neutrophil elastase and metalloprotease activity

NE activity and MMP activity in soluble fraction of BALF from infected mice or PMN supernatants from 1×10^6 PMNs challenged with 1×10^7 CFU *S. pneumoniae* was determined using a PMN Elastase Fluorometric Activity Assay Kit (Abcam) and Fluorogenic MMP Substrate (Mca-PLAQAV-Dpa-RSSSR-NH₂) (R&D Systems), respectively, following manufacturer's instructions. The area under the curve of kinetic substrate conversion curves over two hours was measured with a Synergy H1 plate reader (BioTek) and normalized to uninfected controls.

2.4.11. Opsonophagocytic (OPH) killing

The ability of neutrophils to kill pneumococci was assessed *ex vivo* as described previously [36], with modification. Briefly, 1×10^6 PMNs were incubated with 5×10^3 *S. pneumoniae* grown to mid-log phase and pre-opsonized with 10 μ l rabbit complement (Pel-Freez) in 100 μ l reactions in HBSS with Ca²⁺ and Mg²⁺. The reactions were incubated for 45 minutes at 37°C. Percent killing in comparison to incubations with no PMNs was determined by plating serial dilutions on blood agar plates.

2.4.12. Reactive oxygen species (ROS) production

Neutrophils were resuspended in HBSS with Ca²⁺ and Mg²⁺ containing 10 μ M 2',7'-dichlorodihydrofluorescein diacetate (DCF) (Molecular Probes) to a final concentration of 1×10^7 cells/ml and gently agitated for 10 minutes at room temperature. 1×10^6 DCF-containing neutrophils were challenged with *Sp* at an MOI of 10 for 30

minutes at 37°C, then washed and resuspended in FACS buffer for analysis by a FACSCalibur flow cytometer (BD Biosciences). Collected data were analyzed using FlowJo software (v10.7, BD) to determine the numbers of ROS-producing DCF-positive cells.

2.4.13. Neutrophil extracellular trap formation (NETosis) and apoptosis by flow

1×10^6 neutrophils were challenged with *Sp* at an MOI of 10 for 30 minutes at 37°C, then washed and resuspended in FACS buffer. For NETosis analysis, cells were stained with a plasma membrane-impermeable DNA-binding dye, SYTOX™ AADvanced™ (Life Technologies, Carlsbad, CA), rabbit anti-myeloperoxidase (Abcam ab45977), and secondary goat anti-rabbit-Alexa Fluor 568 antibody (Invitrogen). For apoptosis analysis, cells were stained with FITC-conjugated Annexin V (BioLegend, San Diego, CA, USA), and propidium iodide (PI). Samples were read on a FACSCalibur flow cytometer (BD Biosciences), and collected data were analyzed using FlowJo software (v10.7, BD) to determine percent NETosis (MPO⁺ Sytox⁺), and percent apoptosis (Annexin⁺ PI⁺).

2.4.14. Presentation of data and statistical analyses

Statistical and correlation analysis was carried out using GraphPad Prism (GraphPad Software, San Diego, CA). *p* values <0.05 were considered significant in all cases. For bacterial burdens, geometric mean $\pm$ geometric SD is shown; for all other graphs, the mean values $\pm$ SEM are shown. Due to intrinsic donor-to-donor variability of human PMN transmigration efficacy, experiments involving human donors were normalized within each experiment before pooling individual experiments. The conclusions drawn were those found to be reproducible and statistically significant across independent experiments.

2.5. Contributions

S.X and J.L conceived the hypothesis and designed the experiments. S.X performed the experiments and analyses for Figures 2.1 to 2.3, and Figures 2.5 to 2.7. S.X and P.R performed experiments and analyses for Figure 2.4. J.L.R, Y.Z, J.V and H.M developed the BSC-derived ALI system and provided reagents used in Figures 2.2-2.4. M.M and J.M developed the PMN functional profiling assays used in Figure 2.5. S.X generated figures and wrote the manuscript with assistance from J.L. J.V, J.M and S.T assisted with manuscript editing.

Chapter 3: Pore-forming activity of *S. pneumoniae* pneumolysin disrupts the paracellular localization of the epithelial adherens junction protein E-cadherin³

³ Xu, S.; Mo, D.; Rizvi, F. Z.; Rosa, J. P.; Ruiz, J.; Tan, S.; Tweten, R. K.; Leong, J. M.; Adams, W. Pore-forming activity of *S. pneumoniae* pneumolysin disrupts the paracellular localization of the epithelial adherens junction protein E-cadherin. *Infect Immun* 2023, 91 (9), e0021323. DOI: 10.1128/iai.00213-23. Published.
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3. Pore-forming activity of *S. pneumoniae* pneumolysin disrupts the paracellular localization of the epithelial adherens junction protein E-cadherin

3.1. Introduction

Streptococcus pneumoniae (the pneumococcus, or SPN) is the most frequently isolated causative pathogen of pneumonia, with over 150,000 hospitalizations annually in the U.S. alone and a mortality rate of 5-7% [110]. An ominous development in the course of pneumococcal disease is the translocation of bacteria from the lung into the bloodstream. Indeed, the case fatality rate of pneumococcal disease increases to 15-20% upon the development of bacteremia [118]. The respiratory epithelium, like other mucosal surfaces, serves as a first line of defense against invading pathogens by maintaining a physical barrier comprised of tight junction (TJ) and adherens junction (AJ) multiprotein complexes. [185]. TJ proteins, such as scaffolding proteins zonula occludens (ZO) and transmembrane proteins claudins, occludins, and junctional adhesion molecules (JAM), localize to the apical region of cell-cell junctions [186] where they form an intercellular membrane fence [187]. AJ proteins, such as E-cadherin and catenins, are located basolateral to TJs and are essential for the formation and maturation of cell-cell contacts [188]. Orchestration of junction protein abundance, localization, coupling to the cytoskeleton, and interaction with multiple cellular signaling pathways are all essential for effective epithelial barrier function [185].

Models for pneumonia and for other pulmonary diseases such as acute lung injury, asthma, and acute respiratory distress syndrome feature loss of pulmonary epithelial integrity and concomitant deleterious outcome [189, 190]. Indeed, *S. pneumoniae* infection reduces epithelial junction organization in explanted human lung tissue [186], and murine models suggest that *S. pneumoniae* modulates airway epithelial junctions, influencing the degree of paracellular bacterial migration [157, 191]. *S. pneumoniae* pneumolysin (PLY), a major *S. pneumoniae* virulence factor that belongs to

the family of cholesterol-dependent cytolysins (CDCs) that form ~25 nm diameter pores in mammalian cell membranes, may play a critical role in this process. Pore formation by CDCs activates cytoskeletal rearrangement [73] and calcium flux [192], two processes that can lead to the breakdown of cellular junctions. Two separate studies have implicated PLY in damaging essential TJs [12, 74], such as ZO-1. In addition, other *S. pneumoniae* factors such as TLR stimulants [157] and pneumococcal neuraminidase [193] have been reported to downregulate tight junction components.

In addition to directly disrupting epithelial integrity, PLY is a potent activator of inflammation and massive influx of neutrophils (polymorphonuclear leukocytes, or PMNs) into airspaces [117, 194], a hallmark of *S. pneumoniae* lung infection. PLY triggers the production of neutrophil chemoattractants [195, 196], including epithelial production of the eicosanoid hepoxilin A₃ (HXA₃), resulting in PMN transepithelial migration [59, 136]. Intranasal and intratracheal challenge of mice with *S. pneumoniae* has demonstrated an important role for PMNs in the clearance of pneumococci early in the infection [44, 194], in part reflecting their diverse range of anti-pneumococcal effector mechanisms such as the production of reactive oxygen species (ROS), proteases and antimicrobial peptides, and neutrophil extracellular traps [194, 197-199]. However, these same effector molecules can damage intercellular junctions and increase epithelial permeability [91, 200, 201], with prolonged inflammatory PMN responses leading to epithelial barrier disruption, pulmonary edema, and significant lung damage [126, 127]. In some mouse models, retention of high numbers of pulmonary PMNs after the initial acute infection stage is associated with bacteremia and lethality, indicating that inappropriate PMN responses exacerbate pneumococcal disease [44, 59]. For example, upon pneumococcal lung challenge in mice, diminishing PMN pulmonary infiltration by blocking HXA₃ production effectively decreased bacteremia and promoted 100% survival of an otherwise uniformly fatal infection [59, 80].

The impact of these diverse PLY functions is reflected in the observation that in several pulmonary infection mouse models, PLY is required for maximal inflammation, tissue damage, and bacteremia [13, 67, 202]. Although the majority of the known effects of PLY on host cells is linked to PLY's pore-forming activity [7], PLY interacts with non-cholesterol receptors such as the mannose receptor C type 1 (MRC-1) on macrophages and dendritic cells [58] and is capable of altering functions of both immune and non-immune cells at sub-cytolytic concentrations [203]. In addition, PLY-deficient strains are capable of causing bacteremia [131], suggesting that *S. pneumoniae* encodes multiple factors that contribute to bacterial dissemination. Here, we subjected polarized respiratory epithelial monolayers to apical infection by *S. pneumoniae* and systematically examined the role of infectious dose, PLY-mediated pore formation, and PMN transmigration on the integrity and organization of the AJ protein E-cadherin, as well as on bacterial translocation.

3.2. Results

3.2.1. High dose *S. pneumoniae* infection elicits PLY-dependent PMN recruitment, which promotes epithelial detachment and bacterial translocation

Previously, we reported that PMN transmigration, is required for retrograde translocation of *S. pneumoniae* across polarized airway epithelial monolayers *in vitro* and for bacteremia in mice [59, 80]. We further showed that PMN transmigration is promoted by PLY-induced secretion of the inflammatory eicosanoid HXA₃ [136]. Here, we utilized polarized H292 cells grown on 5 µm pore size Transwell filters to directly test whether PLY-promoted PMN transmigration promotes barrier disruption and subsequent bacterial translocation. We first infected monolayers apically with TIGR4 at a calculated multiplicity of infection (MOI) of 20 using 1×10⁷ total bacteria per Transwell (See Materials and Methods), an infectious dose used in previous reports [59, 136]. This

resulted in 5×10^5 total bound bacteria per Transwell with indistinguishable binding efficiency between wild type (WT) TIGR4 and *PLY* deficient TIGR4 Δply , as visualized by immunofluorescence (IF) microscopy (Supplemental Figure 2.1A). Infection with WT TIGR4 triggered the basolateral-to-apical transmigration of $\sim 30 \times 10^4$ human PMNs, accounting for 30% of PMNs added to the basolateral chamber (Figure 3.1A, “WT”). This induction of PMN transmigration was partially *PLY*-dependent, as the TIGR4 Δply strain was associated with a ~ 1.5 -fold reduction in PMN movement compared to the WT strain ($p < 0.01$) (Figure 3.1A, “ Δply ”). Nevertheless, the level of TIGR4 Δply induced PMN transmigration was 14-fold ($p < 0.0001$) above the mock-infected background level, confirming that PMN transmigration can be elicited in *PLY*-dependent and -independent manners [59, 80].

To examine the influence of *PLY*-dependent and -independent PMN transmigration on barrier dysfunction, we measured the apical-to-basolateral flux of horseradish peroxidase (HRP) and the apical-to-basolateral movement of bacteria. Infection by WT TIGR4 in the presence of PMNs was associated with a three-fold increase in HRP flux ($p < 0.001$) (Figure 3.1B, “WT”) and the detection of 4.8×10^6 *S. pneumoniae* in the basolateral chamber by quantitation of colony-forming units (CFUs) ($p < 0.0001$) (Figure 3.1C, “WT”), which was approximately 7000-fold more than the number of translocated bacteria in the absence of PMNs (Figure 3.1C, “No PMNs”, “WT”). The WT TIGR4 translocation index, defined as the number of bacteria in the basolateral chamber at five hours (2.5 h infection followed by 2.5 h of PMN co-incubation) post-infection divided by the initial inoculum, was 4.8 in the presence of PMNs, as opposed to ~ 0.0007 in the absence of PMNs (Figure 3.1C), indicating efficient translocation only in the presence of PMNs, consistent with our previous findings [59, 80]. (The calculated translocation index of greater than 1 indicates that there is some degree of bacterial replication during the five-hour incubation). Infection with TIGR4 Δply ,

which elicited ~1.5-fold less PMN migration than WT TIGR4 (Figure 3.1A), led to an equivalently lower level of HRP flux, but this difference did not reach statistical significance (Figure 3.1B). The degree of HRP flux induced by TIGR4 Δ ply was two-fold greater than baseline HRP flux in mock-infected monolayers, but this difference also did not reach statistical significance (Figure 3.1B). Barrier disruption by PMNs in response to TIGR4 Δ ply infection was most clearly revealed by bacterial translocation, because 10×10^6 TIGR4 Δ ply were present in the basolateral chamber, resulting in a translocation index of 1, and an approximately 1000-fold increase in bacterial transmigration in comparison to that in the absence of PMNs (Figure 3.1C). Thus, PMN transmigration, whether induced in a PLY-dependent or PLY-independent manner, promotes *S. pneumoniae* transmigration.

To visualize how PMN transmigration to *S. pneumoniae* disrupted the epithelial barrier, we assessed monolayer confluency after *S. pneumoniae* infection and PMN transmigration by immunofluorescence (IF) staining of host cell nuclei (DAPI) and F-actin (phalloidin). At a MOI of 20, infection by WT TIGR4 or TIGR4 Δ ply did not alter monolayer confluency (Figure 3.1D, top row), but did result in approximately 50% of cells becoming permeable to propidium iodide (PI) upon WT TIGR4 infection (Supplemental Figure 2.1B). TIGR4 Δ ply infection, on the other hand, did not result in significant PI staining, suggesting that PLY permeabilized cells during infection (Supplemental Figure 2.1C). Although we and others have shown that H292 cells initiate membrane repair processes to prevent cell death following PLY insult [136, 204]), it is possible that PLY-permeabilized cells are particularly vulnerable to dislocation from the monolayer upon subsequent addition of PMNs. We thus examined monolayer confluency by IF again post-PMN transmigration. PMN infiltration to WT TIGR4 infected monolayers can be identified by their multi-lobed nuclei (Figure 3.1D, white arrowheads), and PMN migration was associated with loss of 62% of epithelial cells from the monolayer (Figure

3.1D, bottom row, “Mock” vs. “WT SPN”, with image analysis quantitation on right). In contrast, infection by TIGR4 Δ ply resulted in a 20% loss of epithelial cells, significantly less than the loss associated with infection by WT TIGR4 ($p < 0.01$) (Figure 3.1D).

Because PLY can also permeabilize PMNs, we used flow cytometry to evaluate the degree of PI staining after direct infection of PMN with WT or TIGR4 Δ ply and

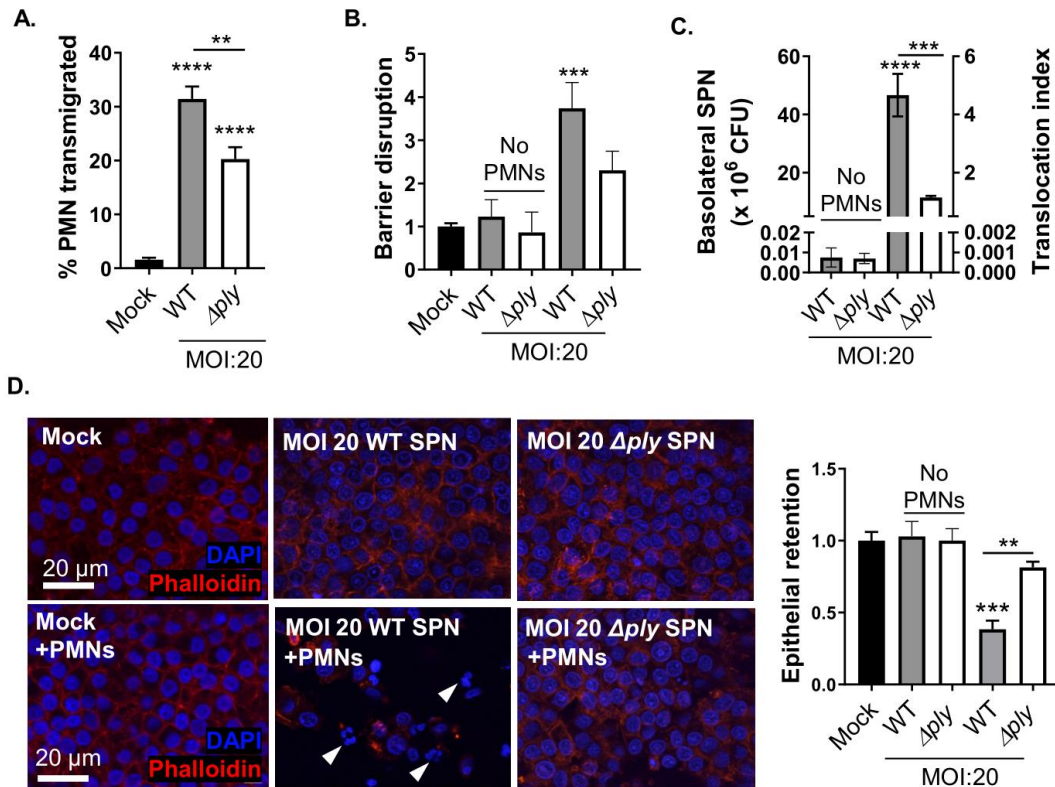


Figure 3.1. High dose *S. pneumoniae* infection elicits PLY-dependent PMN recruitment, which promotes epithelial detachment and bacterial translocation. **A.** 1×10^6 human PMNs were added to the basolateral side of Transwell seeded polarized H292 monolayers that were mock-infected (with HBSS, “Mock”), infected apically with wild type TIGR4 (“WT”), or infected apically with a pneumolysin-deficient mutant (“ Δ ply”) at the indicated MOI for 2.5 h. Subsequently, PMN transepithelial migration after 2.5 h was quantified by MPO activity. **B.** Barrier integrity of monolayers was measured by HRP flux post-infection, normalized to mock-infected. **C.** Transepithelial migration of bacteria was quantitated by plating for CFUs, and the translocation index was determined by normalizing SPN present in the basolateral compartment to the infection inoculum. **D.** Fluorescence microscopy of monolayers stained with DAPI (blue) and phalloidin (red). White arrows indicate PMN nuclei. Scale bar = 20 μ m. Epithelial cell retention was quantified by epithelial cell nuclei number per field of view. Each panel shown is representative of three independent experiments in triplicates, or pooled data from at least three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: * p -value ≤ 0.05 , ** p -value ≤ 0.01 , *** p -value ≤ 0.001 , **** p -value ≤ 0.0001 .

observed a PLY-dependent permeabilization of 2% of PMNs (Supplemental Figure 2.1D). We also evaluated PMN permeabilization after traversing the monolayer by measuring MPO activity in the apical buffer immediately after PMN transmigration. We observed a 2-fold and significant increase in MPO activity in WT TIGR4 infected Transwells (Supplemental Figure 2.1E), which corresponds to if approximately 10% of transmigrated PMNs released MPO. No MPO activity above background was observed with TIGR4 $\Delta p/y$ infection. This raises the possibility that at an MOI of 20, PMN release of effector molecules post transmigration may contribute to epithelial detachment. These findings implicate PMN transmigration, augmented by PLY, as a key factor that drives monolayer disruption, barrier integrity compromise, and *S. pneumoniae* translocation.

3.2.2. Low dose infection elicits PLY-dependent PMN recruitment, which drives *S. pneumoniae* translocation in the absence of epithelial detachment

PMN transmigration in response to TIGR4 infection is highly MOI-dependent [59, 80]. Hence, we performed a similar series of experiments infecting H292 monolayers with a 10-fold lower infection dose, which resulted in approximately 1×10^5 total bacteria bound/Transwell (Supplemental Figure 2.1A). WT TIGR4 infection at MOI 2 triggered transmigration of $\sim 15 \times 10^4$ human PMNs (i.e., 15% of total PMNs added; Figure 3.2A, “WT”), approximately two-fold lower than that observed at a MOI of 20 (Figure 3.1A, “WT”), yet 30-fold higher ($p < 0.01$) compared to mock-infected monolayers. Infection with TIGR4 $\Delta p/y$ resulted in an approximate six-fold (and not statistically significant) increase in PMN transmigration compared to mock-infected monolayers, indicating that PMN transmigration remains PLY-promoted at the lower MOI (Figure 3.2A, “ $\Delta p/y$ ”). Notably, this PLY-dependent effect was not due to more efficient bacteria-host cell interaction because monolayer binding by TIGR4 $\Delta p/y$ was indistinguishable from wild type (Supplemental Figure 2.1A), nor due to host cell killing by PLY because epithelial and

neutrophil viability was not affected at this MOI (Supplemental Figure 2.1B-E). Disruption of barrier integrity correlated with PMN transmigration, because HRP flux was elevated 2.2-fold ($p < 0.01$) upon infection with WT TIGR4 but not significantly by TIGR4 Δply compared to mock-infected monolayers (Figure 3.2B). Correspondingly, at MOI 2, 0.9×10^6 WT TIGR4 and 0.2×10^6 TIGR4 Δply bacteria migrated to the basolateral chamber respectively (Figure 3.2C). While the lower number of bacteria translocated was expected given the lower inoculum, it is notable that translocation was still PMN-promoted, because in the absence of PMNs, translocation of WT TIGR4 and TIGR4 Δply was diminished 700- and 300-fold, respectively (Figure 3.2C). Monolayer confluency was retained despite robust PMN and *S. pneumoniae* transmigration (Figure 3.2D). Hence, at a MOI of 2, PMN and PMNs promoted bacterial migration in a manner that did not require overt destruction of the monolayer.

