Lung epithelial cells: therapeutically inducible effectors of antimicrobial defense

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Lung epithelial cells are increasingly recognized to be active effectors of microbial defense, contributing to both innate and adaptive immune function in the lower respiratory tract. As immune sentinels, lung epithelial cells detect diverse pathogens through an ample repertoire of membrane-bound, endosomal, and cytosolic pattern-recognition receptors (PRRs). The highly plastic epithelial barrier responds to detected threats via modulation of paracellular flux, intercellular communications, mucin production, and periciliary fluid composition. Epithelial PRR stimulation also induces production of cytokines that recruit and sculpt leukocyte-mediated responses, and promotes epithelial generation of antimicrobial effector molecules that are directly microbicidal. The epithelium can alternately enhance tolerance to pathogens, preventing tissue damage through PRR-induced inhibitory signals, opsonization of pathogen-associated molecular patterns, and attenuation of injurious leukocyte responses. The inducibility of these protective responses has prompted attempts to therapeutically harness epithelial defense mechanisms to protect against pneumonias. Recent reports describe successful strategies for manipulation of epithelial defenses to protect against a wide range of respiratory pathogens. The lung epithelium is capable of both significant antimicrobial responses that reduce pathogen burdens and tolerance mechanisms that attenuate immunopathology. This manuscript reviews inducible lung epithelial defense mechanisms that offer opportunities for therapeutic manipulation to protect vulnerable populations against pneumonia.

INTRODUCTION

The lung epithelium has long been perceived as a passive conduit for bulk airflow or an inert barrier to gas exchange, seldom encountering microbes and irrelevant to host–pathogen interactions. However, modern molecular techniques have revealed the complexity of the lower respiratory tract microbiome[1] and accumulating evidence demonstrate that lung epithelial cells function as important mediators of host defense.[2] Lung epithelial cells express an expansive complement of pattern-recognition receptors (PRRs) with oligospecificity for conserved microbial and host motifs. PRR activation by pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) initiates signaling cascades that can promote pathogen exclusion or expulsion, recruit and activate leukocyte-mediated defenses, directly kill microbes, and restore host homeostasis. These varied mechanisms provide manifold theoretical opportunities for intervention, and recent studies confirm that epithelial defenses can be therapeutically manipulated to protect the host, even in the setting of immunosuppression or leukodepletion.[3] This review addresses important lung epithelial pathogen detection and response mechanisms that may be therapeutically manipulated to prevent and treat lower respiratory tract infections in healthy and immunocompromised populations.

INDUCIBLE BARRIER DEFENSES

Cellular junctions and cytoskeletal elements

The histological complexity of the lung epithelium portends the specialized functions of its component cells. The pseudostratified airway epithelium is predominantly comprised of ciliated cells and secretory cells, interspersed with regenerative basal cells and neuroendocrine cells (**Figure 1a,b**). The vast majority of the alveolar epithelial surface area is contributed by exceptionally thin, broad type I pneumocytes that are optimized for gas exchange, while the considerably more numerous type II pneumocytes are principally responsible for