To determine whether the pro-transmigration functions of PMN were restricted to PMN-producing bacteria or could function in *trans*, we infected H292 monolayers with either a 1:1 or 1:9 mixture of WT TIGR4 and TIGR4 Δply , where TIGR4 Δply can be identified through chloramphenicol resistance. To avoid the epithelial detachment that is associated with the MOI 20 dose (Figure 3.1D), we utilized the MOI of 2 dose. As shown previously in Figure 3.2A, WT TIGR4 elicited a stronger PMN response than TIGR4 Δply (Figure 3.2E). Both the 1:1 and the 1:9 mixed infections led to neutrophil recruitment similar to that of WT TIGR4-infected monolayers (Figure 3.2E), consistent with previous reports of PMN-mediated PMN chemoattractant HXA₃ secretion by host cells [136]. In the presence of transmigrating PMNs, bacteria from both the 1:1 and 1:9 mixed infections efficiently migrated to the basolateral compartment with efficiencies similar to that of infections with WT TIGR4 only (Figure 3.2F). Differential antibiotic resistance permitted distinction between the two strains and revealed that the transmigration of TIGR4 Δply was significantly ($p < 0.05$, indicated by '#') increased in the presence of WT TIGR4

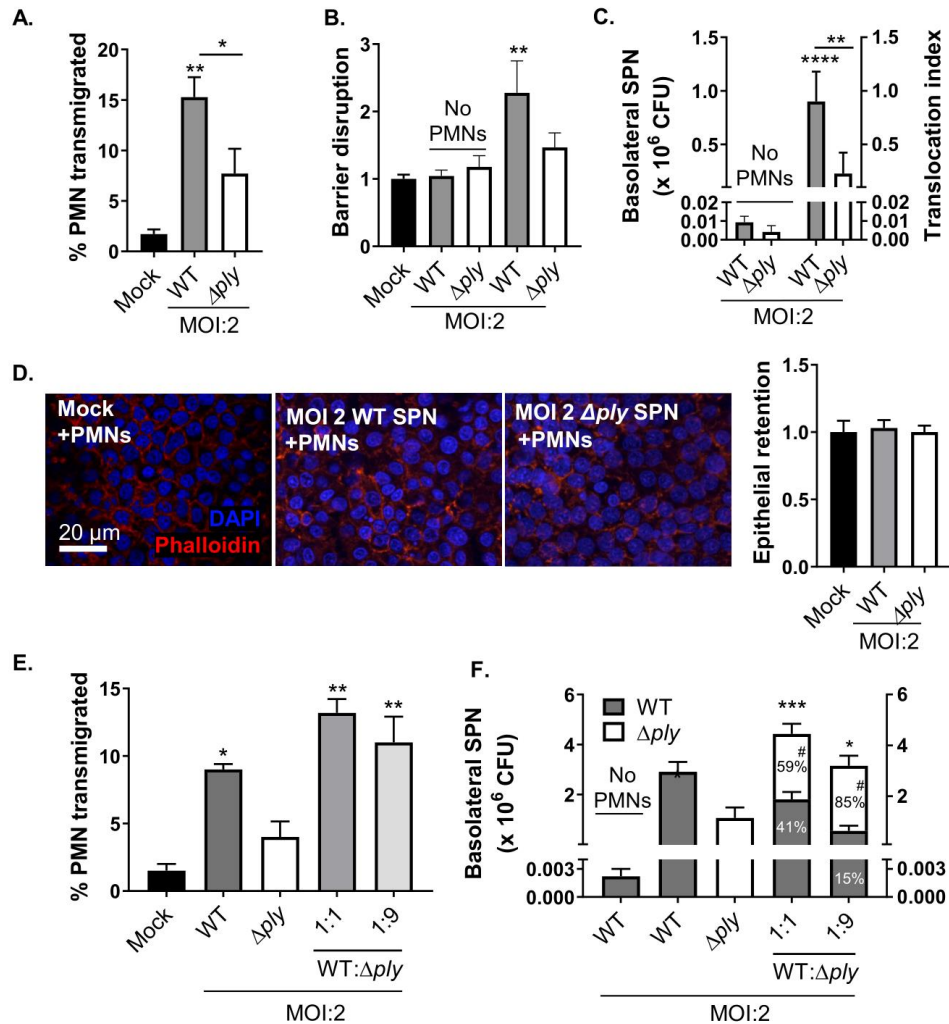


Figure 3.2. Low dose infection elicits PLY-dependent PMN recruitment, which drives *S. pneumoniae* translocation in the absence of epithelial detachment. **A.** 1×10^6 human PMNs were added to the basolateral side of monolayer mock-infected (with HBSS “Mock”) or infected apically with WT or Δply SPN at indicated MOI. PMN transepithelial migration was quantified by MPO activity. **B.** Barrier integrity of monolayers was measured by HRP flux post-infection, normalized to mock-infected. **C.** Transepithelial migration of bacteria was quantitated by plating for CFUs, and translocation index was determined by normalizing SPN present in basolateral chamber to infection inoculum. **D.** Fluorescence microscopy of monolayers stained with DAPI (blue) and phalloidin (red). Scale bar = 20 μm . Epithelial cell retention was quantified by epithelial cell nuclei number per field of view. **E.** Monolayers were mock-infected (HBSS) or apically infected apically with WT, Δply , a 1:1 mix of WT and Δply , or a 1:9 mix of WT and Δply SPN at indicated MOI. PMN transepithelial migration was quantified by MPO activity. **F.** Transepithelial migration of bacteria was quantitated by plating, and translocation index was determined by normalizing SPN migration to infection inoculum. Statistical analysis comparing total SPN migration between respective groups indicated with ‘*’, statistical analysis comparing TIGR4 Δply migration between respective groups indicated with ‘#’. Each panel shown is representative of three independent experiments in triplicates, or pooled data from at least three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value $\leq$ 0.05, **p-value $\leq$ 0.01, ***p-value $\leq$ 0.001.

(Figure 3.2F). Moreover, the ratio of WT: Δply bacteria in the basolateral chamber was similar to input ratios upon apical infection, i.e., the 1:1 WT: Δply mixture resulted in a 1:1.4 output and the 1:9 input resulted in a 1:5.7 output (Figure 3.2F). Thus, the pro-translocation activity of PLY promotes migration of both PLY-producing and PLY-deficient bacteria, likely by eliciting epithelial cell HXA₃ production and concomitant PMN migration, which contributes to barrier disruption [136].

3.2.3. *S. pneumoniae* infection results in cleavage and mislocalization of the AJ protein E-cadherin via PLY-dependent and PLY-independent pathways

S. pneumoniae have been reported to target endothelial and epithelial junctional components to compromise respiratory barrier function [186]. To determine if *S. pneumoniae* targets junctional components in our model, we infected H292 cell monolayers and stained with an antibody that recognizes the extracellular domain of E-cadherin, a transmembrane AJ protein capable of homotypic binding to promote barrier formation that is robustly expressed by H292 cells. When visualized by IF microscopy, E-cadherin is located in circumferential rings at the cell periphery (Figure 3.3A, “Mock”; Supplemental Figure 2.2). Addition of PMNs to a mock-infected monolayer had no effect on E-cadherin localization of staining intensity visualized by IF (Figure 3.3A, “Mock+PMNs”).

To examine whether H292 cell E-cadherin complexes are disrupted upon PMN migration induced by *S. pneumoniae*, monolayers were apically infected with WT TIGR4 or TIGR4 Δply at an MOI of 20 or 2. WT TIGR4 infected monolayers lost all cell periphery E-cadherin, regardless of infection dose (Figure 3.3A, top row, “WT SPN”; supplemental Figure 2.2). In contrast, TIGR4 Δply was able to induce loss pericellular E-cadherin localization at a MOI of 20 but not at a MOI of 2 (Figure 3.3A, bottom row, “ Δply SPN”). Quantification of E-cadherin localization specifically at the cell periphery was carried out

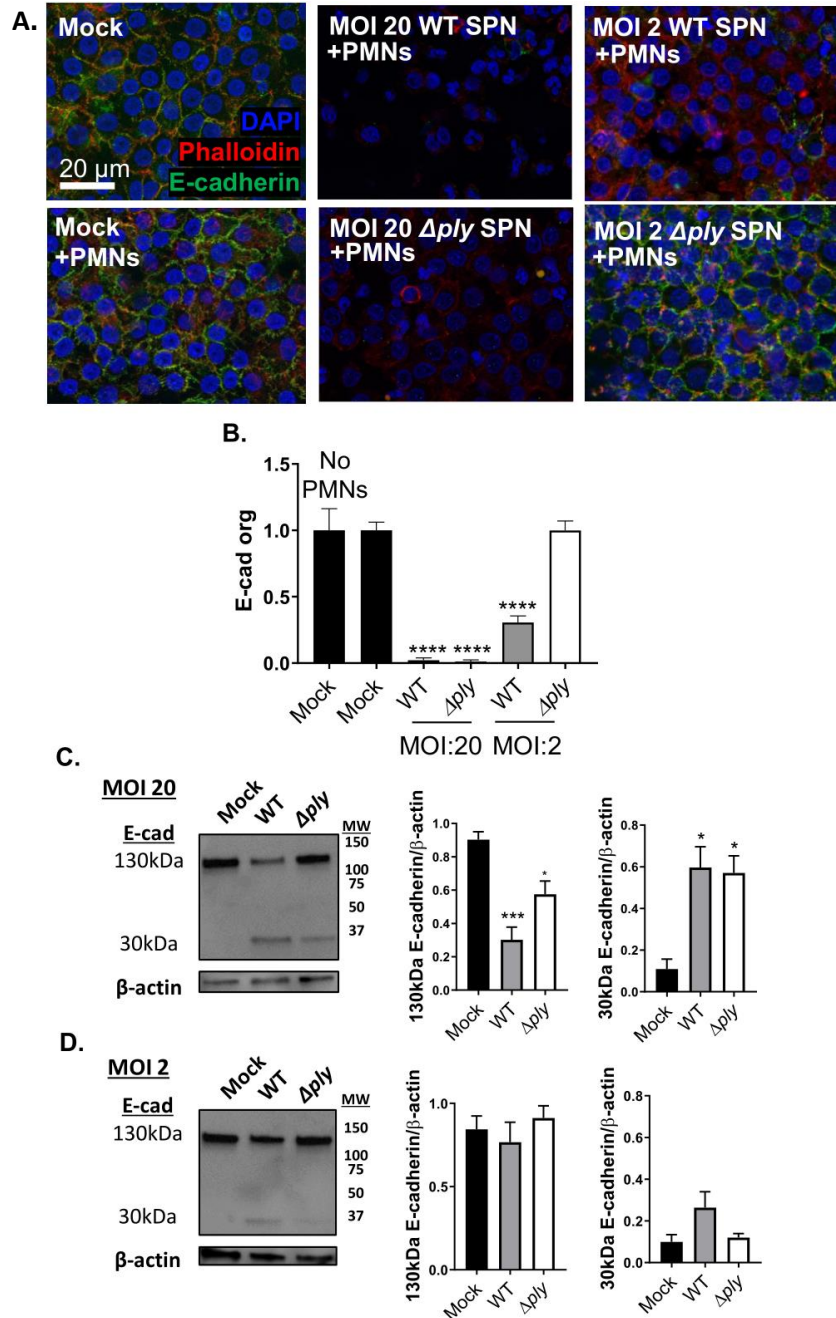


Figure 3.3. *S. pneumoniae* cleaves and disrupts the localization of E-cadherin via **PLY-dependent and -independent pathways.** **A.** 1×10^6 human PMNs were added to the basolateral side of monolayers mock-infected (HBSS) or infected apically with WT or Δ ply SPN at indicated MOI. Monolayers were stained with ectodomain E-cadherin antibody (green) before permeabilization and with DAPI (blue) and phalloidin (red) after permeabilization. Scale bar = 20 μ m. **B.** Peripheral E-cadherin organization was quantitated by image analysis with intercellular junction organization quantification (IJOQ). Each panel shown is representative of three independent experiments in triplicates. **C.** MOI 20 and **D.** MOI 2 infected monolayers were lysed for western blotting of E-cadherin and densitometry analysis normalized to β -actin. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: ****p-value ≤ 0.0001 .

with the Intercellular Junction Organization Quantification (IJOQ) script [205], which quantifies the continuity in pericellular staining. This analysis showed both WT TIGR4 and TIGR4 Δply infection at a MOI of 20 eliminated E-cadherin organization ($p < 0.0001$), whereas at a MOI of 2, only WT TIGR4 infection induced a significant (60%) reduction in E-cadherin organization ($p < 0.0001$) (Figure 3.3B).

To determine if E-cadherin structure is altered by infection by *S. pneumoniae*, we performed western blotting of E-cadherin in total cell lysates from uninfected or infected monolayers. This revealed a PLY-dependent decrease in the 130 kDa full length E-cadherin and the appearance of an E-cadherin-related 30 kDa species after infection with WT TIGR4 at MOI 20, (Figure 3.3C). At MOI 2, infection by WT TIGR4 also resulted in apparent E-cadherin cleavage, although densitometric analysis did not reveal statistical significance (Fig. 3.3D). E-cadherin cleavage appeared to be partially dependent on PLY, because infection by TIGR4 Δply at either MOI 2 or 20 resulted in less cleavage than during WT infection (Figs. 3.3C-D, " Δply "). Thus, E-cadherin cleavage and mislocalization is triggered via both PLY-dependent and PLY-independent pathways, with the PLY-dependent pathway capable of triggering these changes at a lower infectious dose.

3.2.4. PMNs are not required for E-cadherin mislocalization by *S. pneumoniae* at either low or high infectious dose

Independent of PMNs, *S. pneumoniae* has been reported to downregulate epithelial junctional components during infection through mechanisms such as TLR2 engagement [157] and pneumococcal neuraminidase activity [193]. To determine whether PMNs are required for the PLY-dependent and -independent E-cadherin mislocalization described above, we infected monolayers with WT TIGR4 and TIGR4 Δply at a MOI of 20 or 2 in the absence of PMNs. At a MOI of 20, both WT TIGR4

and TIGR4 Δply depleted all pericellular E-cadherin (Figure 3.4A), just as when PMNs were present (Figure 3.3A). Lowering the MOI to 2, we observed that WT TIGR4 induced a 38% ($p < 0.01$) decrease in E-cadherin localization to cell periphery (Figure 3.4B, top row, “MOI 2 WT SPN”, and right, solid bars). In contrast, TIGR4 Δply infection at a MOI of 2 had no effect on E-cadherin localization (Figure 3.4B, top row, “MOI 2 Δply SPN”, and right, solid bars).

To determine if *S. pneumoniae* disruption of E-cadherin integrity could be augmented by the addition of purified PLY, we added recombinant PLY at a sublytic PLY concentration of 0.01 toxin activity units (Materials and Methods) [136], a concentration that by itself was incapable of disrupting E-cadherin localization (Figure 3.4B, “Mock, 0.01U PLY”, and right, open bars). We found that, when combined with infection by WT TIGR4, this level of exogenous PLY enhanced E-cadherin disruption from 38% to 81% ($p < 0.01$; Figure 3.4B, bottom row, “WT SPN, 0.01U PLY”, and right, open bars). Whereas TIGR4 Δply at a MOI of 2 was insufficient to perturb E-cadherin on its own, the addition of 0.01U PLY to monolayers infected with TIGR4 Δply resulted in a 53% ($p < 0.001$) reduction in E-cadherin cell periphery organization (Figure 3.4B, “Mock+0.01U PLY”, and right, open bar).

Because PLY promotes PMN transmigration [136, 195], we hypothesized that the addition of PMNs to monolayers infected with WT TIGR4 may further compromise E-cadherin integrity. Indeed, the addition of PMNs to the basolateral chamber of monolayers infected with WT TIGR4 at a MOI of 2 resulted in a two-fold decrease ($p < 0.05$) in E-cadherin organization (Figure 3.4C, “WT” vs. “WT + PMN”). As expected, PLY was critical to this augmentation, because the addition of PMNs to monolayers infected with TIGR4 Δply had no effect (Figure 3.4C, “ Δply ” vs. “ Δply + PMN”).

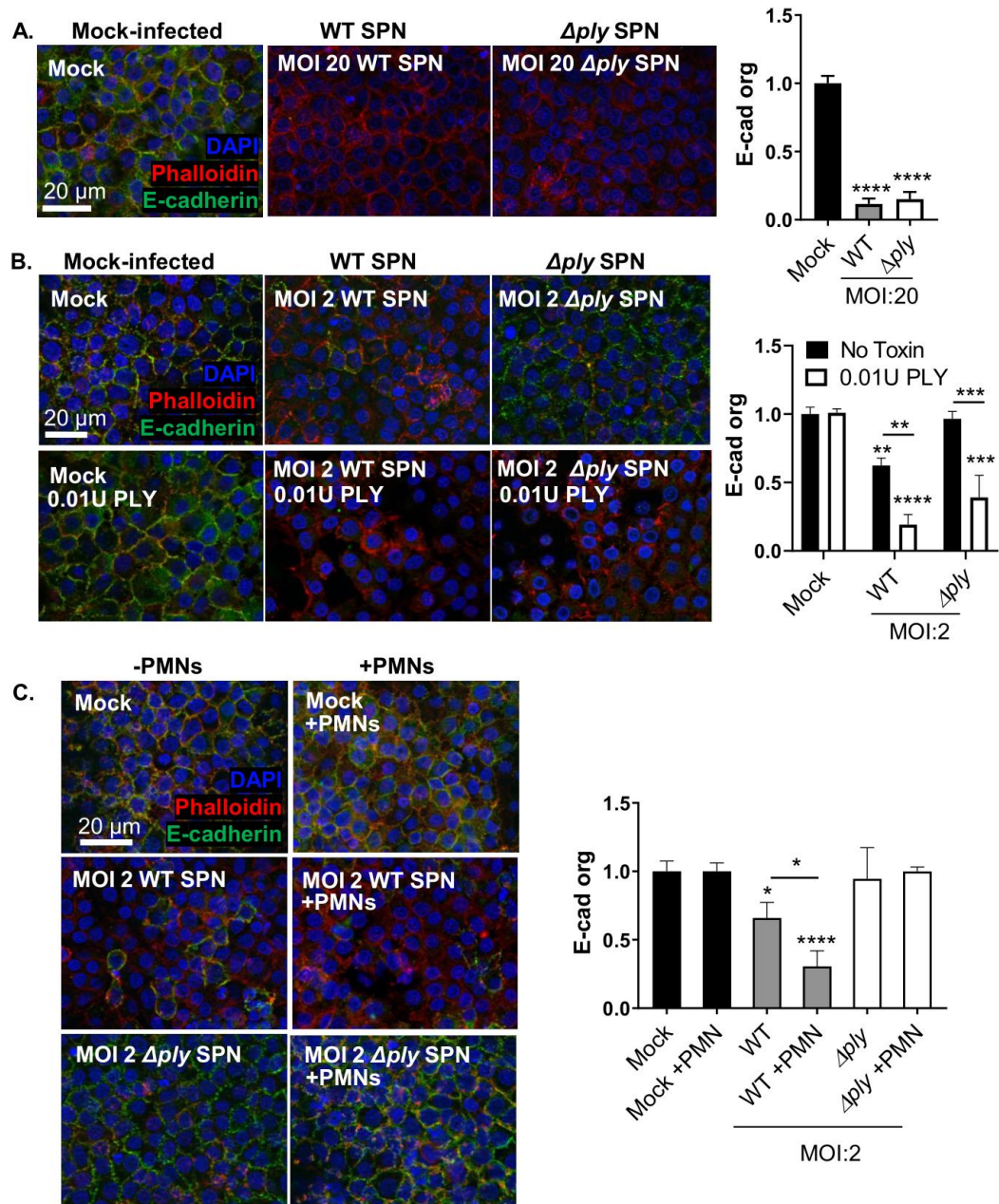


Figure 3.4. PMNs are not required for E-cadherin mislocalization by *S. pneumoniae* at either low or high infectious dose. **A.** Fluorescence microscopy of monolayers mock-infected (HBSS) or infected apically with WT or Δply SPN at indicated MOI in the absence of PMNs, stained with ectodomain E-cadherin antibody (green) before permeabilization and with DAPI (blue) and phalloidin (red) after permeabilization. Scale bar = 20 μ m. Peripheral E-cadherin organization was quantitated by image analysis with IJOQ. **B.** Fluorescence microscopy of monolayers mock-infected (HBSS) or infected apically with WT or Δply SPN at indicated MOI, and complemented with recombinant PLY where indicated. Monolayers were stained with ectodomain E-cadherin antibody (green) before permeabilization and with DAPI (blue) and phalloidin (red) after permeabilization. Scale bar = 20 μ m. Peripheral E-cadherin organization was quantitated by image analysis with IJOQ. **C.** Fluorescence microscopy of monolayers mock-infected

with HBSS (mock) or infected apically with WT or Δply SPN at indicated MOI in the presence or absence of PMNs, stained with ectodomain E-cadherin antibody (green) before permeabilization and with DAPI (blue) and phalloidin (red) after permeabilization. Scale bar = 20 μ m. Peripheral E-cadherin organization was quantitated by image analysis with IJOQ. Each panel shown is representative of three independent experiments in triplicates. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value $\leq$ 0.05, **p-value $\leq$ 0.01, ***p-value $\leq$ 0.001.

3.2.5. Pore formation by PLY and related toxins is necessary and sufficient for E-cadherin mislocalization.

To investigate whether purified PLY is sufficient for disrupting E-cadherin localization, we examined E-cadherin organization after apical treatment of H292 monolayers with various concentrations of PLY. 1U of PLY, a concentration resulting in transient permeabilization of 50% of host cell followed by membrane repair [136], ablated pericellular E-cadherin (Figure 3.5A). A 10-fold lower PLY concentration also significantly ($p < 0.05$) reduced E-cadherin organization compared to mock treatment (Figure 3.5A). 0.01U of PLY had no effect on E-cadherin organization (Figure 3.5A). Thus, purified PLY mimics E-cadherin disruption by live *S. pneumoniae*, and in an infection dose-dependent manner. Western blotting of E-cadherin confirmed no cleavage of E-cadherin with 0.01U PLY treatment, partial diminution of full-length E-cadherin and the appearance of a very faint 30 kDa species with 0.1U PLY treatment, and a dramatic reduction in full length E-cadherin and the appearance of the 30 kDa cleavage product with 1U PLY treatment (Figure 3.5B).

Matrix metalloproteases (MMPs), which can be released by epithelial cells or PMNs during infection, are capable of cleaving extracellular matrix and junctional proteins, including E-cadherin [69, 91, 206]. The pore-forming *Staphylococcus aureus* α -toxin [207, 208] and *Pseudomonas aeruginosa* exolysin [209] trigger E-cadherin cleavage in infected cells by activating host matrix metalloproteases (MMPs). PLY also has been shown to trigger MMP activation in A549 cells [207]. We found that treatment

of either epithelial cells or PMNs with 1 U of purified PLY triggered an increase of MMP activity in cell supernatants (Supplemental Figure 2.3A-B). This activity was inhibited by the broad spectrum MMP inhibitor GM6001. However, the MMP inhibitor GM6001 did not prevent E-cadherin cleavage or disruption of E-cadherin organization when monolayers were treated with 1U PLY or infected with WT TIGR4 at MOI 2 (Supplemental Figure 2.3C-D), implying that E-cadherin disruption observed was not mediated by MMPs.

Many of the biologic activities of PLY are mediated through its pore-forming activity [7], but PLY is also capable of altering cell behavior independent of pore-formation [58, 67]. The mutant $\text{PLY}^{\text{T459G}\cdot\text{L460G}}$ (which we herein term “ PLY_{CBS} ”) is mutated at two critical residues in the cholesterol binding site in PLY domain 4 and incapable of pore-formation [54, 136]. H292 monolayers treated with PLY_{CBS} at equimolar concentration to 1U PLY had no effect on E-cadherin integrity by microscopy (Figure 3.5C-D). Likewise, apical infection with TIGR4 mutant producing PLY_{CBS} (PLY_{CBS} SPN), which is incapable of H292 cell permeabilization, failed to disrupt H292 E-cadherin organization (Figure 3.5E-F), indicating that pore-formation by PLY is essential for E-cadherin disruption.

PLY belongs to a family of cholesterol-dependent cytolysins (CDCs) that share substantial structural and functional conservation. We investigated whether other CDCs could also induce E-cadherin disruption. H292 monolayers apically treated with 1U of perfringolysin O (PFO) or intermedilysin (ILY), resulted in almost complete ablation of E-cadherin staining (Figure 5G-H), like that observed with 1U of PLY. These results indicate that the ability to disrupt pericellular localization of the epithelial cell AJ protein E-cadherin is a property conserved among CDCs and may be utilized by many CDC-expressing bacterial pathogens to target host cell junction networks.

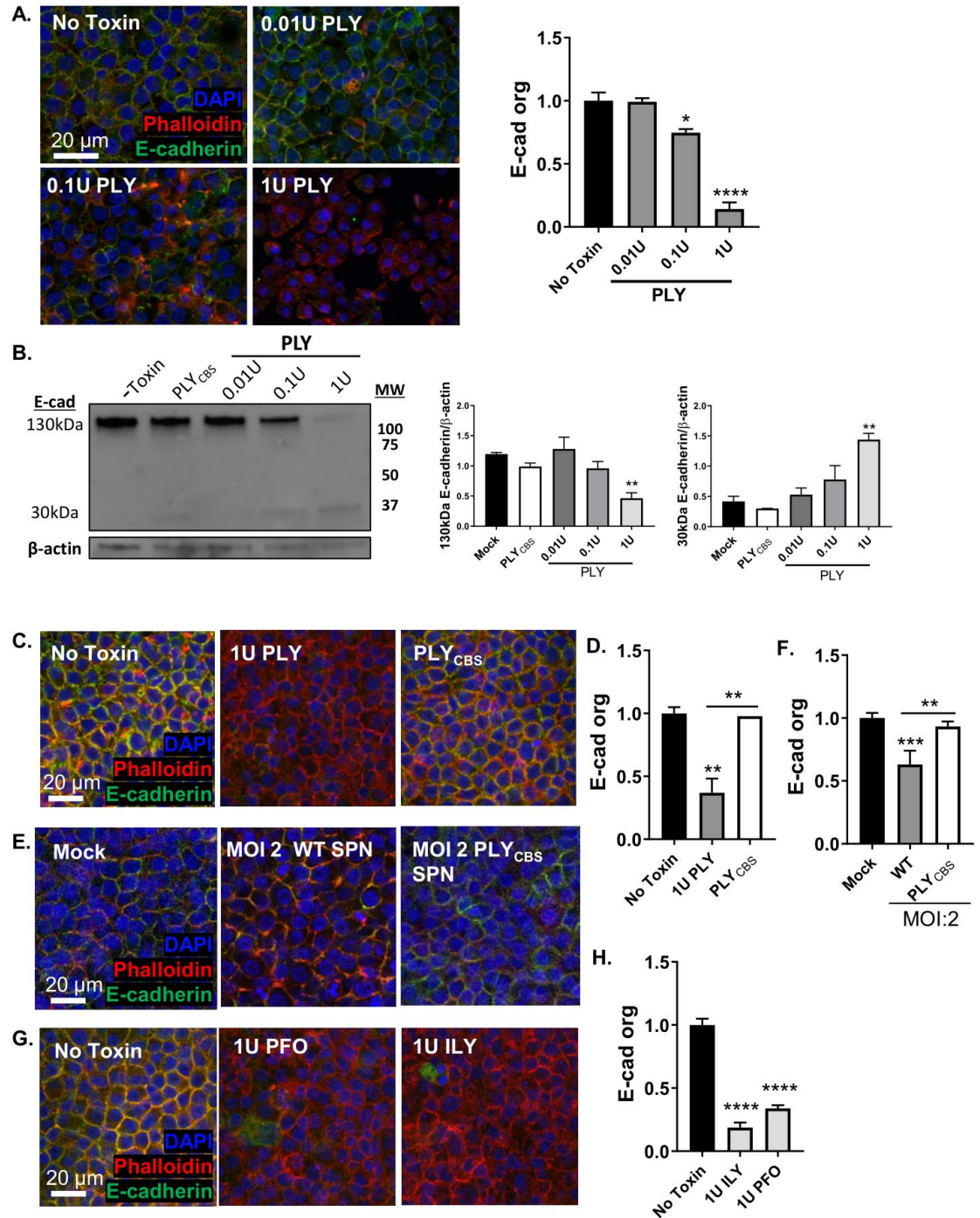


Figure 3.5. Pore formation by PLY and related toxins is necessary and sufficient for E-cadherin mislocalization. Monolayers were treated with HBSS (No Toxin) or the indicated units of recombinant PLY. **A.** Monolayers were stained with ectodomain E-cadherin antibody (green) before permeabilization and with DAPI (blue) and phalloidin (red) after permeabilization. Scale bar = 20 μ m. Peripheral E-cadherin organization was quantitated by image analysis with IJOQ. **B.** Monolayers were lysed for western blotting of E-cadherin and densitometry analysis normalized to β -actin. **C.** Fluorescence microscopy of monolayers treated apically with purified PLY_{CBS} protein at equimolar concentrations to 1U of PLY. Monolayers were permeabilized and stained with E-

cadherin antibody (green), DAPI (blue), and phalloidin (red). Scale bar = 20 μ m. **D.** Peripheral E-cadherin organization was quantitated by image analysis with IJOQ. **E.** Fluorescence microscopy of monolayers infected apically with PLY_{CBS} -expressing SPN (PLY_{CBS} SPN) at indicated MOI. Monolayers were permeabilized and stained with E-cadherin antibody (green), DAPI (blue), and phalloidin (red). Scale bar = 20 μ m. **F.** Peripheral E-cadherin organization was quantitated by image analysis with IJOQ. **G.** Fluorescence microscopy of monolayers treated with the indicated units of purified PLY , intermedilysin (ILY) of *S. intermedius*, or perfringolysin O (PFO) of *C. perfringens*. Monolayers were permeabilized and stained with E-cadherin antibody (green), DAPI (blue), and phalloidin (red). Scale bar = 20 μ m. **H.** Peripheral E-cadherin organization was quantitated by image analysis with IJOQ. Each panel shown is representative of three independent experiments in triplicates. Error bars represent mean $\pm$ SEM. Statistical analysis performed using ordinary one-way ANOVA: *p-value $\leq$ 0.05, **p-value $\leq$ 0.01, ***p-value $\leq$ 0.001, ****p-value $\leq$ 0.0001.

3.3. Discussion

The mucosal epithelium, sealed by a well-organized system of intercellular junctional complexes, is a physical line of defense against microbial invasion. Pathogens that cross epithelial barriers often disrupt these epithelial junctions, which facilitates tissue invasion by paracellular movement [210]. *S. pneumoniae* has been shown to target airway junction proteins and compromise airway epithelial barrier function [157, 186, 191]. Pneumococcal infection reduces alveolar occludin, ZO-1, claudin-5, and VE-cadherin in human lung explants [186], and downregulates claudin-7 and -10 in murine models [157]. The reduction in critical junctional molecules can increase epithelial permeability and is likely an important step in facilitating pneumococcal dissemination [211]; mice deficient in type I interferon, which show junctional defects in response to bacterial infection, are highly susceptible to *S. pneumoniae* bacteremia. [191]. We thus examined the epithelial barrier and junctional integrity during *S. pneumoniae* infection of polarized H292 monolayers to systematically reveal requirements for *S. pneumoniae* translocation across the respiratory epithelium. We show that PLY and transmigrating PMNs 1) individually and cooperatively perturb localization and integrity of the AJ protein E-cadherin, 2) cause distinct degrees of epithelial damage depending on infection dose,

PLY-mediated pore formation, and PMN presence, and 3) only when combined promote maximal bacterial cross-epithelial dissemination.

PLY, a *S. pneumoniae* virulence factor present in almost all clinical isolates of the species, increases alveolar permeability and alveolar epithelial cell injury [77]. In human adenoid epithelial explant cultures, PLY ablates tight junctions and exposes host receptors for bacterial attachment [74]. The notable invasiveness of serotype 1 pneumococci has been attributed to its rapid and robust release of PLY, which promotes ZO-1 disruption [12]. Here, we found that *S. pneumoniae* at an MOI of 2 triggers both cleavage and mislocalization of the junctional protein E-cadherin in a manner dependent on PLY-mediated pore forming activity. Two other cholesterol-dependent cytolysins, ILY and PFO, induce intercellular junction disruption, suggesting that junction disruption is a conserved function of CDCs [212, 213]. Other pathogens, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Serratia marcescens*, produce non-CDC pore-forming toxins that disrupt junctions *in vitro* [207, 209].

The ability of *S. pneumoniae* at an MOI (of 2) that did not compromise host cell membrane integrity indicates that E-cadherin disorganization is not a simple consequence of host cell death [136]. PLY, like other pore-forming toxins, triggers membrane repair [204, 214-216], and, by activating GTPases that regulate actin assembly [73], may destabilize actin-associated junctional protein complexes [71, 217]. PLY, although not a protease itself, has been shown to increase metalloprotease activity in A549 cells [207]. Pore-forming toxins of *P. aeruginosa* and *S. marcescens* induce an influx of calcium ions, thus activating the calmodulin-regulated metalloproteinase ADAM-10, which cleaves E-cadherin [218]. *S. aureus* toxin Hla directly binds and activates ADAM-10. Western blotting revealed a 30 kDa cleavage product was generated upon *S. pneumoniae* infection or PLY treatment, suggesting disruption of E-cadherin organization is associated with its proteolytic cleavage. Consistent with previous reports

[207, 208], we found that infection of PMNs or epithelial cells by PLY-producing SPN or treatment with purified PLY was associated with an increase in MMP activity in culture supernatants. However, inhibition of MMP did not prevent E-cadherin cleavage or mislocalization. Identification of proteases responsible for PLY-dependent cleavage of E-cadherin organization warrants future studies.

At an MOI of 20, both PLY-producing and PLY-deficient *S. pneumoniae* disrupted junctional organization of H292 cell monolayers. This PLY-independent pathway augments PLY-mediated disruption, as a PLY concentration (0.01U) that was insufficient for E-cadherin loss in isolation, in combination with PLY-deficient *S. pneumoniae* facilitated complete junctional disruption. TLR stimulants [157] and pneumococcal neuraminidase [193], which downregulate junctional components, are potential non-PLY bacterial factors that contribute to E-cadherin loss.

S. pneumoniae-mediated junctional disruption of monolayers alone was insufficient to promote bacterial translocation. Neutrophils secrete proteolytic enzymes capable of cleaving extracellular matrix and junctional proteins [81, 91, 201, 219] and we previously showed PMN transmigration triggered by HXA₃ was required for barrier breach of pulmonary epithelium by *S. pneumoniae* [59, 80]. PLY, which triggers epithelial production of HXA₃ [136] and may enhance bacterial translocation. Indeed, a highly invasive *S. pneumoniae* serotype 1 strain, which releases high levels of PLY, induces severe inflammation of the lung, with ~4-fold more neutrophils than a serotype 2 strain tested in parallel [12].

Although PLY clearly promotes PMN transmigration, monolayer infection with TIGR4Δ*p/ly* also triggered (lower levels of) neutrophil movement, presumably due to the production of chemoattractants in response to (non-PLY) bacterial products [220, 221]. The level of barrier breach by TIGR4Δ*p/ly* in the presence of PMNs appeared elevated compared to background levels (Figures 3.1 and 3.2) but these higher levels did reach

statistical significance. Interestingly, at a MOI of 20, PMN transmigration in response to *S. pneumoniae* infection resulted in the PLY-dependent loss of more than 50% of the epithelial monolayer, a loss that correlated with the efficient transepithelial movement of the marker HRP and the apparently unimpeded barrier breach by pneumococci. Epithelial detachment by activated neutrophils has been observed for several cell lines [222, 223] and has been correlated with secreted neutrophil elastase, which, during pneumococcal pneumonia, causes extensive tissue damage in both mouse models and humans [96, 129]. Although dramatic epithelial loss provides an explanation for PLY- and PMN-promoted bacterial transmigration at a MOI of 20, at a lower MOI, wild type TIGR4 was capable of barrier breach in the absence of monolayer destruction, indicating that frank epithelial loss is not required for PLY-promoted bacterial movement in this experimental system.

In summary, by manipulating infectious dose and the presence or absence of PLY or PMNs during apical infection of polarize monolayers, we found that *S. pneumoniae* alters the epithelium and its interaction with PMNs in multiple ways. The bacterium utilizes PLY-dependent and PLY-independent mechanisms to induce cleavage and mislocalization of E-cadherin, as well to recruit PMNs across respiratory epithelial cells monolayers. Notably, E-cadherin disruption alone was insufficient to induce significant bacterial movement across epithelium, which instead depended on PMN transmigration. These studies provide further insight into the multifaceted role of PLY in pulmonary inflammation and barrier breach during *S. pneumoniae* lung infection.

3.4. Materials and Methods

3.4.1. Bacterial strains and growth conditions

Mid-exponential growth phase aliquots of *S. pneumoniae* TIGR4 (serotype 4) were grown in Todd-Hewitt broth (BD Biosciences) supplemented with 0.5% yeast

extract in 5% CO₂ and Oxyrase (Oxyrase, Mansfield, OH), and frozen in growth media with 20% (v/v) glycerol. Bacterial titers in aliquots were confirmed by plating serial dilutions on Tryptic Soy Agar plates supplemented with 5% sheep blood agar (Northeast Laboratory Services, Winslow, ME). The TIGR4 pneumolysin-deficient mutant (Δply) and GFP-expressing WT and Δply TIGR4 were gifts from Dr. Andrew Camilli (Tufts University, Boston, MA). The cholesterol-binding activity-deficient pneumolysin mutant (CBS) was generated by transformation of wild type TIGR4 with linear CBS DNA amplified from *Escherichia coli* plasmid gifted by Dr. Rod Tweten (University of Oklahoma, OH), and screened for non-hemolytic transformants. Insertion of the CBS *ply* gene at the original locus was confirmed by sequencing. For experiments, *S. pneumoniae* strains were grown in Todd-Hewitt broth (BD Biosciences), supplemented with 0.5% yeast extract and Oxyrase, in 5% CO₂ at 37°C and used at mid-log phase to late log phase.

3.4.2. Growth and maintenance of epithelial cells

Human pulmonary mucoepidermoid carcinoma-derived NCI-H292 (H292) cells were grown on the underside of collagen-coated Transwell filters (5 μ m pore size, 0.33cm², Corning Life Sciences) in RPMI 1640 medium (American Type Culture Collection, Manassas, VA) supplemented with 2 mM L-glutamine, 10% FBS, and 100 U penicillin/streptomycin.

3.4.3. Preparation and assessment of polarized H292 monolayers

Polarized H292 monolayers were prepared as previously described [224]. The transepithelial resistance of lung epithelial monolayers is typically very low (unpublished data). Hence, to assess the generation of intact, confluent H292 monolayers, for a sampling of polarized monolayers, we measured the ability of horseradish peroxidase

(HRP) added to the basolateral compartment to be detected in the apical chamber after 20 minutes, as previously described [153]. Potential defects in Transwell monolayer integrity were assessed after collagen coating via detecting excessive buffer loss and after cell seeding via changes in pH of cell media. Approximately 3% of H292 monolayers were excluded from our experiments.

3.4.4. Toxin purification

The expression and purification of recombinant toxins and toxin derivatives from *Escherichia coli* were carried out as previously described [225] with the following modifications. Growth of *E. coli* XL-1 Blue containing pRT20 or derivatives thereof was initiated by inoculating 1 L of sterile TB broth with a 1:100 inoculum of an overnight culture grown at room temperature with 100 µg/mL ampicillin. The 1 L culture was incubated at 37°C with shaking at 200 rpm. At OD₆₀₀ 0.5-0.6, expression of toxin was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG, Fisher Scientific) to a final concentration of 1 mM. Ampicillin was also added to a final concentration of 100 µg/mL. The induced culture was grown overnight at room temperature and pelleted by centrifugation. Cell pellets were resuspended in a total of 30 mL of PBS. Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) was added to prevent proteolytic degradation of the toxin. Cells were lysed by two passages through a microfluidizer, and cell debris was removed by centrifugation at 10,000 x *g* for 30 min at 4°C. The supernatant containing the polyhistidine-tagged toxin was loaded onto a Ni-NTA agarose column (Qiagen). The column was then washed with a 20–120 mM gradient of imidazole to remove additional contaminating proteins. Bound toxin was then eluted (2 mL/min) with 10 mL of PBS containing 500 mM imidazole. SDS-PAGE was performed on the collected fractions to confirm toxin purity. 10% (v/v) glycerol and 5 µM Tris(2-

carboxyethyl)phosphine hydrochloride (TCEP) was added to toxin-containing fractions, which were then flash-frozen and stored at -80°C until use.

3.4.5. Toxin activity and cell viability by FACS

Toxin aliquots were thawed on ice and then spun at 14,000 rpm in a microcentrifuge at 4°C to remove protein precipitate for 10 min. Toxin concentration was determined using Bradford Reagent (BioRad) per the manufacturer's instructions. 5×10^5 H292 cells were placed in 100 μ l volumes in a non-adherent 96 well plate and were treated for 15 minutes at 37°C with toxin at varying concentrations for activity determination, or with *S. pneumoniae* at MOI of 2 or 20 to assess the effect of bacterial infection on membrane permeability. Addition of 5% Triton-X 100 and HBSS+ were used as positive and negative lysis controls, respectively. For PMN viability assessment, 5×10^5 human peripheral blood PMNs were plated per well instead. The plate was then spun at 1200rpm for 5 minutes and cells were resuspended in 1 mg/ml of propidium iodide (PI). Cells were filtered through 100 μ M filters into 100 μ l of PBS with 10% serum, placed on ice, and kept in the dark until analysis by flow cytometry. Cells were run through a FACSCalibur flow cytometer (BD Biosciences) and a minimum of 5×10^4 events were analyzed per replicate. For determining toxin activity unit, collected data were analyzed using FlowJo software (Tree Star, Inc.) to determine the toxin concentration that resulted in 50% of the H292 cells becoming PI+. This concentration was defined as "1 Unit" of toxin activity.

3.4.6. Infection and toxin treatment of polarized H292 monolayers

S. pneumoniae grown to log phase were washed and resuspended to indicated concentrations in Hanks' balanced salt solution (HBSS) supplemented with Ca^{2+} and Mg^{2+} . 25 μ l of bacterial suspension was added to the apical surface of inverted

Transwells and incubated at 37°C with 5% CO₂ for 2.5 hours to allow for attachment and infection of monolayers. To assess for bacterial adhesion, monolayers were infected with GFP-expressing *S. pneumoniae* for 2.5 h hours then fixed with 4% PFA. Adhered bacteria were visualized by IF microscopy and bound bacteria per field of view was used to determine total bound bacteria per monolayer. For toxin treatments, 50 µl of the indicated units of toxin were added to the apical surface of inverted Transwells for 1 h and incubated at 37°C with 5% CO₂. After treatment, Transwells were washed and placed in 24-well plates containing HBSS+Ca²⁺/Mg²⁺ and incubated for an additional 2.5 h to allow for bacteria translocation. Permeability to horseradish peroxidase (HRP) was used to assess monolayer barrier integrity post treatment. Buffer in the basolateral chambers were sampled and bacterial translocation across the monolayer was evaluated by serial dilutions and plating on blood agar. Bacterial translocation index was calculated as total colony forming units (CFUs) in basolateral chamber normalized to the initial inoculum.

3.4.7. PMN transepithelial migration assay

1×10⁶ PMNs, isolated from whole blood obtained from healthy human volunteers, were added to the basolateral chamber after infection of the monolayer with *S. pneumoniae* and allowed to transmigrate for 2.5 hours. PMNs in the apical chamber were quantified by myeloperoxidase (MPO) assay, as described [226]. Briefly, monolayers and their underlying Transwell filters were removed from individual wells of a 24-well plates, leaving the 24-well plate containing the apical buffer and migrated neutrophils for each sample and control well. To assess viability of transmigrated PMNs in the apical chamber, 20 µl of apical chamber buffer was sampled and incubated with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate for detection of released MPO activity. Then, 50 µl of 10% Triton X-100 was added to the apical chamber and gently rocked for

20 min at 4°C to lyse all transmigrated PMNs. 50 µl of citrate buffer was added to each sample and the 24-well plate was gently rocked for 20 min at 4°C. ABTS solution was freshly prepared and 50 µl of hydrogen peroxide was added to the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) solution. 100 µl from each well was transferred to a 96-well plate and 100 µl of ABTS solution was added to each sample in the 96-well plate. The 96-well plate was incubated in the dark at room temperature for 5-10 min until it was read on a microplate reader for absorbance at a wavelength of 405 nm. Absorbance measurement was converted to neutrophil number using a standard curve and used to determine percent PMN transmigrated.

3.4.8. Fluorescence microscopy assessment of monolayer viability and integrity

Monolayer viability and integrity were assessed by fluorescence microscopy of DAPI for cell confluency, E-cadherin for adherens junction organization, and PI for cell permeabilization. To prepare samples for fluorescence microscopy, monolayers were fixed with 4% PFA and stained with 1 mg/ml PI. Another set of fixed monolayers were permeabilized with 0.1% Triton-X 100 in PBS plus 3% BSA, and stained with DAPI, phalloidin, and anti-E-cadherin (24E10), followed by α -rabbit-FITC secondary antibody, and visualized on excised filters. To visualize cell surface exposed E-cadherin, monolayers were stained with ectodomain E-cadherin antibody (DCMA-1) followed by α -rat-FITC secondary antibody, and then permeabilized with 0.1% Triton-X 100 in PBS plus 3% BSA and stained with DAPI (blue) and phalloidin (red). Quantification of E-cadherin junction organization was carried out with the python script IJOQ as previously described [205]. Briefly, the script quantifies spatial distribution of a junctional molecule along the continuous contacts with neighboring cells by determining the degree of intersection between networks of the molecule to a predefined grid-like network.

Percentage epithelial cell loss was quantitated by count of DAPI stained epithelial cell nuclei per field of view.

3.4.9. Western blot

Transwell monolayers were washed three times in HBSS, excised, and lysed in 100 μ l of Pierce radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific) containing protease inhibitors (cOmplete Mini; Roche Diagnostics, Indianapolis, IN). Proteins were resolved by SDS-PAGE under reduced conditions and transferred onto a polyvinylidene difluoride (PVDF) membrane for immunoblotting. Membranes were blocked in Tris-buffered saline–1% Tween (TBS-T) with 5% nonfat milk, and subsequently incubated with the E-cadherin antibody (24E10) at 1:1000. Following washing, immunoblots were probed with the appropriate secondary α -rabbit antibody conjugated to horseradish peroxidase at a dilution of 1:5000. Immune complexes were detected using the SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific), following the manufacturer's instructions, and imaged using a Syngene G:Box XR5 imager. Densitometry analysis was carried out with ImageJ FIJI, normalized to the respective beta actin loading control.

3.4.10. Matrix metalloprotease (MMP) activity

PLY-treated H292 monolayers or PMNs in suspension were treated with 1U PLY and incubated for 30 min at 37°C with 10 μ M fluorogenic peptide MMP substrate (Mca-PLAQAV-Dpa-RSSSR-NH₂, R&D Systems) diluted in 25 mM Tris buffer, pH 8.0. Fluorescence intensity over time was read on a BioTek Synergy HT plate reader and area under the curve of kinetic substrate conversion was used to determine MMP activity.

3.4.11. Ethics statement

All work with human donors was approved by the Tufts University Human Investigation Review Board (IRB). Written informed consent was obtained from all donors and collection was carried out with the assistance of services provided by the Tufts Clinical and Translational Research Center (CTRC).

3.4.12. Presentation of data and statistical analysis

Due to intrinsic donor-to-donor variability of human PMNs and their transmigration, efficiency of transmigration was compared within individual experiments but not between experiments. The conclusions drawn were those found to be reproducible and statistically significant across independent experiments. Statistical analysis was carried out using the program GraphPad Prism (GraphPad Software, San Diego, CA). p values <0.05 were considered significant in all cases. For all graphs, the mean values $\pm$ SEM are shown.

3.5. Contributions

S.X, J.L, and W.A conceived the hypothesis and designed the experiments. S.X and W.A performed the experiments and analyses for Figures 3.1-3.4. S.X performed the experiments and analyses for Figure 3.5A-E. D.M, and F.R performed the experiments and analyses for Figure 3.5G-H. S.X, J.R, and J.P.R performed Western blotting for Figures 3.3 and 3.5. D.M and J.P.R generated and validated the IJOQ Python script used for image analysis in Figures 3.3-3.5. R.T generated PLY toxoids used in Figure 3.5. S.X generated figures and wrote the manuscript with assistance from J.L and W.A. R.T and S.T assisted with manuscript editing.

Chapter 4: Age-associated defect in airway epithelial junctions promotes susceptibility to pulmonary barrier breach by neutrophils following pneumococcal pneumonia⁴

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To be submitted.

4. Age-associated defect in airway epithelial junctions promotes susceptibility to pulmonary barrier breach by neutrophils following pneumococcal pneumonia

4.1. Introduction

Streptococcus pneumoniae (*Sp*; pneumococcus) is a common asymptomatic colonizer of the nasopharynx but can cause serious infections including pneumonia, bacteremia, and meningitis [227], particularly in the elderly population [228]. Despite the availability of vaccines and antibiotics, *Sp* causes ~1.6 million deaths annually, most of which occur in individuals > 65 years of age [229]. Hence, complementary treatment approaches targeting detrimental age-related changes in host response to *Sp* infection are needed to limit complications beyond those observed during pneumococcal infection of young and healthy individuals [121].