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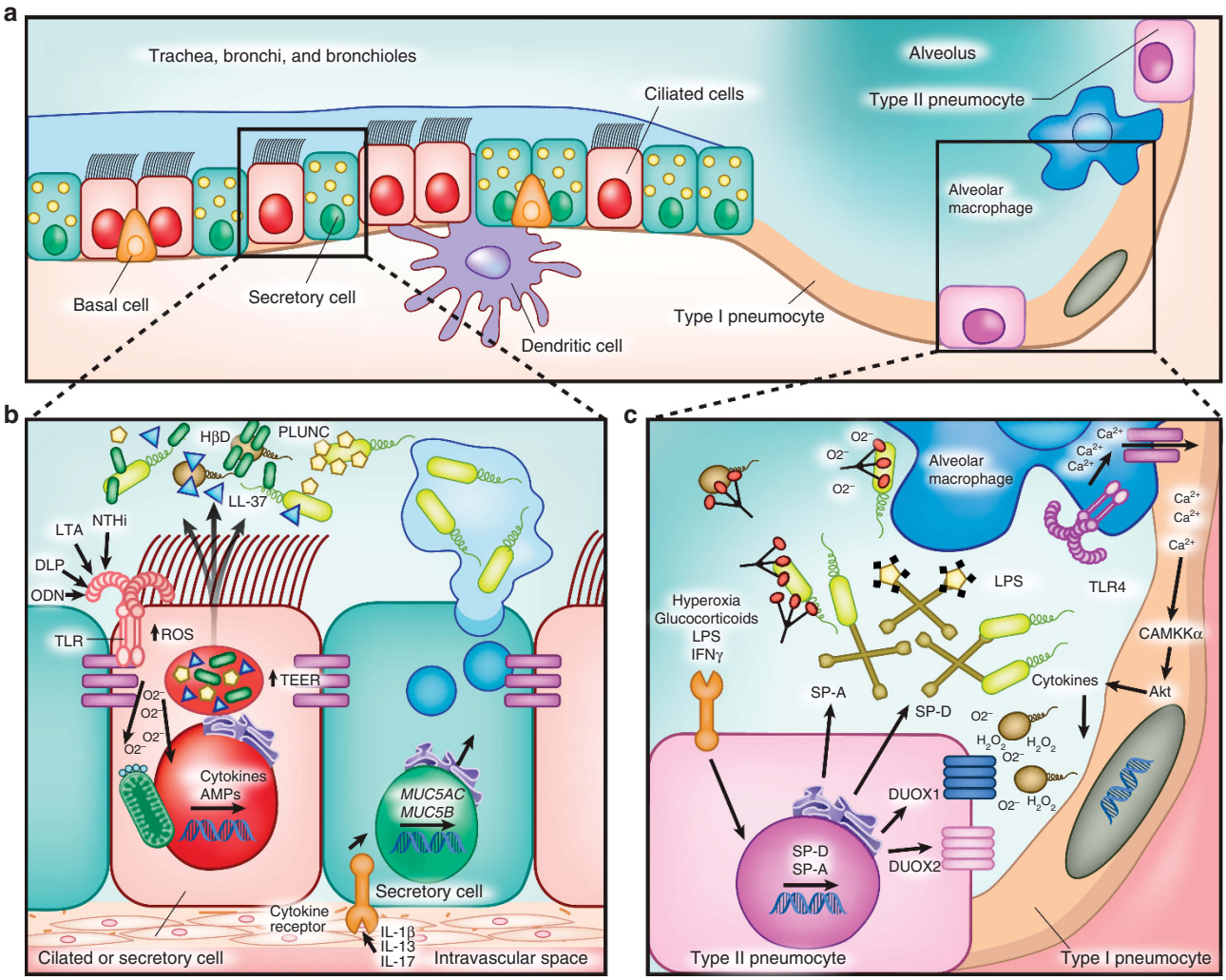

Figure 1 Inducible antimicrobial resistance mechanisms of lung epithelial cells. (**a**) Cells contributing to inducible epithelial airspace defense. (**b**) Inducible responses in the conducting airways. Pattern-recognition and cytokine receptors detect local danger signals in the conducting airways, responding with enhanced barrier and mucociliary functions to improve pathogen exclusion, increased production of microbicidal antimicrobial peptides and volatile species, and secretion of mediators of leukocyte recruitment and activation. (**c**) Inducible responses in the alveolar compartment. Epithelial cells in the gas exchange units of the lungs detect pathogen-associated molecular patterns, perceive stress signals and communicate with lung resident leukocytes, and respond through inducible modulation of barrier function, enhanced production of antimicrobial peptides, collectins and volatile species, and secretion of leukocyte-active cytokines. LTA, lipotechtoic acid; DLP, diacylated lipopeptides; HβD, human β-defensin; NTHi, non-typeable *Haemophillus influenza*; ODN, oligodeoxynucleotide; ROS, reactive oxygen species; SP, surfactant protein; TLR, Toll-like receptor; TEER, transepithelial electrical resistance.