A first line barrier against *Sp* invasion and spread is the integrity of the respiratory epithelium. Excessive and/or sustained PMN presence in the airways is associated with severe tissue damage, including to the epithelium [130, 230, 231]. We have previously shown that apical *Sp* infection of a polarized epithelial monolayer triggers production of heparanase (HXA₃), a potent PMN chemoattractant that both boosts PMN tissue-destructive potential and promotes *Sp* transepithelial migration, thus disrupting the epithelial barrier [232]. Inhibition of HXA₃ production or the release or activity of neutrophil elastase (NE) diminishes lethal bacteremia following lung infection [59, 80]. In addition to age-associated functional defects in PMN functions, such as chemotactic accuracy [233], phagocytosis [39], and ROS production [234], the aged host experiences inflammaging, the chronic elevation of inflammatory signaling [22, 40]. *Sp*-infected elderly patients experience higher levels of PMN pulmonary influx [41, 42], which is associated with increased bacterial burden and mortality.

Baseline epithelial barrier function is fostered by apical junction complexes (AJC), comprised of tight junctions (TJ) and adherens junctions (AJ) [185]. TJ proteins,

such as zonula occludens (ZO), claudins, occludins, and junctional adhesion molecules (JAM), localize to the apical region of cell-cell junctions [186] and form an intercellular membrane fence [187]. AJ proteins, such as E-cadherin and catenins, are located basolateral to TJs and are essential for the formation and maturation of cell-cell contact [188]. Orchestration of the abundance and localization of junction proteins, as well as their interactions with the cytoskeleton and signaling modules, is essential for maintaining optimal epithelial barrier function [185]. Loss of junctional integrity is associated with barrier permeability changes, and AJC malfunction of the pulmonary barrier often has deleterious outcomes during diseases of the lung, including pneumonia, acute lung injury, chronic obstructive pulmonary disease (COPD), and asthma [190].

During infection, *Sp* targets AJC proteins for disruption and compromises function of the airway epithelial barrier [157, 186, 191]. Pneumococcal infection reduces alveolar ZO-1, occludins, claudins, and cadherins in human lung explants [186] and murine models [235] [12] [157, 205]. Mice deficient in type I interferon show junctional defects in response to bacterial infection and are highly susceptible to severe bacteremia following lung challenge [191]. Previously, we reported that during apical infection of polarized epithelial monolayers, pneumolysin (PLY) is a key *Sp* virulence factor that results in E-cadherin dissolution [236] and promotes neutrophils, or polymorphonuclear cells (PMNs), to release neutrophil elastase (NE; [69, 232]), a protease frequently implicated in AJC damage during infection and inflammation [69, 91, 98]. Localized disruption of intercellular junctions can generate PMN transmigration 'hotspots' [237, 238], and PLY-mediated junction disruption may contribute to PLY promoted pro-inflammatory activities, including excessive PMN transmigration [13, 134, 239].

Many genes associated with the assembly of AJCs are amongst those with the greatest age-associated decrease in expression in the lungs [31]. Among them is *CDH1*, which encodes E-cadherin, a key regulator of age-related epithelial barrier defects [32]. E-cadherin dysfunction promotes cell turnover and mucin accumulation by activating the epidermal growth factor receptor and β -catenin, contributing to loss of barrier integrity in asthma and COPD [240]. Whether these age-related defects in AJC integrity contribute to susceptibility to *Sp* cross-epithelial dissemination, however, remains to be elucidated.

In this study, we intratracheally challenged young or aged mice with *Sp*, assessing AJC disruption of the lung epithelium and subsequent bacteremia. We also apically infected respiratory stem cell-derived air-liquid interface (ALI) epithelial monolayers derived from young or aged mice, assessing E-cadherin organization, barrier function, and bacterial translocation. Our findings support a model in which the age-associated defect in AJC integrity is greatly amplified by *PLY*-producing *Sp*, resulting in significant increases in PMN transmigration, barrier disruption, and bacterial translocation. Pharmacologic fortification of AJC, particularly in combination with a NE inhibitor, ameliorated barrier loss and age-related susceptibility to systemic *Sp* infection.

4.2. Results

4.2.1. Upon pulmonary *Sp* challenge of mice, *PLY* alters E-cadherin organization prior to promoting PMN influx, epithelial barrier disruption, and bacteremia

The airway epithelium depends on intact AJCs to maintain proper barrier function and defense against microbial invasion. To examine whether *PLY*-expressing *Sp* damages airway epithelial junctions during pulmonary infection, we challenged 2-month-old BALB/c mice intratracheally (*i.t.*) with 1×10^7 CFU of wild type (WT) or *PLY*-deficient Δply *Sp*. *PLY* had no effect on *Sp* fitness in the lungs as lung burden between WT and Δply *Sp*-infected mice were indistinguishable from each other during an 18-hour infection

course (Figure 4.1a). To assess epithelial junction integrity, we performed immunofluorescence (IF) confocal microscopy of lung sections, visualizing localization of E-cadherin as well as the nuclear dye DAPI, the F-actin stain phalloidin, and a PMN marker Ly6G. In uninfected animals, E-cadherin staining primarily localized to the bronchial epithelium and a few dispersed and rounded (likely alveolar type II) cells along the alveolar wall (Figure 4.1b, “Uninf.”). Upon infection with either WT or Δply *Sp*, E-cadherin signal was progressively enhanced over time and lined the alveolar walls more continuously (Figure 4.1b), consistent with literature reporting airway epithelial AJC upregulation in response to *Sp* invasion [191]. Interestingly, by 6 hours post infection (h.p.i.) and 18 h.p.i, E-cadherin levels in WT *Sp*-infected mice was lower and lagging behind that of Δply -infected mice (Figure 4.1b, “WT *Sp*” vs. “ Δply *Sp*”). We quantitated E-cadherin signal intensity at these time points and found that presence of PLY resulted in significant 4-fold and 1.5-fold reductions in alveolar E-cadherin by 6 and 18 h.p.i respectively (Figure 4.1c), suggesting that PLY-expressing *Sp* disrupted airway AJCs during mouse pulmonary challenge.

Infection of lung mucosa by PLY-producing *Sp* drives robust PMN infiltration into the airways and can damage the airway epithelial barrier [59, 136]. We thus examined whether AJC damage was a result of PMN recruitment or damage to the tissue barrier promoted by PLY. To quantitate PMN infiltration to the lungs, we stained lung homogenates for the PMN marker Ly6G and analyzed by flow cytometry. As quickly as 6 h.p.i, WT and Δply *Sp* infected animals showed higher than baseline levels of Ly6G⁺ cells in the lungs (Figure 4.1d, “6h”). However, to our surprise, significant PLY-promoted PMN infiltration was only observed at 18 h.p.i. (Figure 4.1d, “18h”), well after significant E-cadherin disruption had already taken place (Figure 4.1b-c). Similarly, assessment of tissue barrier damage by pulmonary leakage of intravenously (*i.v.*) administered 70 kDa FITC-dextran showed no substantial leakage out to 6 h.p.i. (Figure 4.1e, “6h”). Only by

18 h.p.i. did PLY promote significant pulmonary permeability, with WT *Sp* infection resulting in a 2.5-fold increase in dextran leakage compared to Δply (Figure 4.1e, “18h”). Correspondingly, at 6 h.p.i. WT and Δply *Sp* infected mice sustained similar levels of bacteremia as an indicator of *Sp* escape across the airway barrier into the bloodstream (Figure 4.1f, “6h”), while significant PLY-promoted bacteremia was observed only at 18 h.p.i. (Figure 4.1f, “18h”). This suggests that E-cadherin disruption by PLY precedes the toxin’s actions in promoting PMN influx, pulmonary barrier damage, and bacteremia.

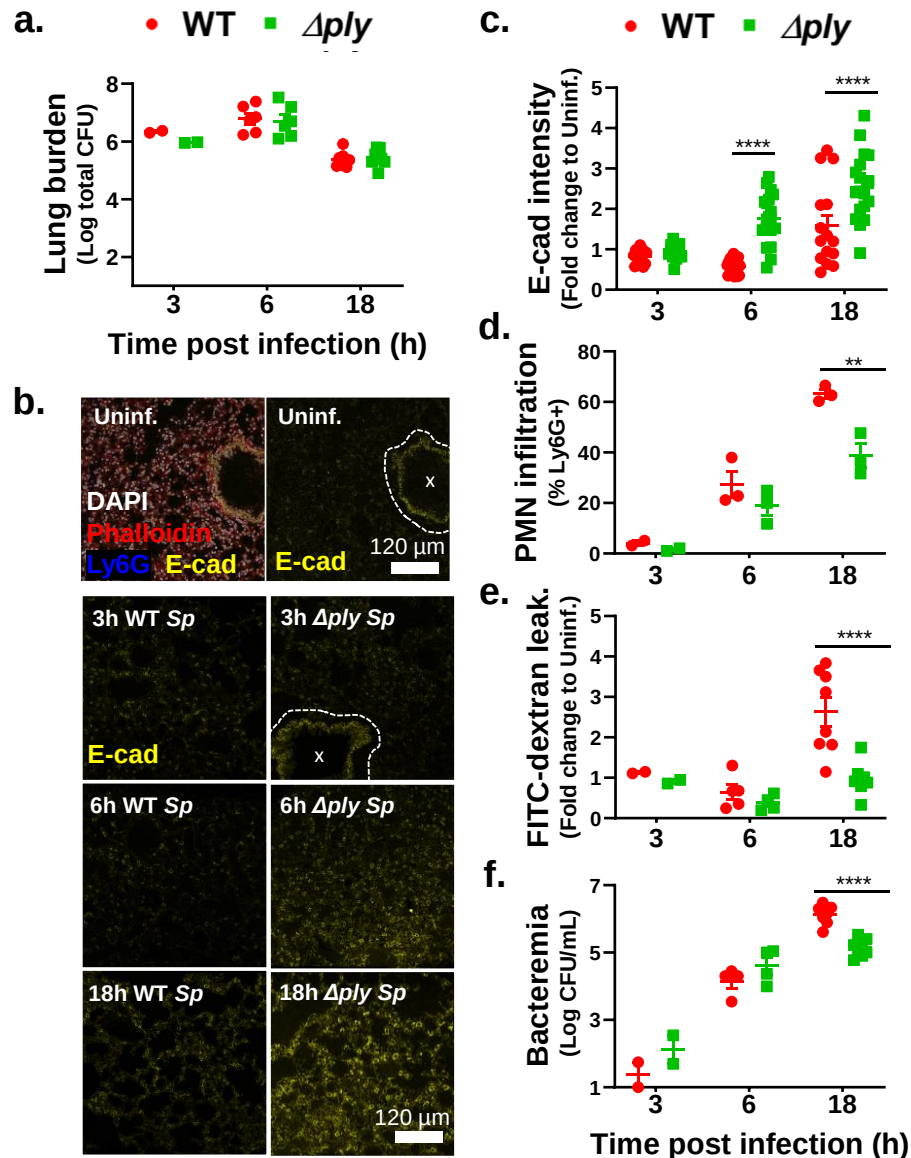


Figure 4.1. Upon pulmonary *Sp* challenge of mice, PLY alters E-cadherin organization prior to promoting PMN influx, epithelial barrier disruption, and bacteremia. 2-month-old young BALB/c mice were infected i.t. with 1×10^7 wild type (WT) or PLY-deficient mutant (Δply) *Sp* for 3, 6, or 18 hours. **(a)** Bacterial lung burden determined by measuring CFU in lung homogenates. **(b)** Lung section immune fluorescence (IF) microscopy images visualizing E-cadherin, Ly6G, DNA, and F-actin localization, bronchial epithelium excluded from analysis marked by dotted line. Scale bar = 120 μm . **(c)** Alveolar E-cadherin quantitated by signal intensity analysis in Image J, **(d)** PMN infiltration determined by flow cytometric enumeration of Ly6G⁺. **(e)** Lung permeability quantitated by measuring the concentration of 70 kDa FITC-dextran in the lung relative to serum after *i.v.* administration. **(f)** Bacteremia measured by enumerating CFU in serum. Each panel is representative of three independent experiments, or pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis between WT and Δply groups at each time point was performed using ordinary one-way ANOVA: **p-value < 0.01, ****p-value < 0.0001.

4.2.2. PLY-producing *Sp* directly disrupts E-cadherin organization and promotes PMN transmigration and barrier disruption *in vitro*

Previously, we reported that PLY triggers E-cadherin dissolution of polarized H292 lung carcinoma cell monolayers through direct PLY pore-forming action on epithelial cells independent of PMN presence [236]. However, the pathological implications of diminished AJC integrity during *Sp* infection were difficult to conclude, confounded by other pro-bacterial dissemination activities of PLY, such as epithelial secretion of the PMN chemoattractant HXA₃ [59, 136] and PMN release of the tissue-damaging protease NE [232]. To elucidate the effects of PLY-triggered AJC dissolution on PMN transmigration and *Sp* cross-epithelial dissemination, we performed apical infection of Transwell-seeded airway basal stem cell (BSC)-derived ALI epithelial monolayers [160], a genetically tractable culture model recapitulating important aspects of *Sp* pathogenesis during mucosal infection [232]. After 2 hours of infection with 1×10^7 WT or Δply *Sp*, we fixed monolayers and visualized E-cadherin localization by IF confocal microscopy (Figure 4.2a). In uninfected ALI monolayers, E-cadherin localized to cell periphery forming a connected network of circumferential rings (Figure 4.1a, “Uninf.”). Upon infection, we observed a loss of cell periphery E-cadherin that was more severe with infection by WT *Sp* than with Δply *Sp* (Figure 4.1a, “WT” vs “ Δply ”), consistent with PLY inducing E-cadherin dissolution more efficiently than other PLY-independent pathways of AJC disruption by *Sp* [236]. To quantitate loss of E-cadherin organization, we performed Intercellular Junction Organization Quotient (IJOQ) determination in Python, which measures the continuity in pericellular junction distribution rather than just signal intensity [205]. IJOQ quantification confirmed that E-cadherin organization was significantly reduced upon WT *Sp* infection (~70% reduction), a significantly higher degree of disruption than the mild 35% reduction upon infection by

Δply Sp (Figure 4.1b). Thus, ALI monolayers, like the previously reported H292 monolayer model [236], sustained AJC injury upon infection by PLY-producing *Sp* independently of PMN presence.

Sites of AJC disorganization are often exploited by infiltrating PMNs to generate “hotspots” of transmigration [237, 238]. To investigate whether PLY-triggered AJC dissolution facilitates PMN transmigration and/or compromises epithelial barrier function, we decoupled the effects of PLY-induced AJC disruption and PLY-induced PMN chemoattractant secretion by the infected epithelium. To do this, 12-LOX deficient *Alox15^{-/-}* ALI monolayers lacking the ability to produce HXA₃ were either left uninfected or primed by infecting with 1×10⁷ WT or *Δply Sp* to induce different degrees of AJC disorganization. We then harvested *Sp* infection supernatant from apically infected WT B6 mouse-derived ALI monolayers and transferred this supernatant to the apical chambers of infection-primed *Alox15^{-/-}* ALI monolayers. 1×10⁶ PMNs were added basally. Infection supernatant induced robust PMN transmigration across WT *Sp* primed *Alox15^{-/-}* ALI monolayers (Figure 4.2c, “WT + *Sp* inf. supe”), whereas supernatant collected from uninfected ALI monolayers had no chemotactic activity (Figure 4.2c, “Uninf. supe”). Compared to the degree of transmigration across WT *Sp* primed *Alox15^{-/-}* ALI monolayers, transmigration crossing uninfected or *Δply* primed *Alox15^{-/-}* ALI monolayers, which have intact junctions and less perturbed junctions respectively, was 2-fold and significantly lower (Figure 4.2c, “Uninf.+ *Sp* inf. supe”, “*Δply* + *Sp* inf. supe”). This suggests that even when responding to the same chemotactic supernatant, PMNs transmigrate more efficiently through ALI monolayers with disorganized junctions (WT *Sp* infection primed) than ALI monolayers with intact junctions (uninfected and *Δply Sp* infection primed).

In agreement with the level of PMN transmigration, when we measured barrier disruption by cross-epithelial leakage of the protein marker horseradish peroxidase

(HRP) and *Sp* translocation, only WT primed *Alox15^{-/-}* ALI monolayers sustained significant HRP flux and *Sp* translocation post-PMN transmigration to infection supernatant (Figure 4.2d-e, “WT+ *Sp* inf. supe”). Uninfected *Alox15^{-/-}* ALI monolayers remained impermeable to HRP flux post-PMN transmigration (Figure 4.2d, “Uninf.+ *Sp* inf. supe”), while $\Delta p/ly$ infected *Alox15^{-/-}* ALI monolayers sustained a mild level of HRP flux and *Sp* translocation that did not reach statistical significance (Figure 4.2d-e, “ $\Delta p/ly$ + *Sp* inf. supe”) when compared to uninfected supernatant supplemented *Alox15^{-/-}* ALI monolayers that remained completely intact regardless of *Sp* priming or basal PMNs (Figure 4.2d-e, “uninf. supe”). These data suggest that AJCs may be acting as gate keepers to PMN influx, and that PLY-dependent junction disorganization permitted excessive PMN transmigration which subsequently damaged the epithelial barrier and promoted *Sp* translocation.

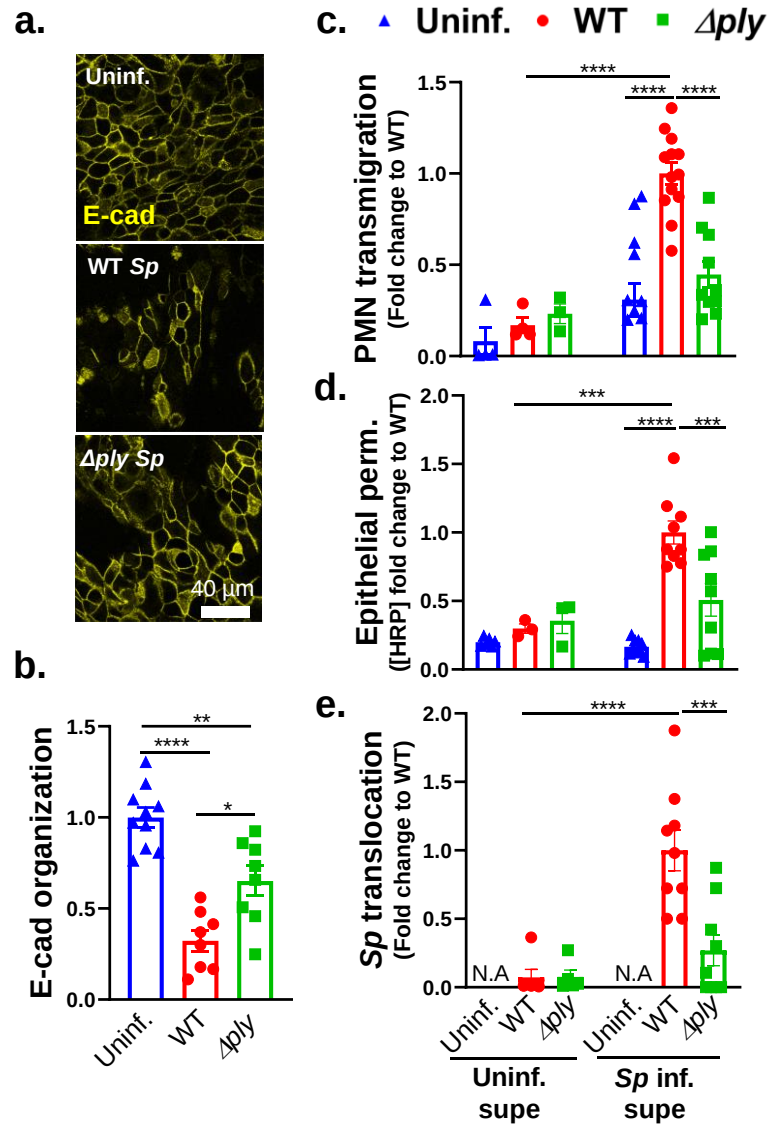


Figure 4.2. PLY-producing *Sp* directly disrupts E-cadherin organization and promotes PMN transmigration and barrier disruption *in vitro*. Healthy adult human BSC-derived ALI epithelial monolayers were apically infected with 1×10^7 WT or Δply *Sp*. **(a)** IF microscopy images of fixed and permeabilized monolayers visualizing E-cadherin localization. Scale bar = 40 μm . **(b)** E-cadherin organization quantitated by image analysis via the InterCellular Junction Organization Quotient (IJOQ) script in Python. **(c)** 12-LOX-deficient *Alox15^{-/-}* mouse-derived ALI monolayers were apically infected with 1×10^7 WT or Δply *Sp* and transferred into apical chambers containing apical supernatant harvested from uninfected or WT *Sp*-infected WT B6 mouse-derived ALI monolayers. 1×10^6 PMNs were added basally to monolayers; after 2 hours of PMN migration, the degree of transmigration as determined by MPO activity in the apical chamber. **(d)** Epithelial permeability measured by HRP flux. **(e)** *Sp* translocation quantitated by measuring basolateral CFU. Each panel is representative of three independent experiments, or pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value ≤ 0.05 , ***p-value ≤ 0.001 , ****p-value ≤ 0.0001 .

4.2.3. Aged mice show diminished E-cadherin, and increased lung permeability and barrier disruption following pulmonary *Sp* challenge

A decrease in TJ and AJ gene expression occurs in the aging human lung [31]. To examine age-related changes in airway AJC integrity and their potential role in promoting susceptibility to *Sp* infection, we performed parallel *i.t.* infection of young (2-month-old) or aged (>20-month-old) BALB/c mice with 1×10^7 CFU of WT or Δply *Sp*. Consistent with previous reports in the literature [43], we found that aged mice were highly susceptible to pneumococcal infection. At 18 h.p.i., WT and Δply *Sp* infected aged mice suffered 100-fold and 40-fold higher lung burden than young mice, respectively (Figure 4.3a). Epithelial AJC integrity assessment by IF revealed significantly lower alveolar E-cadherin in both WT and Δply infected aged mice compared to the young by 18 h.p.i (Figure 4.3b). Image quantification revealed that while young mice exhibited robust E-cadherin upregulation in response to *Sp* and a counteracting *PLY*-dependent E-cadherin loss, E-cadherin levels in infected aged mice, regardless of infection by WT or Δply *Sp*, was indistinguishable from the low basal E-cadherin level of uninfected mice (Figure 4.3c).

Compared to infected young mice, both WT and Δply *Sp* infected aged mice recruited significantly more PMNs into the airways (Figure 4.3d). WT *Sp* infection of aged mice resulted in 5-fold higher FTIC-dextran leakage compared to uninfected aged mice, which was 2.5-fold higher than infected young mice (Figure 4.3e, “WT”). Δply *Sp* remained unable to inflict substantial damage to the pulmonary barrier despite host age, as no change in FTIC-dextran leakage was observed in Δply infected young or aged mice (Figure 4.3e, “ Δply ”). Correspondingly, aged mice suffered higher levels of bacteremia than the young, resulting in 5-fold and 20-fold higher blood burden upon WT and Δply infection respectively (Figure 4.3f). Together, these data suggest that age-

related decline in AJC integrity correlated with susceptibility to excessive PMN infiltration, pulmonary barrier leakage, and *Sp* systemic spread in the aged hosts.

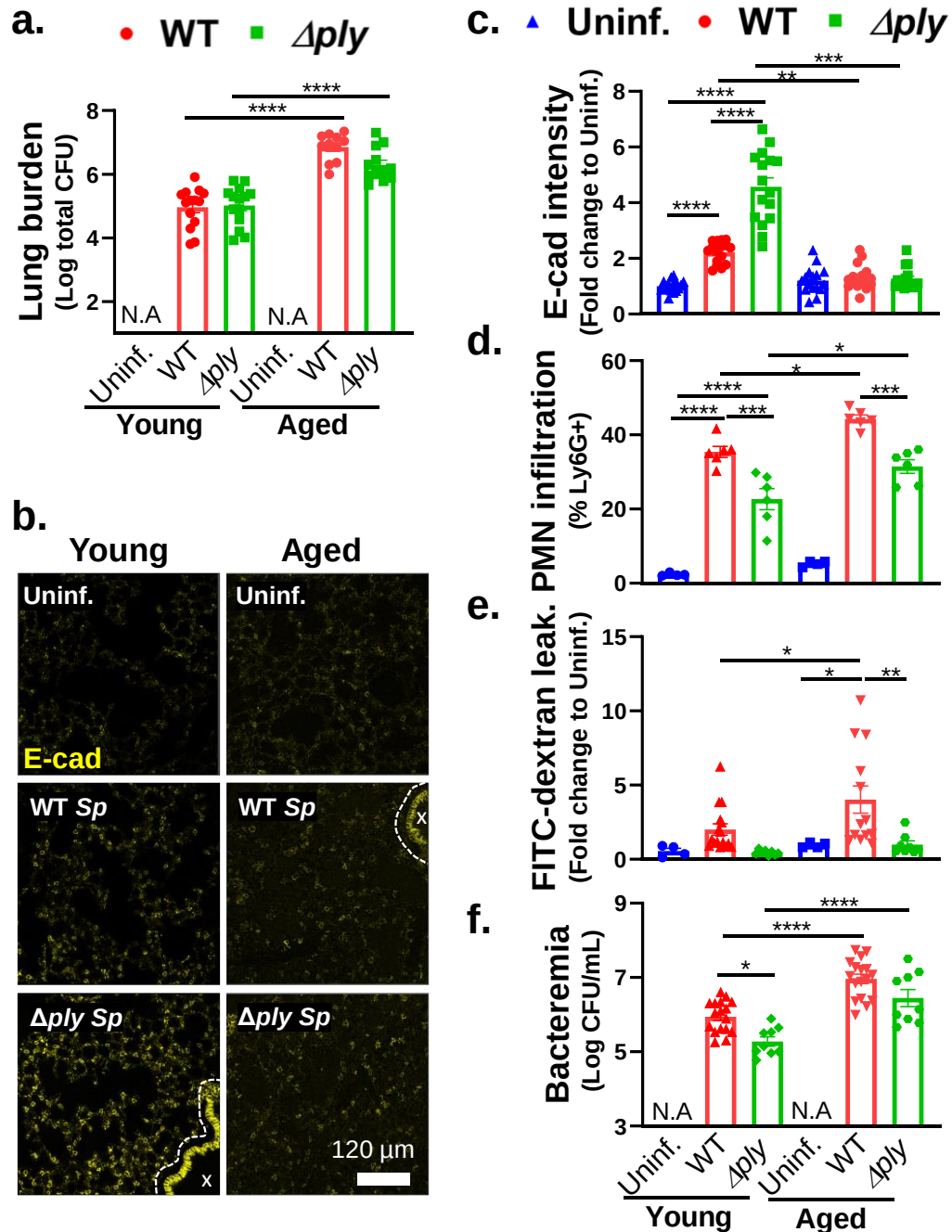


Figure 4.3. Aged mice show diminished E-cadherin, and increased lung permeability and barrier disruption following pulmonary *Sp* challenge. 2-month-old (young) and 22-month-old (aged) BALB/c mice were infected i.t. with 1×10^7 WT or Δply *Sp* for 18 hours. **(a)** Bacterial lung burden determined by measuring CFU in lung homogenates. **(b)** Lung section IF microscopy images visualizing E-cadherin localization, with bronchial epithelium excluded from analysis marked by dotted line. Scale bar = 120 μm . **(c)** Alveolar E-cadherin quantitated by signal intensity analysis in

Image J. **(d)** PMN infiltration determined by flow cytometric enumeration of Ly6G⁺. **(e)** Lung permeability quantitated by measuring the concentration of 70 kDa FITC-dextran in the lung relative to serum after *i.v.* administration. **(f)** Bacteremia measured by enumerating CFU in serum. Each panel is representative of three independent experiments, or pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value < 0.05, **p-value < 0.01, ****p-value < 0.0001.

4.2.4. ALI epithelium derived from aged mice are more susceptible to E-cadherin disruption and barrier breach during *Sp* infection

To investigate whether age-related AJC defects are cell intrinsic, we isolated airway BSCs from young and aged mice to generate young and aged ALI epithelial monolayers. All ALI monolayers regardless of age passed quality control of reaching a TEER of at least 1000 Ohms and being impermeable to HRP flux before being used for infection experiments. We first imaged basal levels of AJC in uninfected ALI monolayers. E-cadherin was robustly expressed and localized to circumferential rings at cell peripheries in young ALI monolayers, but was weaker and less continuous in aged ALI monolayers, with IJOQ quantification showing a significant 25% reduction in E-cadherin organization compared to the young (Figure 4.4a-b, “Uninf.”). We then infected young and aged ALI monolayers with 1×10^7 WT or Δply *Sp* to visualize the degree of AJC disruption upon infection. In young ALI monolayers, we observed substantial loss of E-cadherin with WT *Sp* infection but less so with Δply *Sp* (Figure 4.4a, “Young”). In contrast, both WT and Δply infection resulted in near complete dissolution of cell periphery E-cadherin in aged ALI monolayers (Figure 4.4a, “Aged”). IJOQ quantification confirmed that WT *Sp* infection resulted in 42% E-cadherin organization reduction in young ALI monolayers, but a significantly higher 87% reduction in aged ALI monolayers (Figure 4.4b). Similarly, disruption by Δply *Sp* infection was merely 19% in young but as high as 47% in aged ALI monolayers (Figure 4.4b). This suggests that AJCs of the aged

epithelium are less robust and more prone to disruption by both PLY-dependent and independent pathways of E-cadherin disruption.

To examine whether defective epithelial AJCs promoted PMN infiltration and/or *Sp* movement across the mucosal barrier, we added young PMNs to the basolateral chamber of *Sp* infected young or aged ALI monolayers and measured PMN transmigration, HRP flux, and *Sp* translocation. PMNs did not move across uninfected young or aged ALI monolayers (Figure 4.4c, “Uninf.”). Upon *Sp* infection, PMN transmigration was promoted by PLY in both young and aged ALI monolayers, and only 1.3-fold ($p=0.091$, not significantly) higher across aged ALI monolayers (Figure 4.4c). This similar level of PMN influx was in spite of the apical supernatant harvested from aged versus young ALI monolayers, which had 1.3-fold ($p=0.056$) lower chemotactic activity when transferred to *Alox15^{-/-}* ALI monolayers (Supplemental Figure 3.1a). Nonetheless, HRP flux was 1.8-fold ($p<0.01$) and 2.5-fold ($p<0.05$) higher across aged ALI monolayers following PMN transmigration, upon infection with WT or Δply *Sp* respectively (Figure 4.4d). WT *Sp* translocation was 3-fold and significantly higher in aged ALI monolayers compared to the young, whereas Δply *Sp*, which translocated only minimally across young ALIs, translocated 30-fold higher across aged ALI monolayers (Figure 4.4e). Differences in HRP flux and *Sp* translocation were not a result of different epithelial secretions by young and aged ALI monolayers upon *Sp* infection, as infection supernatant collected from young or aged ALI monolayers triggered indistinguishable levels of HRP flux and *Sp* translocation across *Alox15^{-/-}* ALI monolayers (Supplemental Figure 3.1b-c). Instead, it is likely that direct PMN contact with the aged epithelium and its defective AJC resulted in heightened susceptibility to barrier breach by *Sp*.

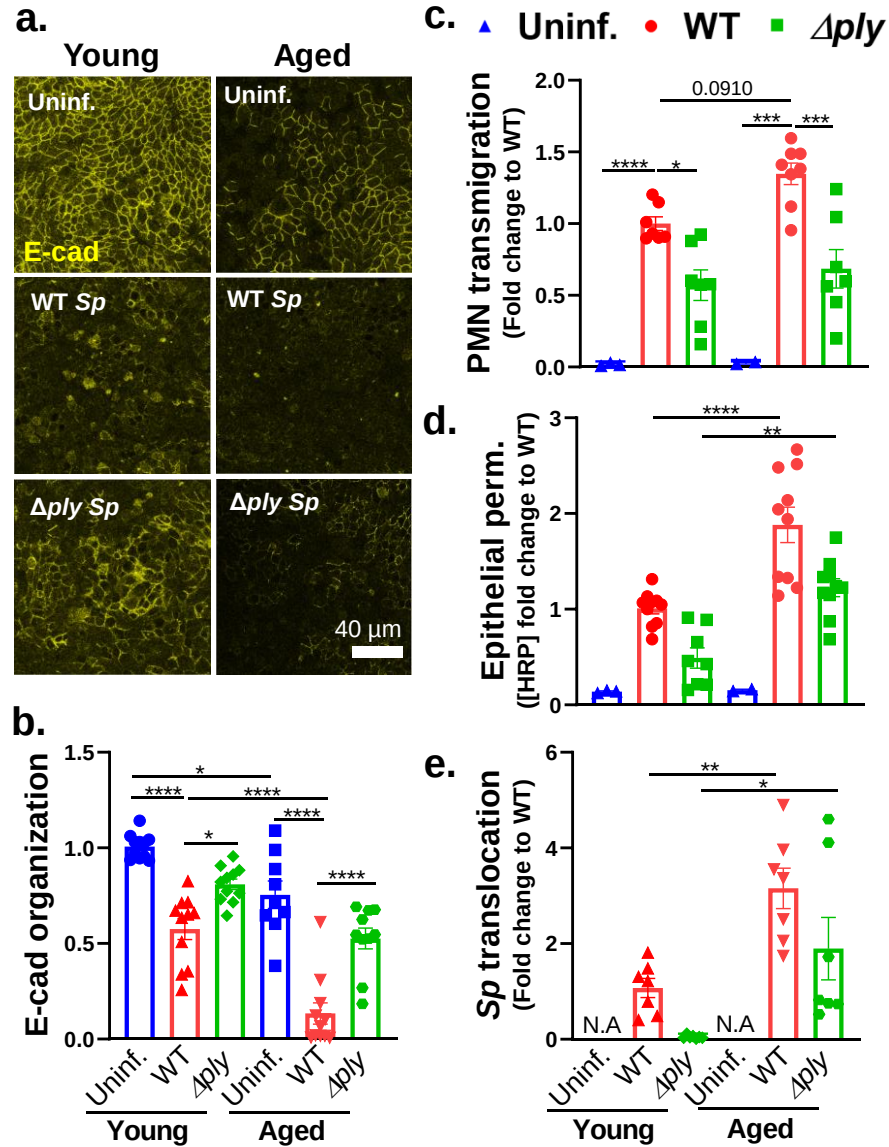


Figure 4.4. ALI epithelium derived from aged mice are more susceptible to E-cadherin disruption and barrier breach during *Sp* infection. 2-month-old (young) and 22-month-old (aged) mouse BSC-derived ALI monolayers were apically infected with 1×10^7 WT or Δ ply *Sp*. (a) IF microscopy images of fixed and permeabilized monolayers visualizing E-cadherin localization. Scale bar = 40 μ m. (b) E-cadherin organization quantitated by image analysis via the IJOQ script in Python. (c) 1×10^6 PMNs were added basally to monolayers; after 2 hours of PMN migration, the degree of transmigration as determined by MPO activity in the apical chamber. (d) Epithelial permeability measured by HRP flux. (e) *Sp* translocation quantitated by measuring basolateral CFU. Each panel is representative of three independent experiments, or pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value $\leq$ 0.05, **p-values $\leq$ 0.01, ***p-values $\leq$ 0.001, ****p-value $\leq$ 0.0001.

4.2.5. Junction fortification diminishes PMN transmigration, barrier disruption, and bacterial translocation upon *Sp* infection of ALI epithelium derived from young or aged mice

In mouse and ALI models of acute lung injury [241, 242], Nrf2 activation by agonists such as bardoxolone methyl (CDDO) promotes expression of AJC proteins, particularly E-cadherin, and preserves AJC integrity [243, 244]. Given that E-cadherin is a postulated key regulator of age-related barrier defect [32], we examined if CDDO pretreatment could protect against AJC disruption and barrier breach upon *Sp* infection. CDDO enhanced E-cadherin organization in uninfected ALI monolayers (Figure 4.5a-b, “Veh.” vs “CDDO”) and resisted E-cadherin dissolution upon *Sp* infection (Figure 4.5a-b, “*Sp*+Veh.” vs “*Sp*+CDDO”) in young ALI monolayers. CDDO pretreatment of aged ALI monolayers did not substantially improve E-cadherin organization at the basal level (Figure 4.5a-b, “Veh.” vs “CDDO”), but protected against E-cadherin loss upon *Sp* infection (Figure 4.5a-b, “*Sp*+Veh.” vs “*Sp*+CDDO”). CDDO treated ALI monolayers were diminished in PMN transmigration by 90% in the young and 40% in the aged (Figure 4.5c). Correspondingly, CDDO protected against barrier integrity loss and reduced HRP leakage by 70% and 25%, and *Sp* translocation by 90% and 60% respectively in young and aged ALI monolayers (Figure 4.5d-e).

To determine whether the protective effects of CDDO treatment is specific to its action on AJC or through modulation of epithelial secretions such as HXA₃ [232], we pretreated *Sp* infected *Alox15^{-/-}* ALI monolayers with CDDO and supplemented the apical chamber with either infection supernatant or 10 nM purified HXA₃ methyl ester. PMN infiltration to both infection supernatant and purified HXA₃ was significantly diminished when across CDDO-treated *Alox15^{-/-}* ALI monolayers (Supplemental Figure 3.2a). In accordance, both HRP flux and *Sp* translocation was significantly reduced upon CDDO treatment (Supplemental Figure 3.2b-c). Thus, CDDO fortification of epithelial

AJCs limited PMN transmigration and barrier breach during *Sp* infection, acting independently of 12-LOX activity and was effective for both infection of young or aged epithelial cultures.

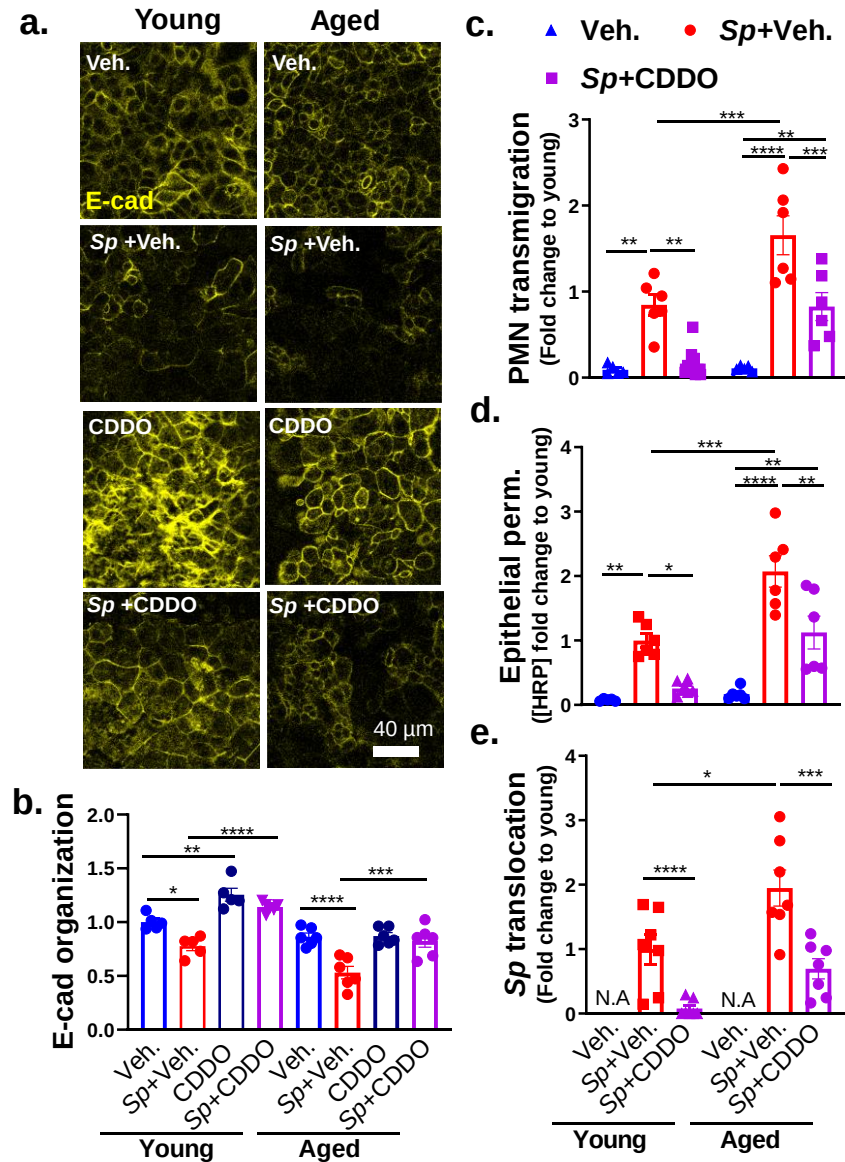


Figure 4.5. Junction fortification diminishes PMN transmigration, barrier disruption, and bacterial translocation upon *Sp* infection of ALI epithelium derived from young or aged mice. Young and aged mouse BSC-derived ALI monolayers were pretreated with vehicle (DMSO) or 100nM bardoxolone methyl (CDDO) before apical infection with 1×10^7 WT *Sp*. **(a)** IF microscopy images of fixed and permeabilized monolayers visualizing E-cadherin localization. Scale bar = 40 μ m. **(b)** E-cadherin organization quantitated by image analysis via the IJOQ script in Python. **(c)** 1×10^6 PMNs were added basally to monolayers; after 2 hours of PMN migration, the degree of transmigration as determined by MPO activity in the apical chamber. **(d)** Epithelial

permeability measured by HRP flux. **(e)** *Sp* translocation quantitated by measuring basolateral CFU. Each panel is representative of three independent experiments, or pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value $\leq$ 0.05, ***p-value $\leq$ 0.001, ****p-value $\leq$ 0.0001.

4.2.6. Aged PMNs are associated with higher levels of NE in response to *in vitro* and *in vivo* *Sp* infection

While CDDO diminished barrier integrity loss during *Sp* infection of both young and aged ALI monolayers, HRP flux and *Sp* translocation across CDDO-treated aged ALI monolayers remained substantially higher than that of treated young ALI monolayers (Figure 4.5d-e). This suggests that there are additional factors contributing to age-related susceptibility to barrier breach during *Sp* infection. PMNs from elderly humans are elevated in NE activity upon exposure to *Sp* [245] and NE is a key driver of lung barrier breach during pulmonary *Sp* challenge in mice [232]. Bone-marrow isolated PMNs from aged mice were elevated in NE activity upon *Sp* exposure compared to the young (Figure 4.6a). Correspondingly, *Sp* infected aged mice showed higher BALF NE activity compared to their young counterparts (Figure 4.6b, "Veh."). Since E-cadherin is a substrate for NE [98, 156], we targeted NE for inhibition using the clinically approved NE activity inhibitor Sivelestat or the PMN degranulation inhibitor Nexinhib20 (Nex), previously shown to diminish bacteremia following *Sp* lung challenge in young mice [232]. Pretreatment of PMNs with Siv or Nex before loading to the basolateral chamber of ALI monolayers diminished the near complete E-cadherin loss following *Sp* infection and PMN infiltration (Supplemental Figure 3.3a-b). Siv and Nex resulted in moderate reductions of PMN transmigration compared to vehicle control by 38% and 48% respectively (Supplemental Figure 3.3c), as well as significantly decreasing HRP leakage by 53% and 69% respectively, and *Sp* translocation by 58% and 78% respectively (Supplemental Figure 3.3d-e). NE thus promotes AJC loss upon PMN

addition, facilitates PMN transmigration, and contributes to barrier dysfunction triggered by *Sp* infection.

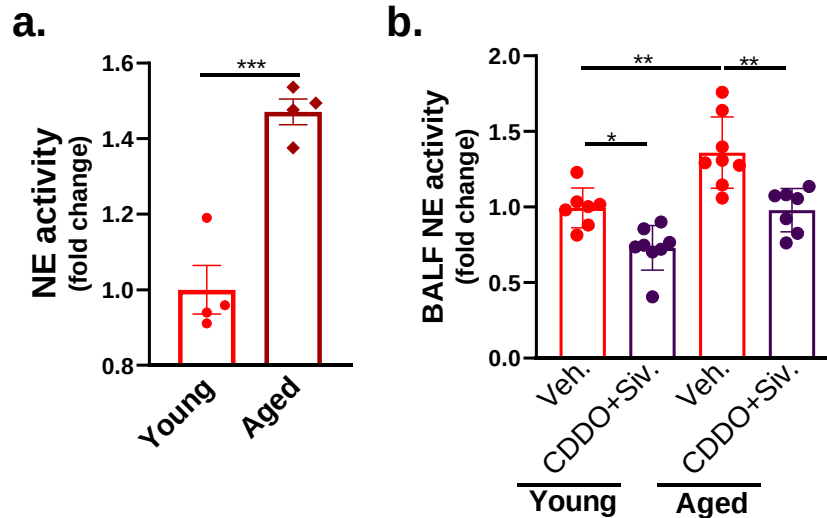


Figure 4.6. Aged PMNs are associated with higher levels of NE in response to *in vitro* and *in vivo* *Sp* infection. (a) 1×10^6 young or aged mouse bone marrow isolated PMNs were infected with 1×10^7 WT *Sp* and released NE activity quantitated by substrate conversion. (b) Young and aged BALB/c mice were treated i.p. with a combination of 100 $\mu\text{g}/\text{mice}$ of CDDO and 500 $\mu\text{g}/\text{mice}$ Siv at 1 hour prior to i.t. infection with 1×10^7 WT *Sp*. NE activity in cell-free BALF determined by substrate conversion. Each panel is representative of three independent experiments, or pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value < 0.05, **p-value < 0.01, ***p-values $\leq$ 0.001.

4.2.7. Barrier disruption and *Sp* bacteremia are diminished by junction fortification and NE inhibition in young and aged mice

Given that aged mice exhibited both defective AJC and elevated NE in response to *Sp* infection, we examined protective effects of either single CDDO treatment or dual CDDO and Siv treatment in young and aged mice upon *Sp* pulmonary challenge. At 18 h.p.i, CDDO single treatment did not significantly affect bacterial lung burden or PMN infiltration, in either young or aged mice (Figure 4.7a-b, “CDDO”). AJC integrity assessment by IF microscopy revealed higher E-cadherin level in both young and old CDDO-treated *Sp* infected mice than in vehicle-treated counterparts (Figure 4.7c-d).

Correspondingly, CDDO significantly decreased lung permeability to FITC-dextran in young and aged mice by 2-fold and 5-fold, respectively (Figure 4.7e), and, as predicted, diminished bacteremia by 5-fold ($p=0.04$) in the young, and 10-fold ($p=0.003$) in the aged (Figure 4.7f).

We then investigated whether adjunctive NE inhibition provided additional therapeutic benefit on top of AJC fortification. Dual CDDO and Siv treatment diminished BALF NE activity in infected aged mice to young mouse levels (Figure 4.6b, “CDDO+Siv”) but had no additional effect on lung burden, PMN infiltration, or FITC-dextran permeability in infected young or aged mice beyond that of CDDO alone (Figure 4.7a-b,e, “CDDO+Siv”). In young mice, dual treatment diminished bacteremia to the same degree as single CDDO treatment (Figure 4.7f, “CDDO+Siv”), suggesting that CDDO and Siv both act to preserve AJCs and are redundant to each other in the young hosts. However interestingly in aged mice, dual treatment resulted in a significant 5-fold additional protection against bacteremia compared to CDDO treatment alone (Figure 4.7f, “CDDO+Siv”). This suggests that elevated NE in aged mice exacerbated pulmonary barrier breakdown during *Sp* infection, and that AJC fortification and NE blockade together is required for maximal protection in the aged and susceptible hosts.