secretory functions of the peripheral lung[4] (**Figure 1a,c**). At baseline, these epithelial populations form a continuous 100 m^2 barrier interface between the host and the external environment. Following PRR activation, this barrier function can be actively adapted to enhance microbial protection. Tight and adherens junctions, connecting cytoskeletons of apposing cells, modulate paracellular flux of ions and macromolecules through structural protein phosphorylation or translation of alternate tight junction protein isoforms to prevent barrier disruption and lung injury in both the airways and alveolar space.[5,6] Inducible modification of paracellular permeability regulates access to epithelial receptors for PAMPs, cytokines, and intraepithelial leukocytes.[7,8] Epithelial tight junctions also contain many potentially targetable signaling molecules, including protein kinase C, Rho proteins, phosphatidyl inositol 3-kinase, transcription factors, and epidermal growth factor receptor family members, such as HER2/3.[7,8] For example, Toll-like receptor (TLR)-2 stimulation of bronchial epithelial cells can activate PKCζ, increasing claudin-1 expression, thereby enhancing transepithelial electrical resistance and tight junctional integrity.[9] Epithelial cytoskeletons can also rearrange to facilitate PRR signaling, and cytoskeletal elements themselves can augment host defenses, as when F-actin released during necrosis activates dendritic cell CLEC9A receptors[10] promoting clearance of dying cells and, ultimately, hastening resolution of infection-related injury.

Perhaps underscoring the importance of these responses, several bacteria possess the ability to enter non-phagocytic cells

via cytoskeletal manipulation and form sequestering compartments that promote their own growth. Mitigating this threat, mammalian PRRs such as NOD1, NOD2, and pyrin can sense pathogenic cytoskeletal protein changes, leading to initiation of proinflammatory responses and entrapment of actin-polymerizing bacteria by septin cages.[11] Thus, although not yet established in practice, manipulation of junctional and cytoskeletal elements may eventually provide valuable opportunities to protect against respiratory infections.

Mucociliary defenses

Particles and microbes entrained into the lower respiratory tract during respiration are impacted into the airway lining fluid by turbulent flow and >90% are expelled from the lungs via the mucociliary escalator within minutes.[12] The largest populations of airway epithelial cells, present in roughly equal numbers, are the ciliated cells that beat the airway lining fluid proximally toward the glottis and the secretory cells that, among other things, contribute mucins to the airway lining (**Figure 1b**). MUC5AC and MUC5B are the most abundantly expressed polymeric secreted mucins in the airways.[13] In addition to optimizing the viscoelastic properties to the airway lining fluid for particle clearance, there is mounting evidence that gel-forming mucins contribute to antimicrobial responses. Stimulated MUC5B production from epithelial cells has been directly linked to antibacterial defense and enhanced alveolar macrophage function,[12] and is inducible by PAMPs such as β-glucan[14] and by some NF-κB-mediated cytokines (e.g., IL-1β, IL-17A).[15] Concordantly, MUC5B-deficient mice have been found to be hypersusceptible to pneumonia and to demonstrate impaired pathogen clearance.[12] MUC5AC is strongly induced by epithelial exposure to IL-13, cathelicidin, and a number of bacterial and viral pathogens,[12,16–18] including specificity protein 1 (Sp1)- and epidermal growth factor receptor-mediated induction by influenza A.[19] Cytosolic PRR stimulation may also enhance airway mucin production, as NLRP6 inflammasome activation promotes mucin secretion during colonic bacterial infections, although this is not yet confirmed in the lung.[20] Active epithelial regulation of periciliary layer pH further augments mucin-facilitated pathogen clearance and defense. The importance of this function is evident in cystic fibrosis, where reduced periciliary pH impairs detachment of submucosal mucins and impedes mucus clearance.[21] The multiple stimuli capable of inducing MUC5AC and MUC5B secretion suggest their potential as therapeutic targets. While membrane-bound mucins (MUC1, MUC4, MUC16) also facilitate mucociliary escalator function, there is limited evidence that their induction by infectious or pharmacological stimuli enhances pathogen clearance.[13] No strategies have definitively shown improved pathogen clearance through ciliary function manipulation in hosts without intrinsic impairments. However, recent data revealing that IL-13-induced bronchial epithelial cell MUC5AC contributes to mucostasis in asthma via tethering of mucus gel domains to the epithelium[22] suggest there may be additional opportunities to augment mucociliarly pathogen clearance in patients with baseline defects, beyond inducing mucus production and moderating periciliary pH.

PATHOGEN DETECTION

PRRs are highly conserved across species, emphasizing their importance to host survival.[23–25] The broad spectrum of PRRs expressed by lung epithelial cells (**Table 1**) allow PAMP sensing within most cellular compartments, facilitating rapid responses to infections, and offering abundant opportunities for therapeutic elicitation of protective epithelial functions.

Toll-like receptors

Lung epithelial cells are reported to express all known human TLRs, including those that localize to the plasma membrane (TLR2/1, TLR2/6, TLR4, TLR5, TLR10) and those that localize to endosomes (TLR3, TLR7, TLR8, TLR9).[26] N-terminal leucine-rich repeat (LRR) domains with varying degrees of N-glycosylation form a characteristic TLR solenoid structure responsible for pathogen sensing.[27] A single transmembrane domain connects leucine-rich repeat domains to a cytoplasmic Toll/IL-1 receptor (TIR) C-terminus tail that recruits TIR adaptor proteins to initiate downstream signaling. Despite the structural similarity of TLRs, their ligand specificity and binding sites vary substantially, allowing them to detect a remarkable spectrum of patterns, including such diverse moieties as lipopeptides, nucleic acids, and bacterial proteins, making TLRs frequent targets of studies investigating epithelial manipulation strategies, as outlined below.

NOD-like receptors

NOD-like receptors (NLRs) are soluble cytosolic proteins composed of a C-terminal leucine-rich repeat domain that confers ligand specificity, a central nuclear oligomerization domain (NOD) and a variable N-terminal effector domain.[28] They are further categorized by their effector domains into caspase recruitment domain (CARD or NLRCs), pyrin domain (PYD or NLRPs) or baculoinhibitor of apoptosis protein domain (NAIPs or NLRBs). The active oligomerization of NLRs, adaptor proteins and caspases comprises inflammasome formation, resulting in cleavage of pro-IL-1β and pro-IL-18 into their active forms.[29]

NLRs expressed by lung epithelial cells include NOD1 and NOD2 which bind bacterial peptidoglycan moieties, activating mitogen-activated protein (MAP) kinase, NF-κB, and autophagic pathways.[3] Deficiency[30] or polymorphisms[31] of these receptors results in increased susceptibility to respiratory infections. NLRP1 enhances resistance to pneumonia by detecting virulence factors such as *Bacillus anthracis* lethal toxin.[32] NLRP3 is an important bronchial epithelial DAMP sensor during infection,[33,34] detecting potassium efflux,[35] excess reactive oxygen species (ROS) production,[36] or mitochondrial dysfunction.[37] NLRC4 initiates inflammasome formation following detection of flagellated bacteria such as *Legionella pneumophila*,[38] *P. auruginosa*,[39] *Klebsiella pneumoniae*, and those expressing type-III secretion systems (T3SS). NLRX1 possesses an N-terminal mitochondrial targeting sequence and appears to contribute to ROS production by

Table 1 PRRs in the pulmonary epithelium

Receptor	Canonical activating ligands	Upregulated by	Compartment	Adaptor protein
Toll-like receptors				
TLR2/1	Triacylated lypopeptides (e.g., $Pam3CSK_4$)		P.M.	MyD88
TLR2/6	Diacylated lipopeptides (e.g., $Pam2CSK_4$)	Diacylated lipopeptides, ODNs, NTHi	P.M.	MyD88
TLR3	dsDNA, poly (I:C)	Poly (I:C)	Endosome	TRIF
TLR4	LPS, LBP		P.M.	MyD88, TRIF
TLR5	Flagellin		P.M.	MyD88
TLR7	ssRNA		Endosome	MyD88
TLR8	ssRNA		Endosome	MyD88
TLR9	CpG ODN	Diacylated lipopeptides, ODNs, NTHI	Endosome	MyD88
TLR11	Profilin, flagellin		P.M.	MyD88
NOD-like receptors				
NOD1	D-glutamyl-meso-diaminopimelic acid		Cytosol	RIP2, ATGL16
NOD2	MDP		Cytosol	RIP2, ATGL16
NLRP1	*B. anthracis* lethal toxin, MDP, ribonucleoside triphosphate	TLR stimulation	Cytosol	ASC inflammasome
NLRP3	*K. pneumonie*, *S. pneumonie*, *S. aureus*, *C. pneumonie*, *M. tuberculosis*, influenza, rhinovirus, RSV, *A. fumigatus*, ROS, mitochondrial dysfunction, K efflux, Ca mobilization, ATP, uric acid, hyaluronan, silica, asbestos	Host GI microbiota, TLR stimulation	Cytosol	ASC inflammasome
NLRP4	Unknown	Unknown	Cytosol	Beclin-1
NAIP	T3SS needle protein (Cpr1), flagellin		Cytosol	NLRP4 inflammasome
NAIP2	*P. aeruginosa*, T3SS (Prg1)		Cytosol	NLRC4 inflammasome
NAIP5	Flagellin		Cytosol	NLRC4 inflammasome
NLRC4	*L. pneumophila*, *K. pneumonia*, flagellum proteins, T3SS		Cytosol	ASC inflammasome
NLRC5	Viruses		Cytosol	ASC inflammasome
NLRX1	RNA		Mitochondria	MAVS
RLRs				
RIG-I	5'ppp-ssRNA, Paramyxoviruses, influenza, Japanese encephalitis virus, reoviruses, flaviviruses	Type 1 IFN	Cytosol	MAVS
MDA5	Poly (I:C), picoraviruses, flaviviruses, reoviruses	Type I IFN	Cytosol	MAVS
Nucleotide				
cGAS	DNA		Cytosol	STING-IRF3
STING	Cyclic di-nucleotides		E.R.	STING-IRF3
IFI16	DNA		Cytosol	STING
AIM2	DNA		Cytosol	AIM2 inflammasome