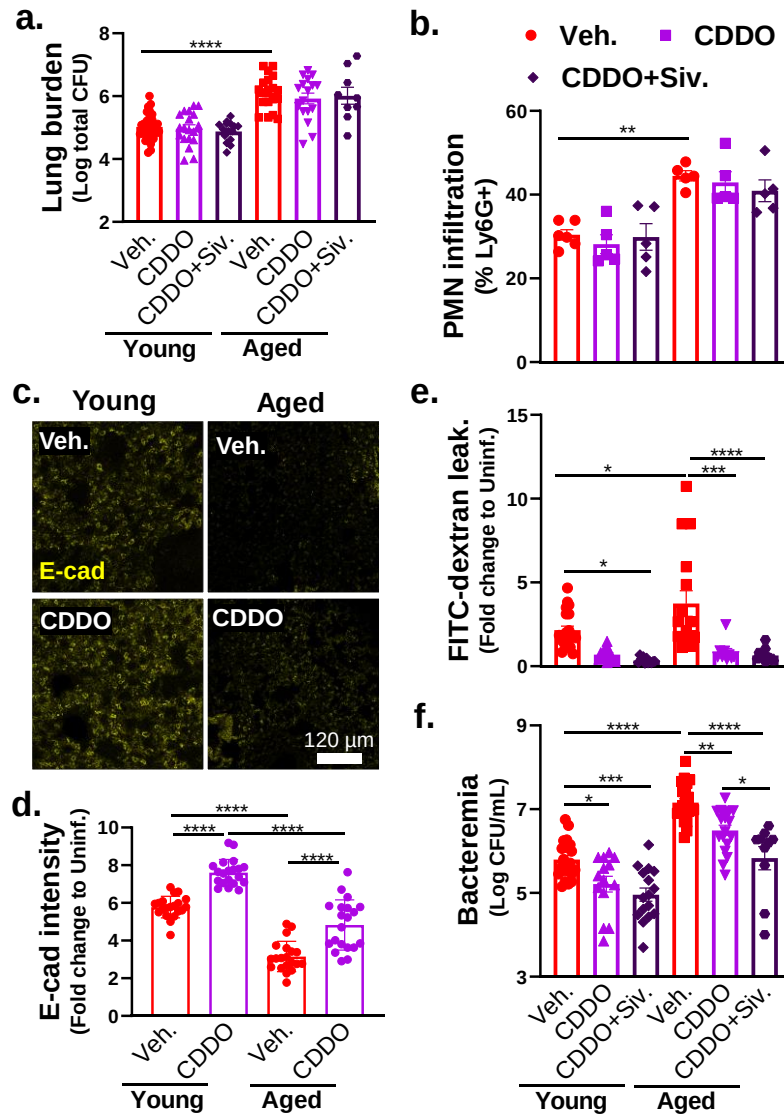


Figure 4.7. Barrier disruption and *Sp* bacteremia are diminished by junction fortification and NE inhibition in young and aged mice. Young and aged BALB/c mice were treated i.p with 100 µg/mice of CDDO or combination of 100 µg/mice of CDDO and 500 µg/mice Sivelestat (Siv) at 1 hour prior to i.t. infection with 1×10^7 WT *Sp*. **(a)** Bacterial lung burden determined by measuring CFU in lung homogenates. **(b)** PMN infiltration determined by flow cytometric enumeration of Ly6G⁺. **(c)** Lung section IF microscopy images visualizing E-cadherin localization. Scale bar = 120 µm. **(d)** Alveolar E-cadherin quantitated by signal intensity analysis in Image J. **(e)** Lung permeability quantitated by measuring the concentration of 70 kDa FITC-dextran in the lung relative to serum after i.v. administration. **(f)** Bacteremia measured by enumerating CFU in serum. Each panel is representative of three independent experiments, or pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value < 0.05, **p-value < 0.01, ***p-value $\leq$ 0.001, ****p-value < 0.0001.

4.3. Discussion

The respiratory mucosa is in direct exposure to the outside environment and is a crucial front line of defense against inhaled foreign microbes. The AJC establishes tight intercellular seals between adjacent airway epithelial cells, maintaining integrity of the mucosal barrier and controlling epithelial permeability. Many respiratory pathogens, bacterial and viral [246], evolved mechanisms to break down AJCs as a strategy to promote infection, as opened junctions not only grant microbes the ability to invade deep into airway tissues, but can also provide access to the bloodstream for systemic dissemination [246, 247].

Sp targets airway AJCs for disruption during infection of epithelial cultures [12, 157, 236], human lung tissue explants [186], and in mouse experimental challenge models [205, 235], with numerous reported mechanisms including protease-dependent cleavage [207], TLR-dependent downregulation [157], and autophagy-dependent degradation [235]. Previously, we showed that a PLY pore-forming activity-dependent pathway was most efficient at E-cadherin cleavage and causing AJC mis-localization even at a low infection dose [236]; however, the lack of junction integrity did not directly result in epithelial permeability changes or *Sp* movement in the absence of infiltrating PMNs. Here, by use of a genetically tractable airway BSC-derived ALI epithelial organoid co-cultured with PMNs [149], we were able to examine the outcome of PLY-triggered AJC dissolution without other confounding PLY pro-disseminating activities, such as HXA₃ production [136, 232]. We found that PLY disrupted AJCs prior to substantial PMN infiltration, and was a means to maximize PMN transmigration and subsequent bacterial movement across the compromised epithelial barrier. Compared to PMN transmigration across ALI monolayers primed with PLY-producing *Sp* and diminished in AJC organization, migration across uninfected monolayers or monolayers primed with PLY-deficient *Sp* was significantly lower, despite the same chemotactic

supernatant used to trigger migration. Correspondingly, *Sp* infection-primed monolayers with disorganized AJCs sustained higher a degree of barrier integrity loss and *Sp* translocation post-PMN transmigration, supporting a functional role of AJC disruption as a prerequisite step to maximal PMN-triggered epithelial barrier breach that facilitate *Sp* cross-epithelial dissemination.

During leukocyte transmigration, AJCs must transiently open to allow the passage of infiltrating cells. PMN transmigration is highly dependent on cleavage of intracellular AJC proteins such as E-cadherin [248]. The active role of AJC integrity on the degree and outcome of transmigration is becoming increasingly appreciated [249, 250]. The architecture of cell-cell contact permitting a tight AJC seal can act as a physiological brake on PMN transendothelial migration, limiting pathology in models of inflammation-associated sterile injury [251]. Furthermore, AJC restrictiveness can influence functions of transmigrated PMNs via activating mechano-sensing, as squeezing through restrictive endothelial junctions enhances PMN bactericidal activity post-transmigration [252]. By prematurely loosening AJCs prior to PMN arrival, *Sp* may not only be enhancing PMN infiltration, but also altering PMN effector functions to a state that promotes infection progression and pathology.

When PMNs infiltrate without check, the sheer mechanical forces and released tissue-damaging PMN products can inflict severe injury of the epithelial barrier [253]. *PLY*, in addition to directly disrupting epithelial AJCs, can act on PMNs to increase its tissue-destructive potential, including enhanced neutrophil extracellular trap (NET) formation, ROS production, and protease secretion [70, 85, 254]. Moreover, *PLY* indirectly alters PMN functions via soluble mediators secreted by the *PLY*-exposed epithelium [232]. *PLY*-triggered HXA₃ production not only triggers PMN trans-epithelial migration, but also increases NE release [232], which we show here to be a factor further diminishing E-cadherin organization and epithelial barrier integrity to facilitate *Sp*

dissemination. The multifaceted means by which PLY targets AJCs and the epithelial barrier highlight the importance of retaining adequate barrier integrity during infection as a physical safeguard against *Sp* spread.

Elderly patients with pneumococcal pneumonia experience exacerbated PMN pulmonary infiltration and delayed inflammation resolution [41, 42], which correlates with high bacterial burden and worse clinical outcomes. Aging of the mucosal epithelium is associated with elevated inflammation and diminished barrier function. Cultures of human pulmonary epithelia exhibit age-dependent decreases in expression of genes for AJC assembly, including genes for ZO-2, EpCAM, and E-cadherin [32]. Similarly in aged mice, pulmonary barrier dysfunction occurs at terminal stages of life [255] and in a model of acute lung injury [256]. Here we found that the pulmonary AJCs of aged mice and aged mice-derived ALI cultures were less robust and more susceptible to disruption by *Sp* challenge, events correlating with increased PMN infiltration and barrier integrity loss post infection. Moreover, PMNs from aged hosts decline in chemotactic accuracy [233], phagocytosis [39], and ROS production [234] and are a critical determinant of *Sp* infection outcome as adoptive transfer of PMNs from young mouse rescued aged-related susceptibility to *Sp* [43]. In accordance, PMNs from aged mice *in vivo* and *ex vivo* released higher levels of NE, a protease capable of AJC destruction, in response to *Sp* stimulation, consistent with previous reports using peripheral blood PMNs isolated from elderly subjects [245].

Given both the decline in epithelial AJC integrity and overproduction of NE during *Sp* infection of aged mice, therapeutic approaches that address these particular age-related mechanisms promoting susceptibility to *Sp* may be of interest. In addition, since *Sp* is capable of multiple mechanisms of AJC perturbation, directly via PLY or via NE released by recruited PMNs, a broad acting AJC fortification agent like the Nrf2 agonist CDDO, may have benefits over those targeting single mechanisms of disruption [246].

Nrf2 activation enhances expression of AJC proteins and shows promising therapeutic potential in preventing injury to the airway epithelial barrier against challenge by bleomycin, LPS, or viral insult [243, 244, 257, 258]. The synthetic Nrf2-activating compound CDDO has been examined in phase III clinical trial for chronic kidney disease, phase I/II trials for various malignancies [259], and a number of preclinical models of inflammation-related diseases including those of the lung [260]. Upon pulmonary *Sp* challenge of young or aged mice, CDDO treatment did not alter lung bacterial burden or PMN infiltration, but specifically diminished the loss of lung barrier integrity and subsequent bacteremia. Interestingly, NE inhibition in addition to CDDO treatment only provided additional protective benefit to aged and not young mice. This suggests that AJC fortification by CDDO alone is sufficient to limit both AJC damage inflicted by *PLY* directly and indirectly via NE in young mice. In contrast, with aged mice the elevated NE release and increased damage to the airway epithelium rendered AJC fortification by CDDO alone insufficient. The need for dual CDDO treatment and NE inhibition to maximize barrier function protection in *Sp*-challenged aged mice underscores the importance of adjuvant therapy tailored to dysregulations of the aging host to most effectively mitigate age-related susceptibility to invasive pneumococcal diseases.

4.4. Materials and Methods

4.4.1. Bacterial strains and growth conditions

Mid-exponential growth phase aliquots of *S. pneumoniae* TIGR4 (serotype 4) were grown in Todd-Hewitt broth (BD Biosciences) supplemented with 0.5% yeast extract in 5% CO₂ and Oxyrase (Oxyrase, Mansfield, OH), and frozen in growth media with 20% (v/v) glycerol. Bacterial titers in aliquots were confirmed by plating serial dilutions on Tryptic Soy Agar plates supplemented with 5% sheep blood (blood agar)

(Northeast Laboratory Services, Winslow, ME). The TIGR4 pneumolysin-deficient mutant ($\Delta p/y$) was a gift from Dr. Andrew Camilli (Tufts University School of Medicine, MA). For experiments, frozen aliquots of *S. pneumoniae* strains were grown in Todd-Hewitt broth, supplemented with 0.5% yeast extract and Oxyrase, in 5% CO₂ at 37°C and used at mid-log to late log phase.

4.4.2. Establishment of epithelial air-liquid interface monolayers

Human bronchial basal cells were isolated and expanded from lung tissue harvested from donors without lung disease through the New England Organ Bank under an IRB-approved protocol (MGH #2010P001354). In brief, using a previously published basal cell isolation protocol [68, 151], EpCAM⁺ epithelial basal cells were isolated from human trachea and mainstem bronchi tissue. Mouse airway basal cells were obtained from young and aged C57BL/6J or young *Alox15^{-/-}* mouse trachea. Harvested basal cells were cultured in complete small airway epithelial growth media (SAGM) (Lonza, Cat. CC-3118), using the dual SMAD inhibition protocol [151]. For the purposes of consistency between experiments performed within this study, basal cells isolated from a single donor were used between passages 2-5. To generate conventional up-right monolayers on Transwells for infection and imaging studies [151], the up-facing side of 6.5 mm Transwell inserts with 0.4 μ m sized pores (Corning product #3470) were pre-coated with 804 G rat bladder cell-conditioned medium as a source of collagen before cell seeding. For studying neutrophil transepithelial migration, the inverted ALI model was adopted [160] where Transwells with permeable (3 μ m pore size) polycarbonate membrane inserts (Corning product #3415) were used and the underside of the Transwells were 804 G medium-coated before cell seeding. Each Transwell was seeded with 80 μ l of the airway basal cells suspension (containing >200,000 cells) in SAGM, resulting in a density of >6000 cells/mm², and submerged in

complete SAGM for airway basal cell recovery and expansion for 1–2 days to ensure monolayer confluence. The media in both chambers was then replaced with complete Pneumacult-ALI medium (StemCell Technology, Cat. 05001) for an additional day. To initiate air-liquid interface, media in the chamber contacting the cell apical surface was removed (designated as day 0) and media in the cell basal-facing chamber was changed every 1-2 days during differentiation.

ALI monolayers used in experiments were cultured for at least 21 days to allow for full maturation of both cilia and goblet cells, but no more than 34 days to avoid overgrowth or loss of epithelial barrier [149]. Transepithelial electrical resistance was assessed using a voltmeter (EVOM2, Epithelial Voltohmmeter, World Precision Instruments, Inc.) prior to migration assays to ensure the establishment of a polarized epithelial barrier.

4.4.3. Infection of ALI monolayers

S. pneumoniae grown to log phase was washed and resuspended to 5×10^8 CFU/ml in Hanks' balanced salt solution (HBSS) supplemented with 1.2 mM Ca^{2+} and 0.5 mM Mg^{2+} . 25 μl of bacterial suspension was added to the apical surface of the ALI monolayers and incubated at 37°C with 5% CO_2 for 2 hours to allow for attachment and infection of the ALI monolayers. After treatment, Transwells were placed in 24-well receiving plates containing HBSS with Ca^{2+} and Mg^{2+} , and to allow for bacteria translocation, incubated for an additional 2 hours with or without the addition of 1×10^6 PMNs to the basolateral chamber. Detection of basally added horseradish peroxidase (HRP) in the apical chamber was used to assess ALI monolayer barrier integrity post-treatment. Buffer in the basolateral chambers were sampled and bacterial translocation across ALI monolayers evaluated by plating serial dilutions on blood agar plates.

Bacterial migration index was calculated as total CFUs in the basolateral chamber normalized to infection inoculum.

4.4.4. PMN transepithelial migration assays

Whole blood obtained from healthy human volunteers under an IRB-approved protocol (Tufts University protocol #10489) was used to isolate neutrophils using the Easysep direct human neutrophil isolation kit (Stemcell), and 1×10^6 PMNs were added to the basolateral chamber after two hours of apical infection of the ALI monolayers with *S. pneumoniae*. Purified HXA₃ methyl ester (Cayman) at 10 nM were supplemented apically as indicated. To test the effect of HXA₃-containing cell supernatants, the apical media was replaced with cell supernatants prepared as described above.

After two hours of transmigration, PMNs in the apical chamber were quantified by MPO activity assay, as described [136]. Briefly, 50 μ l of 10% Triton X-100 and 50 μ l of 1 M citrate buffer were added to lyse transmigrated PMN, and 100 μ l of lysed PMNs from each well was transferred to a 96-well plate. 100 μ l of freshly prepared 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) with hydrogen peroxide solution was added to each well and incubated in the dark at room temperature for 5-10 minutes. Absorbance at a wavelength of 405 nm was read on a microplate reader and measurement was converted to neutrophil number using a standard curve.

4.4.5. Fluorescence microscopy assessment of ALI monolayer integrity

At the end of the two hours of infection, followed by two hours of PMN transmigration across ALI monolayers, the degree of apical junction complex integrity and cell confluency of ALI monolayers on Transwell filters was assessed by fluorescence microscopy. To prepare samples for fluorescence microscopy, monolayers were fixed with 4% PFA, permeabilized with 0.1% Triton-X 100 in PBS plus 3% BSA,

and stained with rabbit anti-E-cadherin (24E10, Cell Signaling), following by Alexa Fluor 488-conjugated anti-rabbit secondary antibody, DAPI, and Alexa Fluor 594-conjugated phalloidin. Transwell filters were then excised and mounted in 25% Vectashield Antifade Mounting Medium (Vector Laboratories) solution [261] for visualization with a Leica SP8 spectral confocal microscope (Leica). Quantification of E-cadherin and ZO-1 junction organization was carried out with the python script IJOQ as previously described (43). Briefly, the script quantifies spatial distribution of a junctional molecule along the continuous contacts with neighboring cells by determining the degree of intersection between networks of the molecule to a predefined grid-like network. Quantitations were normalized to that of untreated controls.

4.4.6. Production of PMN chemoattractant-containing cell supernatants

Epithelial cell supernatants were generated from young (2-month-old) or aged (>20-month-old) C57BL/6J mouse basal stem cell-derived ALI monolayers infected with 1×10^7 WT *S. pneumoniae* for 1 hour at 37°C with 5% CO₂. Infected Transwells were placed in 24-well receiving plates containing HBSS with Ca²⁺ and Mg²⁺ in the apical chamber for an additional 1 hour to allow for polarized chemoattractant secretion. The apical chamber supernatants were then collected and transferred to new Transwells with *Alox15^{-/-}* ALI monolayers for PMN transmigration and barrier integrity assessment assays.

4.4.7. Murine infections

Young (2-month-old) BALB/c and C57BL/6J mice were obtained from Jackson Laboratories. Aged (>20-month-old) BALB/c and C57BL/6J mice were obtained from National Institute on Aging aged rodent colonies. All animal experiments were performed in accordance with Tufts University Animal Care and Use Committee approved

protocols. Bedding transfers were performed between young and old mouse cages of the same mouse strain. Roughly one-quarter of soiled bedding was collected from each cage and the bedding from all cages was mixed in an empty sterile cage before redistribution across all cages. To induce experimental pneumococcal pneumonia, young or aged BALB/c mice were intratracheally challenged with 1×10^7 colony forming units (CFU) of *S. pneumoniae* in 50 μ l phosphate-buffered saline (PBS). Control mice received PBS. The role of preserving junctional integrity during on *S. pneumoniae* infection was investigated by injection of either a Nrf2 agonist bardoxolone methyl (CDDO) at 100 μ g/mice, in 3% DMSO, 3% cremaphor EL (CrEL) in PBS, or in combination with a neutrophil elastase inhibitor Sivelestat at 500 μ g/mice, in PBS, by *i.p.* 1 hour prior to infection. Mice were euthanized at 18 h.p.i.. Blood was obtained by cardiac puncture. Bronchoalveolar lavage fluid (BALF) was collected by washing the lungs with 1 ml PBS via a cannula. Then whole lungs were removed and bacterial burden enumerated by plating lung homogenate on blood agar plates.

4.4.8. Lung barrier integrity by dextran and microscopy

For assessment of lung permeability, mice were intravenously injected with 70 kDa MW FITC-dextran at 5 mg/kg 30 minutes prior to euthanasia. Whole lungs were isolated and homogenized in 1 ml PBS, which were then subjected to fluorescence quantitation using a Synergy H1 plate reader (BioTek). Readouts were normalized to fluorescence in the serum of the same animal, diluted 1:10 in PBS. To visualize alveolar E-cadherin architecture by fluorescent microscopy, lung tissues were harvested from euthanized mice, fixed with 4% paraformaldehyde, and sectioned to a thickness of 250 μ m with a Leica Vibratome (0.145 mm/s, 70 Hz, blade angle 5°). Tissue sections were permeabilized with 0.1% Triton X-100 in PBS plus 3% BSA for 2 hours. Permeabilized sections were stained with primary anti-E-cadherin antibody (24E10, Cell Signaling) and

primary anti-Ly6G antibody (1A8) overnight, followed by Alexa Fluor 514-conjugated anti-rabbit secondary, Alexa Fluor 647-conjugated anti-rat secondary, DAPI, and Alexa Fluor 594-conjugated phalloidin. Samples were mounted with Vectashield antifade mounting medium (Vector Laboratories) and visualized with confocal microscopy (Leica SP8).

4.4.9. Measuring PMN infiltration

For flow cytometric quantitation of lung PMNs, mice were euthanized at 18 h.p.i. and lung tissues were digested with 1 mg/ml Type II collagenase (Worthington) and 50 U/ml Dnase (Worthington) to obtain a single-cell suspension. Cells present in the suspension were stained on ice for 30 minutes with APC-conjugated anti-Ly-6G (clone 1A8) and then washed two times in FACS buffer (Biolegend). Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and the fluorescence intensities of the stained cells were determined. Collected data were analyzed using FlowJo software (v10.7, BD) to determine the numbers of infiltrating (Ly6G⁺) PMNs.

4.4.10. Neutrophil elastase activity

NE activity in soluble fraction of BALF from infected mice or PMN supernatants from 1×10^6 PMNs challenged with 1×10^7 CFU *S. pneumoniae* was determined using a PMN Elastase Fluorometric Activity Assay Kit (Abcam), following manufacturer's instructions. The area under the curve of kinetic substrate conversion curves over 2 hours was measured with a Synergy H1 plate reader (BioTek) and normalized to uninfected controls.

4.4.11. Presentation of data and statistical analyses

Statistical analysis was carried out using GraphPad Prism (GraphPad Software, San Diego, CA), ordinary one-way ANOVA followed by Tukey's multiple comparison test. p values <0.05 were considered significant in all cases. Tissue and blood bacterial burdens were log-transformed; for all other graphs, the mean values $\pm$ SEM are shown. Due to intrinsic donor-to-donor variability of human PMN transmigration efficacy, experiments involving human donors were normalized before pooling individual experiments. The conclusions drawn were those found to be reproducible and statistically significant across independent experiments

4.5. Contributions

S.X and J.L conceived the hypothesis and designed the experiments. S.X performed the experiments and analyses for Figures 4.1-4.5. S.X and T.Z performed experiments and analyses for Figures 4.6-4.7. S.T developed the method of *in situ* tissue imaging used in Figures 4.1, 4.3, and 4.7. H.M developed the BSC-derived ALI system and provided reagents used in Figures 4.2, 4.4, and 4.5. S.X generated figures and wrote the manuscript with assistance from J.L.

Chapter 5: Discussion

5. Discussion

5.1. Summary and significance

This work addresses the basis by which *Sp* infection triggers tissue-destructive inflammation in the lungs and subverts the host immune response to promote disease progression to lethal systemic infection. The respiratory epithelium provides an important barrier to microbial invasion, but *Sp* can translocate from the alveolar space across the lung epithelium to seed the bloodstream, an ominous development in the course of disease [117]. In addition to the intracellular transport [262] and microinvasion [158] pathways of *Sp* transepithelial movement previously reported and observed in our hands at low infection dose *in vitro* (data not shown) or early time points *in vivo* that are independent of PLY, we found that PLY-promoted PMN infiltration into *Sp* infected airways is associated with significant lung damage and poor infection outcome [59, 80], fueling interest in developing PMN- and PMN transepithelial migration-targeted therapy strategies [121]. Using a combination of *in vivo* murine infection models and *ex vivo* modeling of *Sp*-PMN interactions at an ALI culture system of the mucosal epithelium, we found that the eicosanoid chemoattractant HXA₃ is sufficient for driving epithelial barrier disruption and bacterial dissemination by promoting NE release from PMNs transmigrating into the airways [232].