ATP, adenosine triphosphate; E.M., endoplasmic reticulum; GI, gastrointestinal; LBP, lipopolysaccaride-binding protein; LPS, lipopolysaccharide; MDP, muramyl dipeptide; NTHi, non-typeable *Haemophilus influenza*; ODN, oligodeoxynucleotide; P.M., plasma membrane; Poly I:C: poly-inosine:poly-cytosine; RLRs, Rig-I-like receptors; ROS, reactive oxygen species; RSV, respiratory syncitial virus; TLR, toll-like receptor; TTSS, type three secretion system.

the respiratory chain transport when stimulated by RNA.[28] In contrast, stimulation of other lung epithelial NLRs, such as NLRC3 and NLRC5, is associated with anti-inflammatory responses,[40] suggesting that therapeutic targeting of NLRs may allow selective activation or resolution of antimicrobial responses.

Nucleic acid sensors

Microbe- or host-derived nucleic acids, respectively, can serve as PAMPs or DAMPs that initiate antimicrobial responses. In addition to endosomal TLRs, multiple cytosolic receptors detect RNA and DNA in lung epithelial cells.[41] This prominently includes retinoic acid inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), cytosolic RIG-I-like helicases (RLRs) that sense ssRNA and dsRNA with a 5' triphosphate motif.[29] RLR expression is enriched in lung epithelial cells in response to type I interferons[42] and signal through the mitochondrial membrane-bound protein MAVS (also known as IPS-1, VISA, and Cardif) to elicit IRF3, IRF7, and NFκB mediated antimicrobial responses. RLR-MAVS signaling is critical to interferon production during RSV pneumonia,[43] for example, and contributes to infection-induced airway hyperreactivity through increases in metalloproteinase and cathepsin production.[44] Though less studied, lung epithelial MDA5 may also elicit virus-specific protection in other infection models.[45]

Independent of TLRs, NLRs and RLRs, lung epithelial cytosolic detection of microbial DNA and cyclic di-nucleotides[42] frequently involves signaling via stimulator of interferon genes (STING), an endoplasmic reticulum-localized protein that acts both as a direct receptor for bacteria-produced cyclic dinucleotides and as an adaptor molecule for other cytosolic nucleic acid receptors,[46,47] such as IFI16[48] or DDX41 (which can also detect both DNA and cyclic dinucleotides).[49,50] Additionally, 2'3'-cyclic GMP-AMP (cGAMP) is produced by cGAMP synthase (cGAS) in lung cells upon cGAS binding of microbial DNA, resulting in alternate activation of STING by cGAMP,[51] making both STING and cGAS frequent targets of investigation for potential therapeutic manipulation of responses from the lungs and elsewhere. Other STING-independent lung epithelial DNA sensors such as DAI, AIM2 and LRRFIP1[ref. 52] have been described, although their relevance in inducible lung immunity remains less established.

C-type lectins

Pathogen-derived carbohydrates, including mannose, fucose, and glucans, can be detected by plasma membrane localized C-type lectins. These receptors are widely distributed on epithelial and myeloid cells and allow detection of fungi, viruses, and bacteria.[53] Dectin-1 is expressed in bronchial epithelial cells and mediates inflammatory responses to infections caused by non-typeable *Haemophilus influenza* (NTHi)[54] and a number of fungi.[55] Surfactant proteins SP-A and SP-D also contain C-type lectin domains, and broadly contribute to host defense via pathogen detection,[56] in addition to the antimicrobial effects discussed below. Epithelial cells can also detect fungal glucans via the non-lectin containing glycosphingolipid lactosylceramide.[57]

ANTIMICROBIAL EFFECTOR MOLECULES

Upon PRR activation, lung epithelial cells not only generate cytokine signals that initiate leukocyte-mediated responses but they also produce numerous effector molecules that exert directly microbicidal effects (**Table 2** and **Figure 1**) and have been targeted for potential therapeutic induction.

Cathelicidin and defensins

Small cationic antimicrobial proteins (AMPs) are frequently synthetized as pre-pro-peptides that are cleaved before secretion to expose share positively charged moieties that disrupt negatively charged microbial membranes,[58,59] as is the case for lung epithelial defensins and cathelicidin.[60] Defensins are amphipathic molecules that a common γ-core region and are further subdivided into α-defensins and β-defensins based on disulfide bridge arrangement. Although previously thought to express only β-defensins, recent data indicate that murine lung epithelial cells also produce α-defensin 5[ref. 61] and α-defensin 1 following exposure to bacterial lysates.[62] The only human cathelicidin, hCAP18, possess two characteristic hydrophobic α-helix domains.[63] hCAP18 is stored in pre-formed granules and, upon stimulation, is cleaved on its N-terminal domain to its active form, LL37.[3] LL37 is secreted by airway and submucosal gland epithelial cells and is believed to electrostatically disrupt bacterial membranes.[64] The functions of epithelial defensins and LL-37 are multiple, both selectively promoting and attenuating different elements of the inflammatory response.[60,65] When induced by infections, both act as chemoattractants for neutrophils, dendritic cells, T cells, macrophages, and monocytes.[66–68] LL-37 also interacts synergistically with IL-1β to promote local inflammatory cytokine production.[69] However, its binding of negatively charged molecules also allows LL-37 to sequester lipopolysaccaride (LPS) and certain DAMPs (e.g., self-DNA and RNA), thereby attenuating other inflammatory responses.[70–72]

Antiproteases

Lung cells express secretory leukocyte protease inhibitor and elastin-specific inhibitor (elafin) in response to stimuli such as LPS, IL-1β and TNF.[73] Secretory leukocyte protease inhibitor is expressed in the airway mucosa[60] and inhibits neutrophil elastase, chymotrypsin, and cathepsin G, but notably also exerts directly antimicrobial effects on bacteria (*Pseudomonas aeruginosa*, *Stephylococcus aureus*, *Escherichia coli*), fungi (*Candida albicans*, *Aspergillus fumigatus*) and HIV.[74,75] Similarly, elafin has direct antimicrobial activity against *P. aeruginosa* and *S. aureus*.[76] Further, both of these antiproteases demonstrate immunomodulatory activity on macrophages and endothelial cells following epithelial stimulation, decreasing IκBα degradation[77] and increasing production of TGFβ and IL-10.[78] Properties promoting tissue repair and resolution of inflammation have also been attributed to secretory leukocyte protease inhibitor and elafin.[79]