In addition, we found that *Sp* disruption of the epithelial barrier was associated with highly efficient dissolution of the epithelial AJC protein E-cadherin, which was initiated as early as 6 hours *in vivo* and at an infection dose as low as an MOI of 2 *in vitro*. PLY pore-forming activity is necessary and sufficient to induce AJC disorganization in the absence of PMNs, a function conserved by diverse pore-forming toxins including intermedilysin of *S. intermedius*, and perfringolysin O of *C. perfringens* [236]. During infection of 12-LOX-deficient ALI monolayers, PLY-promoted AJC dissolution enhanced PMN transmigration, barrier integrity loss, and *Sp* translocation in response to

exogenous HXA₃, a process contributing to the increased bloodstream-lung permeability and susceptibility to *Sp* systemic infection in the AJC-defective aging hosts. While HXA₃ did not directly affect junctional stability and permeability of the epithelium (data not shown), HXA₃-triggered NE release by infiltrating PMNs aggravated junctional damage and promoted detachment of epithelial cells *in vitro*. Pharmacologic agents enhancing E-cadherin expression (CDDO) or mitigating NE release (Nexhib20) or proteolytic activity (Sivelestat) protected the pulmonary barrier and diminished bacterial translocation during *Sp* infection of ALI monolayers and young and aged mice, illustrating the therapeutic potential of these agents as a host-directed therapy options for invasive diseases following pneumococcal pneumonia.

5.2. Versatile models to study pulmonary barrier dysfunction

The respiratory epithelial barrier is a safeguard against entry of infectious microbes and environmental agents such as allergens and pollutants. Many pulmonary diseases, both of infectious etiologies like pneumonia, and of non-infectious inflammatory etiologies such as asthma, COPD, and cystic fibrosis, involve disturbances to and dysfunction of the pulmonary barrier as a critical step of pathogenesis [185, 246]. The architecture and biological function of the respiratory epithelial barrier is highly complex, encompassing a plethora of cells that are ciliary, mucus-producing, or secreting antimicrobial peptides, cytokines, and chemokines. Upon infection, these cells cooperate with submucosal residing immune cells and infiltrating leukocytes to defend the epithelial barrier and clear invading pathogens. Model systems that faithfully reflect this complexity are crucial for a better understanding of the physiological and biological functions of the pulmonary barrier and its interacting partners during homeostasis and disease [263].

5.2.1. *In situ* pulmonary tissue imaging reveals alveolar barrier architecture during i.t. *Sp* challenge in mice

Compared to B6 mice, BALB/c are more resistant to pneumococcal pneumonia with less severe pulmonary pathology. Upon visualization of infected lung tissue by IF microscopy, B6 mice had high bacterial burdens and colocalized swarms of PMNs associated with areas of high bacterial burden (data not shown), confounding a straightforward assessment of injury specific to the epithelial barrier. Infected lung tissues of BALB/c mice, on the other hand, did not contain localized bacterial lesions, facilitating an overall assessment of injury to the airway epithelium appeared. Moreover, PLY triggers robust PMN infiltration to the airways during *Sp* infection of both strains of mice, and both suffer similar degrees of bacteremia. However, BALB/c mice sustains higher levels of PMN infiltration, and correspondingly, a reproducible phenotype of PLY-promoted bacteremia. We infected BALB/c mice to examine PLY-mediated damage to airway architecture and barrier function after *Sp* challenge.

By examining lung tissues from *Sp*-challenged mice harvested at different infection time points, we found that AJCs of the alveolar epithelium were disrupted in a PLY-dependent manner prior to substantial PMN infiltration or increase in pulmonary permeability. Areas of disrupted AJC included both flat type I and rounded type II alveolar pneumocytes and did not overlap in localization with either the bacterium or PMNs (data not shown), consistent with PLY, a released soluble toxin, as the direct agent of AJC disruption. While it is commonly believed that in cases of pulmonary barrier dysfunction triggered by neutrophilic inflammation, AJCs are lost to PMN proteases such as NE and MMPs [91, 200], we presented evidence that during *Sp* infection of the lung, alveolar AJC disruption was an early event initiated independently of PMN infiltration, an observation that prompted us to examine the biological role of early AJC loss and therapeutic targeting of AJCs in curtailing downstream barrier pathologies.

Visualizing PMN migration in real time has been a challenge *in vivo*. Intravital microscopy (IVM) is the tool of choice for studying dynamic barrier function and cell trafficking in tissues such as the skin and endothelial vasculatures [263]. However, IVM studies of the living lung has unfortunately been limited by its natural breathing motion that requires additional engineering entrapments to improve imaging stability [264]. Advances in fixed tissue processing and fluorescent confocal microscopy techniques have enabled *in situ* visualization of the lung barrier at single cell resolution [265]. In addition, novel engineering techniques such as implantable imaging chest windows on living mice [266], or the crystal ribcage [267] capable of preserving a functional lung in motion *ex vivo*, may allow for monitoring of alveolar architecture and function in real time by IVM in the future.

5.2.2. PMN transmigration across BSC-derived ALI monolayers provides mechanistic insights

While animal models provide an *in vivo* context, *in vitro* models provide the precision and genetic tractability in interrogating specific mechanisms. Primary bronchial epithelial cell-derived ALI culture models recapitulate key aspects of the architecture of *bona fide* airway epithelium, including the diversity of cell types and the formation of mature apical tight junction and adherens junction complexes that facilitate a functional mucosal barrier [151, 160]. Despite being widely used as a model of airway barrier function, the system is hampered by issues such as high cost, limited primary sample availability, and the inability to propagate cells extendedly in culture [151]. BSCs, on the other hand, can be collected from sources ranging from whole organ [149] to aspirated medical waste [268], and under expansion conditions, can generate unlimited tracheobronchial ALI cultures similar in complexity to primary epithelia, permitting their use to be recently extended to systems of PMN transepithelial migration [160]. A

potential limitation of this model in recapitulating host cell interactions in the infected lung is that the ALI monolayers we utilized here mimics small airway epithelium, with a high ciliated cell to goblet cell ratio [160]. Small airway epithelium is adjacent to the alveolar epithelium where most PMN-epithelial interactions during *Sp* infection are predicted to occur. We attempted to model the alveolar epithelium with undifferentiated BSC monolayers but these cells failed to trigger PMN transmigration in response to *Sp* (data not shown).

Upon PMN addition to the basal chamber, the small airway epithelium-mimicking ALI monolayers reproducibly and reliably facilitated apical bacterial infection-induced PMN transepithelial migration, as previously shown with a *P. aeruginosa* infection model [160]. Here we show that *Sp* infection of human and murine BSC-derived ALI monolayers mirror key features of epithelial barrier breach that are found during pulmonary *Sp* challenge in mice [11, 59], including the early PMN-independent AJC loss, the requirement for PMN transmigration to permeabilize barrier and permit *Sp* translocation, and that the process is entirely dependent on 12-LOX and partially dependent on PLY [136]. Generation of ALI monolayers from 12-LOX-deficient mice permitted direct comparison of different degrees of AJC damage or different chemotactic stimuli on the outcome of PMN transmigration in the context of *Sp* infection, which will be discussed in detail in the following section. And lastly, as an unexplored feature of the BSC-derived ALI systems, stem cells retaining differentiation potential remain at the basement of the matured ALI epithelia, and this regenerative capability may be of interest for integration of longer-term infection studies, such as *Sp* biofilm colonization, post-viral *Sp* superinfection, or secondary interaction with PMN byproducts, such as NE, NETs, and other immune mediators association with lung injury and/or healing post injury, making the BSC-derived ALI system a versatile model for studying airway barrier function in response to multiple types of infectious and noninfectious insults.

5.3. PMN functional heterogeneity conferred by interaction with the epithelium

To date, multiple phenotypic and functional variants of PMNs have been identified, both in homeostatic settings arising from different PMN differentiation stages, and in specific disease conditions like cancer and autoimmune diseases [114]. However, PMNs also exhibit remarkable plasticity in functional response to acute stimuli, sensing and adapting to environmental changes via pattern recognition receptors, chemokine receptors, and mechanical receptors, which are all essential for the cell to be equipped with host defense functionalities optimal for the context of the response [161, 269]. Upon infection of a mucosal tissue like the lung, PMNs transmigrate across endothelial and epithelial barriers following chemotactic signals to respond to the invading pathogen in the airways, during which process they experience and are shaped by multiple types of stimuli [113]. Ultimately, the functional profile of these infiltrating PMNs can have immense impact on the outcome of the infection.

5.3.1. AJCs regulate the degree and nature of PMN migration mice

Opening and closing of the AJC is a well-orchestrated process that allows passage of infiltrating cells such as PMNs without compromising overall barrier integrity. The role of barrier cells on the degree and outcome of transmigration is becoming increasingly appreciated [250], owing to the growing body of IVM studies on PMN trans-endothelial migration [249], demonstrating presence of migration hotspots that permit passage of high numbers of PMNs arising from sites of unstable or altered junction architecture [237]. Whether the existence of these hotspots allow for easier repair after migration or promote barrier dysfunction remains debated [237]. Here we found that epithelial AJC dissolution alone did not directly result in epithelial permeability changes or *Sp* movement in the absence of infiltrating PMNs. Experimental utilization of a 12-

LOX-deficient ALI culture allowed us to examine the outcome of this PLY-dependent AJC dissolution without HXA₃ production by the infected monolayer as a confounding PLY-triggered pro-bacterial dissemination activity [136, 232], elucidating that AJC disorganization was a prerequisite step to maximizing PMN transmigration and subsequent bacterial movement across the compromised epithelial barrier. In addition, there is evidence that PMNs migrating through less restrictive endothelial junctions receive insufficient mechanical signals and are defective at bactericidal activity post-migration [252]. By prematurely loosening AJCs prior to PMN arrival, *Sp* may not only be creating migration hotspots enhancing PMN infiltration, but also altering PMN effector functions that further debilitate the cells to respond to infection effectively.

5.3.2. Chemoattractants alter PMN functional profile in response to infection

Although PMN transepithelial migration into the airways during *Sp* infection clearly disrupts the epithelial barrier, it is not clear that simple migration is sufficient to cause barrier dysfunction. PMN response at the mucosal epithelium can be highly context-dependent, ranging from effective infection control to severe tissue damage, and then at other times being critical for healing and tissue regeneration [115]. In many of these instances, the diverse chemotactic cues and environmental stimuli that drove PMNs to the site of their response determine their respective effector functions, antimicrobial activity, and inflammatory potential [133, 161]. For example, in models of sterile lung injury, infiltrating PMNs are apoptotic and produce tissue-repair molecules such as TGF- β , VEGF, and resolvins [162-164]. Conversely, in cystic fibrosis and COVID-19 infection models, PMNs undergo transcriptional changes that reduce bactericidal activity and enhance tissue-damaging degranulation and NE production [147, 148, 165]. Under these pathological conditions where large numbers of hyperinflammatory PMNs infiltrate without check, the sheer mechanical forces, along

with released PMN products that damage AJC and matrix proteins, can inflict severe injuries to the epithelial barrier [253].

Inhibition of 12-LOX activity drastically reduced PMN infiltration, bacteremia, and mortality following *Sp* lung challenge of mice [59, 136], suggesting that barrier disruption and systemic *Sp* disease could be promoted by a chemotactic product of the 12-LOX pathway. Using 12-LOX-deficient ALI monolayers, we definitively identified HXA₃ as being sufficient to induce PMN transmigration and mucosal barrier disruption triggered by *Sp* infection. In addition to its chemotactic activity, HXA₃ triggers PMN intracellular calcium release [143], promoting PMN survival [144], NETosis [145], and release of additional arachidonic acid metabolites [143, 146]. Direct comparison of HXA₃-driven chemotaxis with that triggered by a well-studied control chemoattractant fMLP [133, 166] revealed that different chemotactic cues ultimately lead to divergent infection outcomes: HXA₃ promoted specific pro-inflammatory conditioning of PMNs during *Sp* infection, including diminished apoptosis and enhanced NE and ROS production, activities promoting epithelial tissue destruction.

The observations that purified HXA₃ is sufficient to drive PMNs across ALI monolayers *in vitro*, and that 12-LOX, an enzyme required for the synthesis of HXA₃, is also required to promote migration of PMNs into airway spaces during *in vivo Sp* infection are consistent with the hypothesis that HXA₃ is the major PMN chemoattractant that drives pulmonary inflammation during infection. Early studies using *Salmonella enterica*-infected gut epithelial cultures utilized lipid fractionation, chromatography, and mass spectrometry to identify bioactive lipids, and by a process of elimination identified HXA₃ as the sole lipid mediator secreted by epithelial cells that had substantial PMN chemotactic capability [140]. Since then, blocking the activity of 12-LOX, the enzyme catalyzing the final step of HXA₃ synthesis, has been used to indirectly implicate HXA involvement in many infection models. More recently, inactive HXA₃ structural mimetics

that competitively block HXA₃ activity have been used to implicate HXA₃ as the primary driver of PMN transepithelial migration to *Pseudomonas aeruginosa* [141].

Nevertheless, we cannot rule out the possibility that other 12-LOX-dependent eicosanoids play a major role in promoting acute inflammation *in vivo*. For example, in studies with *P.a.*, HXA₃ is shown to be only required in the initiation of PMN transmigration and that following HXA₃ activation, other PMN-released products such as LTB₄ sustain the migration [146]. The potential effects of chemoattractants produced by HXA₃-activated PMNs during *Sp* infection are yet to be examined. Future studies using HXA₃ mimetics [141] that antagonize HXA₃-mediated signaling may help clarify the role of HXA in PMN-promoted PMN migration and *Sp* translocation. Such reagents would also aid in elucidating the receptor and mechanism of HXA₃ signaling promoting epithelial barrier breach, which in turn would provide avenues to test the essentiality of HXA₃ in *Sp*-induced PMN migration.

While capability to generate HXA₃ is conserved among diverse mucosal epithelia, whether there are specific airway epithelial cell type(s) responsible for HXA₃ production to *Sp* infection *in vivo* or in the ALI model are unclear. By single cell transcriptional profile 12-LOX expression is predominantly localized to ciliated cells and club cells in the lung [270]. *Sp* infection increases 12-LOX protein expression in a whole tissue level [59], but the cell types harboring 12-LOX or actively producing HXA₃ remain unknown and may be elucidated by IF staining of 12-LOX upon infection of mouse or ALI monolayers.

5.4. Dissecting the multifactorial age-related susceptibility to *Sp*

The underlying causes of susceptibility in older adults are complex, with multiple inflammaging- and immune senescence-related processes compromising effective mucosal defense mechanisms as well as diminishing integrity of the pulmonary barrier [116, 271]. We and others have shown that PMN senescence is a major cause of age-

associated susceptibility to pneumococcal lung infection as well as IPD in mice (4). Aberrant PMN response in age can be driven by PMN intrinsic changes, or by extrinsic changes as a result of altered PMN-epithelial interactions in the aging host. Identification and experimental therapeutic interventions of such factors often requires studies that can only be performed in model systems allowing for examining aging of the epithelium and aging of PMNs individually and collectively.

5.4.1. Age-related AJC defect makes for a susceptible pulmonary barrier

Even though mouse models are extensively used for aging studies, mice have a life span of 2-3 years and are costly to use for terminal infection studies like the bacteremic *Sp* infections of focus in this work. The ALI culture system has been successfully used to examine structural and biological properties of primary respiratory epithelial cells isolated from elderly subjects [32]. The additional advantage of a BSC-derived system employed here is the ability to isolate cells from aged mice in sufficient numbers to propagate, expand, and use in multiple experiments. The generation of an *in vitro* model of the aged pulmonary barrier has also allowed us to interrogate barrier function changes in aging independently of other age-related defects promoting susceptibility, such as PMN immune senescence [116]. Upon host aging, many genes associated with the assembly of TJ and AJ complexes are amongst the top genes that decrease in the lungs with increasing age [31]. In particular, *CDH1* (gene for E-cadherin) is downregulated and has been identified as a putative key regulator of age-related defect in epithelial barrier function [32]. Compared to *Sp* infection of ALI epithelium generated from the young, infection airway-derived epithelium from aged mouse (or human; data not shown) suffered greater AJC disruption independent of PMNs and displayed greater barrier compromise and bacterial translocation upon PMN transmigration. This suggest that age-related defects in AJCs contribute to susceptibility

to *Sp* and PMN insult. Unlike barrier tissues such as the skin and the gut where a decline of barrier function with age is well documented [25, 272, 273], the implications of age-related lung barrier deficiency remain relatively unexplored.

The relative paucity of insights into age-related changes of the pulmonary epithelium may reflect the low epithelial cell turnover rate compared to skin or gut epithelium [ref]; the lung epithelium is a tissue at homeostasis, with activation of quiescent barrier regeneration mechanisms only upon damage [274]. Indeed, only after *Sp* challenge were we able to identify an enhanced susceptibility to AJC disruption in the aged mice compared to their young counterparts, suggesting that this defect may lie in AJC stabilizing or repair mechanisms that are more critical during infection than homeostasis. This contrasts with age-related defects in gut epithelial AJC, which is noted both at baseline and when upon microbial or chemical insult.

The mechanism by which airway AJC integrity changes with age remains unanswered. A prior study identified IFN-I signaling as the driver of AJC upregulation in response to *Sp* infection of young mice [191]. Administration of exogenous IFN- β diminished bacteremia and improved survival of young mice [191], but in our hands did not alter infection outcome in aged mice (data not shown). Age-related and prior infection related increase in IFN-I autoantibodies [275] may result in defective IFN-I signaling and the lack of AJC fortification in response to *Sp* infection observed in aged mice. In addition, much of gut AJC defect with age is correlated with age-related dysbiosis of the gut microbiota [272, 276], raising the possibility that commensal flora at local or distant (e.g., gut) epithelium contributes to airway AJC integrity, perhaps via stimulating systemic levels of inflammatory cytokines. Wild-bred or pet store mice, which may be colonized with a greater diversity of microbiota than specific pathogen free-reared mice, and may have an infection history that more closely resembles non-infant

humans, may provide models to test these hypothetical mechanisms of age-related AJC changes.

5.4.2. Age-related changes in PMNs enhance PMN tissue-destructive potential

Neutrophil function is known to decline with age, displaying diminished phagocytosis, reactive oxygen production, neutrophil extracellular trap formation, and chemotaxis [116]. Adoptive transfer of PMNs from young, but not aged, mice prior to pneumococcal infection was effective to control bacterial burden in both lung and blood as well as significantly improve clinical score [43], suggesting that there are neutrophil intrinsic changes responsible for age-related susceptibility to *Sp*. This is consistent with our finding that epithelial supernatant from young or aged *Sp* infected ALI cultures elicited similar degrees of chemotactic and pro-tissue destructive activities when used to stimulate young donor-derived PMNs. *Ex vivo* *Sp* infection of PMNs isolated from aged mouse bone marrow resulted in higher NE production, which, given the detrimental role of NE during *Sp* infection of young mice [232], may contribute to barrier dysfunction and increased *Sp* translocation in the aged hosts as well. The PMN-epithelial interaction studies presented here are limited to examining interactions of young or aged ALI monolayers with young donor-isolated PMNs only. Future parallel work using aged donor-isolated PMNs may help elucidate the degree of epithelium and PMN contribution to age-related susceptibility to barrier breach by *Sp* infection, and whether the two work in a synergistic manner.

5.5. Strategies to develop general or tailored HDTs targeting barrier dysfunction

Documented limitations of pneumococcal vaccine efficacy and an increase in *Sp* antimicrobial resistance indicate a need for adjunctive therapies that diminish lethal invasive diseases following *Sp* infection of the lung. Given the age-related defects in

epithelial AJC integrity and elevation in NE production, host-directed therapies that address these barrier-disruptive mechanisms may be of benefit to the general population but also of particular interest to the elderly, who experience heightened susceptibility to serious *Sp* infection.

5.5.1. CDDO fortifies barrier regardless of the mechanism triggering AJC loss

During *Sp* infection, AJC disruption was an early onset event initiating a feedforward loop of PMN infiltration, furthering AJC dissolution, epithelial destruction, and *Sp* extrapulmonary dissemination. While there are therapeutics targeting pathways of AJC disruption in clinical and preclinical studies [246], they often act on single mechanisms of disruption elicited by specific microbes or microbial products, such as protease inhibition [207, 209], cytoskeletal scaffold stabilization [277, 278], and antioxidants [246, 279-281]. CDDO is a synthetic agonist of Nrf2 and at low nanomolar concentrations, protects the cells against oxidative stress via inhibition of reactive oxygen species generation, while at low micromolar concentrations, modulate the activities of a number of inflammation, redox balance, cell proliferation and programmed cell death pathways via promoting IKK β suppression of nuclear factor- κ B [259]. We found that CDDO enhanced E-cadherin organization, resisted against PLY-triggered AJC disruption, and rescued susceptibility to *Sp* infection in the AJC-defective aging host. NE inhibition in addition to CDDO treatment did not provide additional protective benefit to young mice, suggesting that AJC fortification by CDDO is sufficient to limit both AJC damage inflicted directly by PLY and indirectly via NE. Since *Sp* is capable of multiple mechanisms of AJC perturbation [157, 235, 236], a strategy that fortifies AJC integrity regardless of the mechanism triggering AJC loss is favorable and may have broader applications in other infection- and inflammation-triggered diseases involving barrier dysfunction.

5.5.2. NE inhibitors promote infection tolerance in environments of excessive inflammation

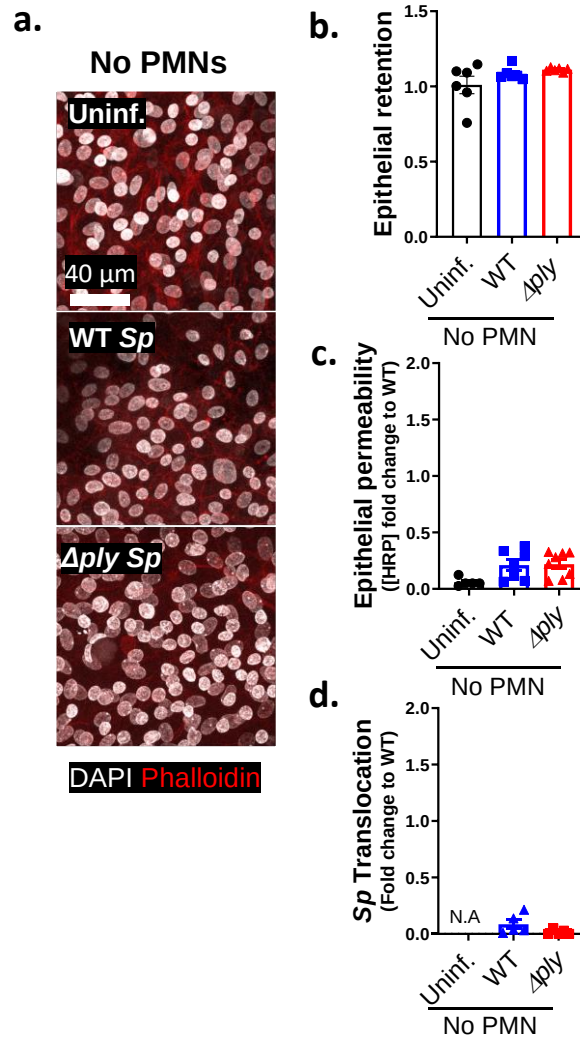
Optimal disease control during *Sp* infection requires both an effective initial acute inflammatory response and rapid resolution of inflammation later in the course of infection. Healthy and young patients with a fully functional immune system can benefit from HDTs that either boost innate immune function or those that modulate the inflammatory response [121]. Elderly patients and patients suffering excessive inflammation, on the other hand, may benefit more from therapeutics that first aim to reduce the inflammatory damage to achieve tolerance of the infection, rather than immediate pathogen clearance at the expense of pulmonary barrier integrity [121, 167]. NE, along with other serine proteases, contributes to *Sp* killing by PMNs *ex vivo* [87]. However, we found that *in vivo* the pathological and tissue-damaging activities of NE outweigh any beneficial role in direct pathogen killing [232]. Blocking NE activity has little impact on pulmonary *Sp* burden but curtails host tissue damage and systemic bacterial dissemination, shedding light on NE regulation as mechanism of disease tolerance that, despite having a neutral impact on pathogen clearance, is host protective by limiting disease progression to the more lethal systemic stages of infection. This is in contrast to degranulation inhibition by Nexinhib20, which, in addition to curtailing bacteremia, mildly diminished lung burden in young mice and significantly in aged mice (data not shown). Thus, additional non-NE PMN granular contents may affect pulmonary bacterial clearance or promote bacterial growth, especially in the aging hosts. *Sp* infection in aged mice triggered a higher degree of NE release and damage to the airway epithelium, to the point that AJC fortification by CDDO alone proved insufficient to diminish subsequent bacteremia in the lung-infected mouse; rather, the combination of CDDO and Sivelestat treatment was required for full protection restoring bacteremia to levels similar to that of

lung-infected young mice. These findings highlight the importance of adjuvant therapy tailored to immune dysregulations of the aging host to most effectively mitigate age-related susceptibility to invasive pneumococcal diseases.

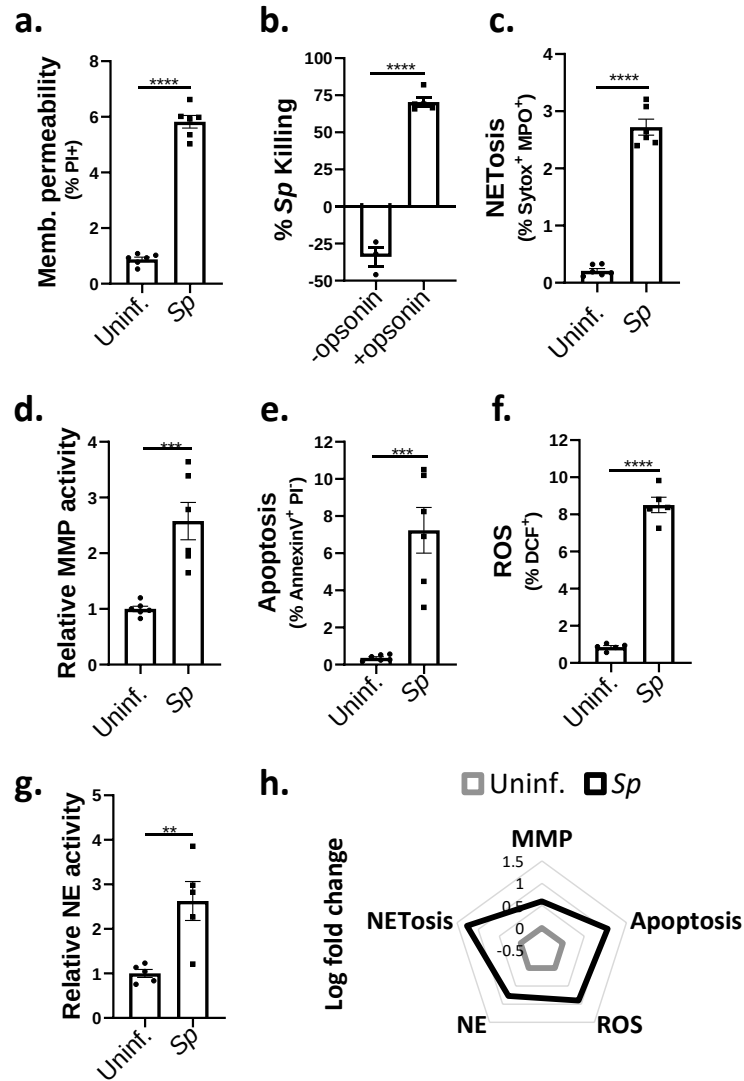
Chapter 6: Appendix

6. Appendix

6.1. Supplemental figures for Chapter 2

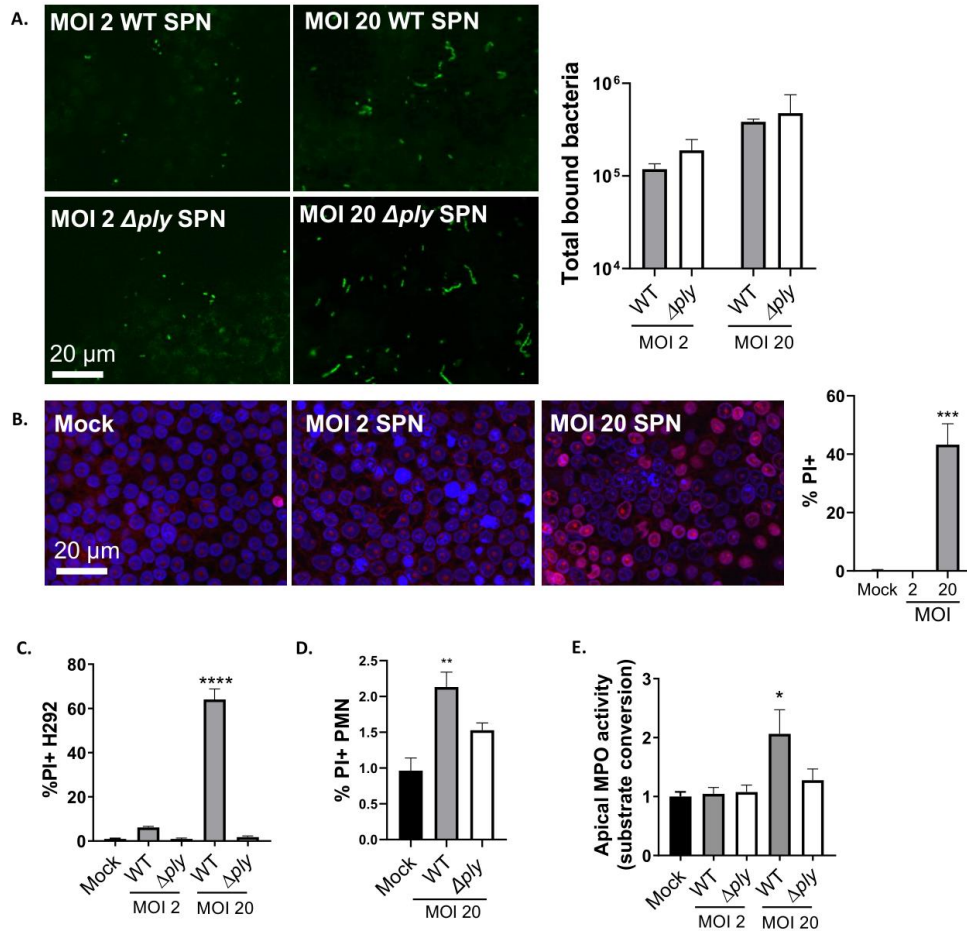


Supplemental Figure 1.1. PMNs are required for epithelial cell detachment and barrier breach. Human BSC-derived ALI monolayers were apically infected with 1×10^7 WT or Δ ply *Sp* without basolateral PMNs. (a) After two hours, monolayer integrity was assessed by fluorescence confocal microscopy after staining nuclei with DAPI and F-actin with fluorescent phalloidin. For clarity, images shown are of extended projections (all z-sections collapsed into 1 plane). Scale bar = 40 μ m for all images. (b) Epithelial retention was quantitated by enumerating epithelial cell nuclei relative to uninfected ALI. (c) Epithelial permeability was measured by HRP flux relative to monolayers infected with WT *Sp*. (d) *Sp* translocation was quantitated by measuring basolateral CFU. Each panel is a representative of three independent experiments. Error bars represent mean $\pm$ SEM.

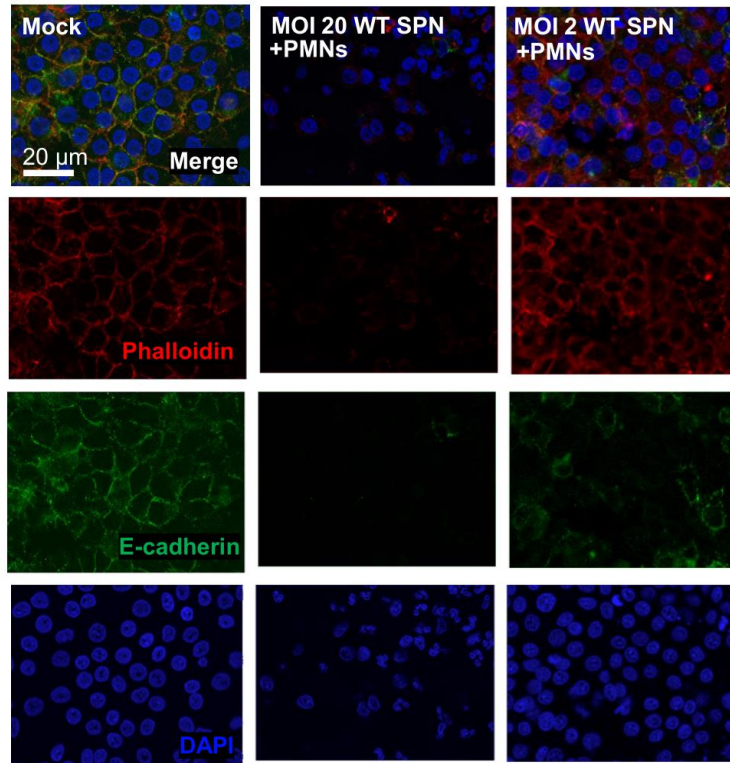


Supplemental Figure 1.2. *Sp* infection alters PMN functional response profile. 1×10^6 PMNs were uninfected or infected with 1×10^7 *Sp* and evaluated for functional performance via (a) PMN membrane permeability determined by propidium iodide staining (PI⁺), (b) opsonophagocytic killing with or without the addition of complement opsonin, quantitated by plating for CFU, (c) NETosis determined by Sytox and anti-MPO staining (Sytox⁺ MPO⁺), (d) released MMP activity by substrate conversion and expressed relative to uninfected PMNs, (e) apoptosis determined by lack of straining by propidium iodide and positive staining of Annexin V (PI⁺ Annexin V⁺), (f) ROS production by intracellular oxidation of substrate (DCF⁺), or (g) released NE activity by substrate conversion and expressed relative to uninfected PMNs (See Materials and Methods). (h) Radar plot summary of log fold change in PMN functional performance in (c-g). Each panel shown is representative of three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: **p-value < 0.01, ***p-value < 0.001, ****p-value < 0.0001.

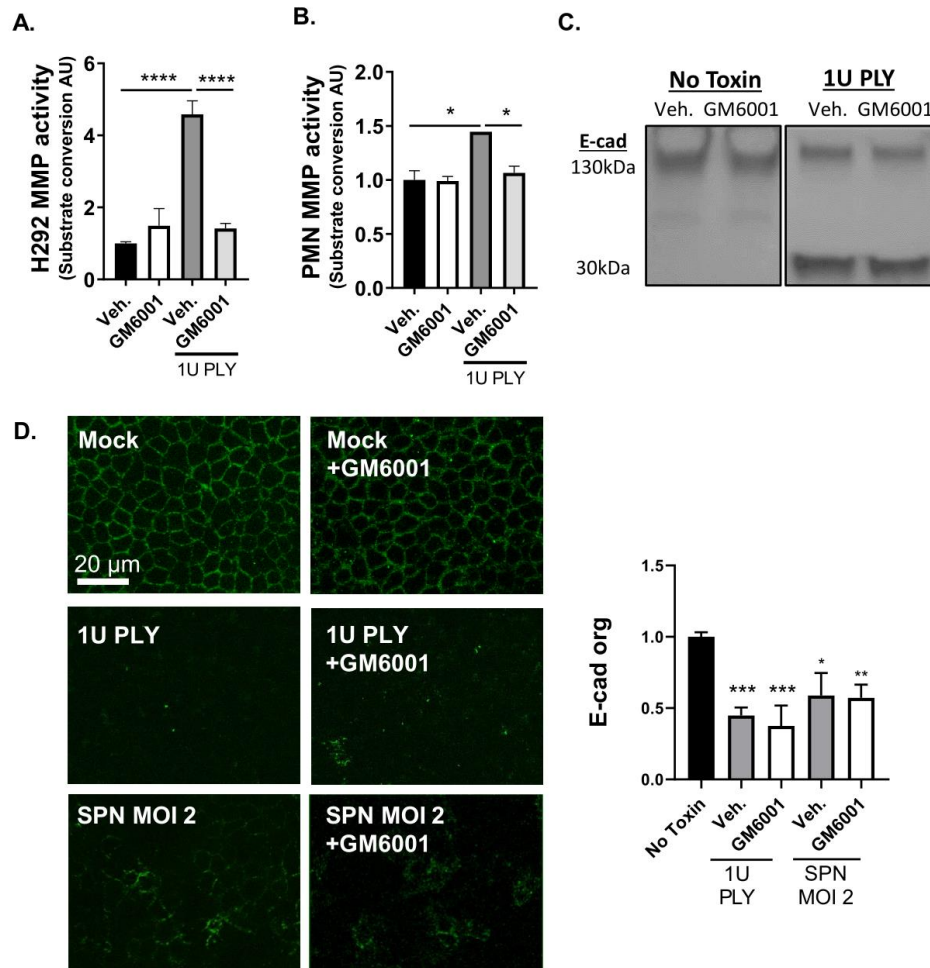
6.2. Supplemental figures for Chapter 3



Supplemental Figure 2.1. PLY-producing *S. pneumoniae* induces membrane permeabilization of epithelial cells and PMNs at high but not low dose infection. **A.** Polarized monolayers of H292 cells on Transwells were infected with the indicated MOI of GFP-expressing WT or Δply SPN, and monolayer adhered SPN visualized by IF microscopy and bacteria adherence per area was quantitated. **B.** Polarized monolayers of H292 cells on Transwells were infected with the indicated MOI of WT SPN and stained with DAPI and PI, and percentage PI permeabilization was determined. **C.** H292 cells in suspension were infected with the indicated MOI of WT or Δply SPN, followed by flow cytometry analysis for PI incorporation. **D.** PMNs in suspension were infected with the indicated MOI of WT or Δply SPN, followed by flow cytometry analysis for PI incorporation. **E.** Released MPO activity in apical buffer of WT or Δply SPN infected monolayers post-PMN transmigration and before lysis by detergent. Each panel shown is representative of three independent experiments in triplicates. Error bars represent mean $\pm$ SEM. Statistical analysis performed using One-way ANOVA: ** P-value $\leq$ 0.01, *** P-value $\leq$ 0.001, **** P-value $\leq$ 0.0001.

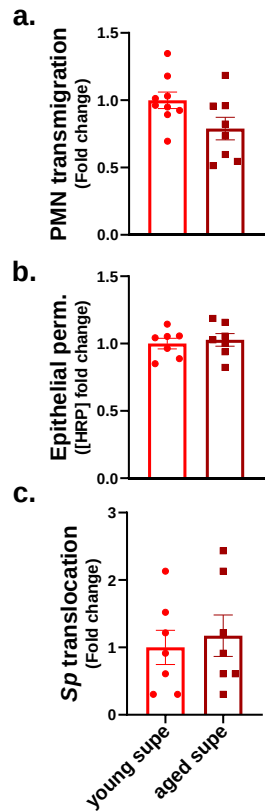


Supplemental Figure 2.2. Single channel IF microscopy images of polarized H292 monolayers infected with the indicated MOI of WT *S. pneumoniae*. Polarized monolayers of H292 cells on Transwells were infected with the indicated MOI of GFP-expressing WT SPN. Monolayers were stained with DAPI (blue), E-cadherin (green), and phalloidin (red). Scale bar = 20 µm.

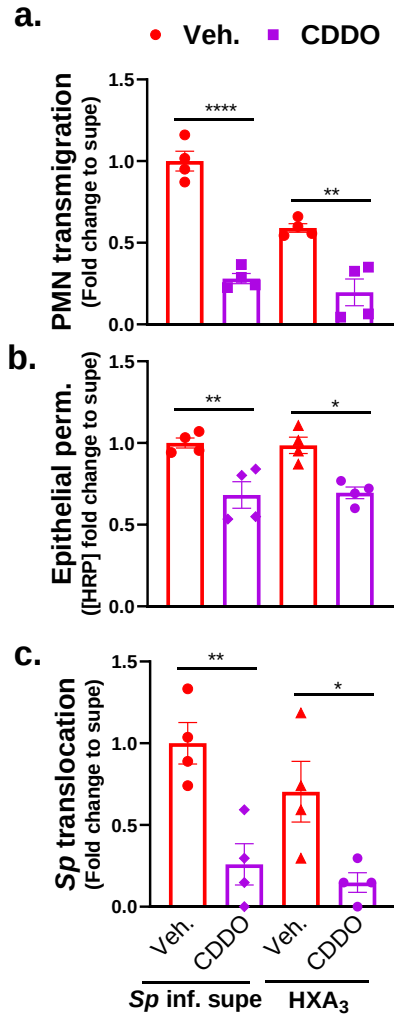


Supplemental Figure 2.3. PLY enhances MMP activity in culture supernatants of both epithelial cells and PMNs but MMP inhibition does not protect against junctional damage. **A.** Polarized monolayers of H292 cells on Transwells were treated with vehicle (Veh.) or a wide-spectrum MMP inhibitor (GM6001) and intoxicated with 1U PLY as indicated, and MMP activity measured by fluorogenic peptide substrate conversion. **B.** PMNs in suspension were treated with vehicle or GM6001 and then intoxicated with 1U PLY as indicated, and MMP activity measured by fluorogenic peptide substrate conversion. **C-D.** Polarized monolayers of H292 cells on Transwells were treated with Veh. or GM6001 and intoxicated with 1U PLY or infected with WT SPN at MOI 2, as indicated. In **C.** monolayers were lysed for western blotting of E-cadherin. In **D.** monolayers were permeabilized and stained with E-cadherin antibody (green), DAPI (blue), and phalloidin (red). Scale bar = 20 μ m. Peripheral E-cadherin organization was quantitated by image analysis with IJOQ. Each panel shown is representative of three independent experiments in triplicates. Error bars represent mean $\pm$ SEM. Statistical analysis performed using ordinary one-way ANOVA: *p-value $\leq$ 0.05, **p-value $\leq$ 0.01, ***p-value $\leq$ 0.001.

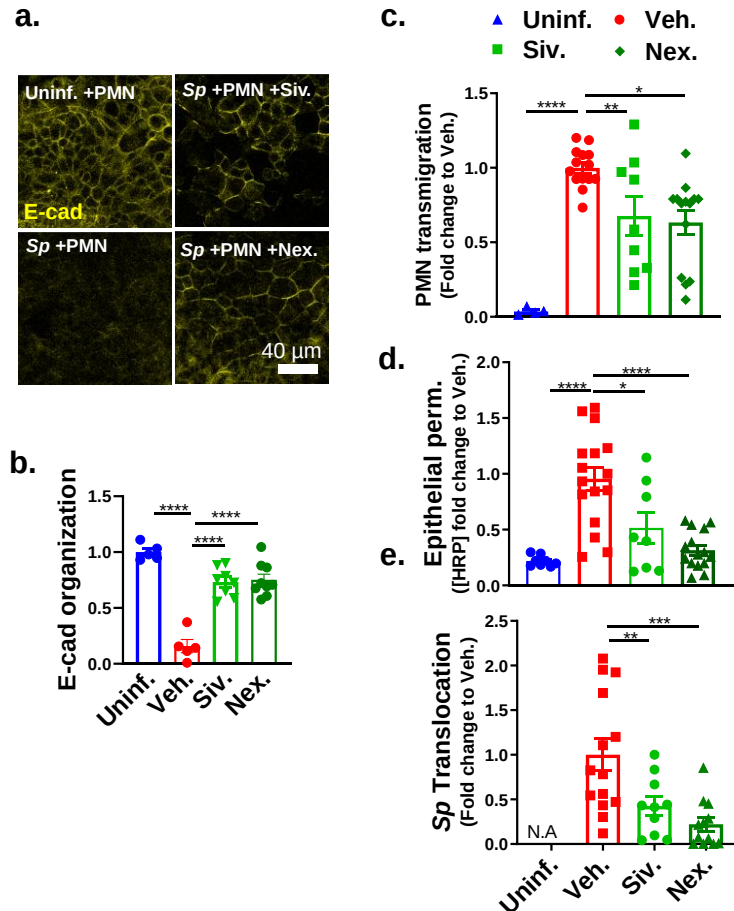
6.3. Supplemental figures for Chapter 4



Supplemental Figure 3.1. Supernatants harvested upon *Sp* infection of ALI monolayers derived from young and aged mice trigger similar levels of PMN transmigration and barrier disruption. *Alox15^{-/-}* mouse-derived ALI monolayers were apically infected with 1×10^7 WT *Sp* and transferred into apical chambers containing apical supernatant harvested from young or aged mouse-derived ALI monolayers apically infected with 1×10^7 WT *Sp*. 1×10^6 PMNs were added basally to monolayers. **(a)** After 2 hours of PMN migration, the degree of transmigration as determined by MPO activity in the apical chamber. **(b)** Epithelial permeability measured by HRP flux. **(c)** *Sp* translocation quantitated by measuring basolateral CFU. Each panel represents pooled data from three independent experiments. Error bars represent mean $\pm$ SEM.



Supplemental Figure 3.2. Junction fortification protects against PMN infiltration and barrier disruption independent of 12-LOX activity. Alox15^{-/-} mouse-derived ALI monolayers were pretreated with vehicle (DMSO) or 100nM CDDO before apical infection with 1×10^7 WT *Sp* and transferred into apical chambers containing *Sp* infection supernatant or 10 nM hepxilin methyl ester (HXA₃). 1×10^6 PMNs were added basally to monolayers. **(a)** After 2 hours of PMN migration, the degree of transmigration as determined by MPO activity in the apical chamber. **(b)** Epithelial permeability measured by HRP flux. **(c)** *Sp* translocation quantitated by measuring basolateral CFU. Each panel represents pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value < 0.05, **p-value < 0.01, ***p-value < 0.0001.



Supplemental Figure 3.3. NE promotes E-cadherin loss of ALI epithelium upon *Sp* infection. Human BSC-derived ALI monolayers were apically infected with 1×10^7 WT *Sp* in the presence of basolateral PMNs treated with 50 μ M Nexinhib20 (Nex) or 100 μ g/mL Siv. (a) IF microscopy images of fixed and permeabilized monolayers visualizing E-cadherin localization. Scale bar = 40 μ m. (b) E-cadherin organization quantitated by image analysis via the IJOQ script in Python. (b) 12-LOX-deficient Alox15^{-/-} mouse-derived ALI monolayers were apically infected with 1×10^7 WT or Δ ply *Sp* and transferred into apical chambers containing apical supernatant harvested from uninfected or WT *Sp*-infected WT B6 mouse-derived ALI monolayers. 1×10^6 PMNs were added basally to monolayers; after 2 hours of PMN migration, the degree of transmigration as determined by MPO activity in the apical chamber. (c) Epithelial permeability measured by HRP flux. (d) *Sp* translocation quantitated by measuring basolateral CFU. Each panel is representative of three independent experiments, or pooled data from three independent experiments. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using ordinary one-way ANOVA: *p-value $\leq$ 0.05, ***p-value $\leq$ 0.001, ****p-value $\leq$ 0.0001.

Chapter 7: Bibliography

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