Table 2 Inducible antimicrobial peptides in the lung

AMP	Mechanism(s)	Induced by	Observed distribution
Small cationic peptides			
α-defensin 1	Membrane disruption	NTHi lysate	Mouse lung homogenates
α-defensin 5	Membrane disruption	Constitutive	Human nasal and bronchial epithelium
β-defensin 1	Membrane disruption, leukocyte chemotaxis	Constitutive	Mouse and human airway cells
β-defensins 2	Membrane disruption, leukocyte chemotaxis	TNF, IL-1β, LTA, LPS, neutrophil elastase, MEF, triacylated lipopeptides, rhinovirus, L-isoleucine, *L. pneumophila*	Mouse and human airway cells
β-defensin-34, -5, -39	Membrane disruption	NTHi lysate	Mouse lung homogenates
Cathelicidin (hCAP18/LL-37, CRAMP)	Membrane disruption, LPS and DAMP binding, PMN chemotaxis	NTHi lysate, vitamin D3, TLRs	Human airways and submucosal glands, mouse lung homogenates
ELR$^-$ CXC chemokines (CXCL-9, -10, -11,-14)	Membrane disruption, inhibition of spore germination	IFNγ	Mouse lungs
Antiproteases			
SLPI	Membrane disruption, inhibition of inflammation	LPS, neutrophil elastase, cytokines, NTHi lysate, TNF, EGF, IL-1, α-defensins	Club, goblet and nasal epithelial cells
Elafin	Membrane disruption, inhibition of inflammation.	LPS, neutrophil elastase, cytokines, NTHi lysate, TNF, IL-1	Airway epithelial cells
Iron modulators			
Lactoferrin	Iron sequestration	NTHi lysate	Submucosal glands
Lipocalin 2	Iron siderophore binding	IL-22, NTHI lysate, LPS, IL-1B, IL-17	Airway epithelial cells
Other AMPs			
Lysozyme	$\beta_{1\text{-}4}$ glycoside hydrolysis of peptidoglycans	NTHi lysate	Airway epithelial cells
Collectins (e.g., SP-A, SP-D)	Opsonization, membrane permeabilization	Glucocorticoids, IFNγ, NTHi lysate, hyperoxia, LPS	Type II pneumocytes, club cells
SPLUNC1 (BPIFA1)	LPS and biofilm inhibition	TLR2 agonists (e.g., Pam3CSK$_4$)	Airway and nasal epithelial cells

AMP, antimicrobial peptide; BPIFA1, BPI fold containing family A member 1; CRAMP, mouse cathelicidin-related antimicrobial peptide; ELR-, not containing glutamate-leucine-arginine sequence; LPS, lipopolysaccharide; LTA, lipotechoic acid; MEF, myeloid ELF-1-like factor; NTHi, non-typeable *Haemophilus influenzae*; SLPI, secretory leukocyte protease inhibitor; SP, surfactant protein; SPLUNC1, short palate, lung, and nasal epithelial clone 1; TLR, Toll-like receptor; TNF, tumor necrosis factor.

Chemokines

Cytokine and chemokine production are essential to epithelial modulation of immune responses, but some IFNγ-induced epithelial chemokines, including CXCL9, CXCL10, and CXCL11, also demonstrate directly bactericidal effects on *B. anthracis*, *E. coli*, and *Listeria monocytogenes*, [80,81] likely via membrane disruption, given their defensin-like α-helical domains. The N-terminal domain of CXCL14 also appears to affect the integrity of Gram-positive and Gram-negative bacterial membranes.[82]

Lysozyme, lactoferrin, and lipocalin 2

Human lysozyme and mouse lysozyme-1 and -2 are abundantly expressed by lung airway and submucosal gland epithelial cells,[83] catalyzing hydrolysis of β_{1-4} glycosidic bonds in bacterial peptidoglycan.[3] This effect is sufficiently robust that transgenic mice overexpressing lysozyme display decreased susceptibility to pneumonias caused by *P. aureginosa* and Group B Streptococci.[84] Lactoferrin is a cationic glycoprotein that can bind both free iron and bacterial membrane components,[60] impairing bacterial growth. While the isolated effects of epithelial lysozyme and lactoferrin are predominantly bacteriostatic at physiological concentrations, together they synergize to exert bactericidal effects.[85] Lysozyme's activity on Gram-negative bacteria is enhanced by lactoferrin increasing the permeability of bacterial outer membranes, facilitating cleavage of peptidoglycan bonds in the periplasmic space. Consistent observations of this positive interaction have promoted numerous studies of therapeutic supplementation with combinations of recombinant antimicrobial effectors.

Lipocalin 2 is a secreted protein that is also induced by DAMPs and cytokines in multiple cell types, including lung epithelial cells,[62,86–88] and has been found to be important in host responses against *K. pneumoniae* and *E. coli*. Similar to lactoferrin, lipocalin 2 has the ability to sequester iron from siderophores, though it appears likely that additional iron-independent mechanisms also contribute to its antibacterial effect.

Collectins

Lung epithelial collectins (collagen-containing C-type lectins), prominently including SP-A and SP-D, function as soluble PRRs in the airspaces that polymerize into large scaffolds and opsonize or aggregate microbes. SP-A and SP-D expression by type II alveolar epithelial cells and club cells increases in responses to LPS, glucocorticoids, hyperoxia, bacterial lysates, and IFNγ,[62,89] suggesting they may be accessible therapeutic targets. SP-A- and SP-D-mediated opsonization enhances microbial clearance by alveolar macrophages (**Figure 1c**), while their deficiency increases susceptibility to Group B Streptococci, Gram-negative bacteria, RSV, influenza A, and adenoviruses.[90] SP-A and SP-D are also reported to disrupt bacterial membrane permeability of some Gram-negative bacteria.[91]

PLUNC proteins

Palate-lung-nasal-clone (PLUNC) family proteins are secreted by cells throughout the lung and nasal mucosa. SPLUNC1 is induced by TLR2 stimulation in a MAP kinase/AP-1 and NF-κB-dependent matter.[92] Their antimicrobial properties are thought to relate to their structural homology with LPS-binding proteins, though they may also function as surfactant proteins, and possibly inhibit biofilm formation.[93] SPLUNC1 deficient mice are susceptible to *P. aeruginosa* and *K. pneumoniae* pneumonia, and demonstrate impaired production of proinflammatory cytokines and other AMPs.[94,95]

Reactive oxygen species and ion transport

ROS are increasingly recognized to function as direct antimicrobial effector molecules, most likely through lipid peroxidation of microbial membranes and DNA damage, in addition to their well-established roles as signaling molecules. Lung epithelial cells generate ROS both constitutively and inducibly, most abundantly as superoxide or hydrogen peroxide. While all NADPH oxidase (NOX) isoforms are reportedly found in the lungs, dual oxidases (DUOX) are the principal ROS generators of lung epithelial cells. DUOX1 produces a relatively consistent amount of ROS, though recent evidence indicates that this production can be moderately enhanced by IL-4 and IL-13 exposure.[96] In contrast, DUOX2-dependent ROS production can be profoundly increased by activation of existing DUOX2 and increased *DUOX2* and *DUOXA2* transcription following exposure to cytokines such as IFNγ,[96] an intervention that has been shown *in vitro* to reduce pathogen burdens. DUOX enzyme activity is regulated by calcium concentrations and they are predominantly expressed apically on ciliated airway epithelial cells and on type II alveolar epithelial cells, approximating the enzymes to microbes that gain access to the airspaces[97] (**Figure 1c**). In addition to toxic effects of the volatile species directly on pathogens, submucosal epithelial cell-derived lactoperoxidase catalyzes a reaction between epithelial hydrogen peroxide and thiocyanate to form the highly microbicidal molecule hypothiocyanate.

As observed in cystic fibrosis, impaired anion transport due to cystic fibrosis transmembrane conductance regulator (CFTR) mutations causes decreased bicarbonate secretion, resulting in reduced periciliary pH and impaired AMP function.[98] In addition, active epithelial ion transport is required to provide halide (chloride, bromide, iodide) and pseudohalide (thiocyanate) substrates for ROS-mediated antimicrobial defenses.[99] Thus, investigators have variously proposed to stimulate DUOX activity in the lungs, modify ion channel activity, and enhance halide/pseudohalide availability as means to enhance antimicrobial defenses of the lungs.

EPITHELIAL MODULATION OF IMMUNE RESPONSES AND TOLERANCE

PRR elicited epithelial cytokines importantly participate in the recruitment and activation of leukocytes in the lung. Beyond simply increasing the number of leukocytes present, epithelial cell responses clearly modulate adaptive immune responses in the lung during infections.

Modulation of inflammation

Lung epithelial cells have long been recognized to demonstrate profound asthmatic/allergic phenotypes when exposed to IL-4, IL-5, IL-9, and IL-13. However, it is now evident that they are also capable of autonomously promoting or initiating type 2 inflammatory responses. A typical example of this is TLR4-mediated detection of house dust mite antigen[100] by club cells, resulting in allergic cytokine secretion and Th2 immune deviation. This epithelial exposure promotes production of IL-33, TSLP, and IL-25, stimulating DCs to subsequently activate type 2 innate lymphoid cells (ILC2), basophils, eosinophils and mast cells.[101] Epithelial granulocyte macrophage colony-stimulating factor (GM-CSF) is also secreted in response to allergens and induces maturation, proliferation, and activation of antigen presenting cells that drive type 2 responses.[102] Infection-released DAMPs also contribute to epithelial type 2 immune deviation. Epithelial cells respond to uric acid and ATP that are released into the airway in response to allergens, further releasing IL-33 and TSLP.[103,104] While type 2 deviation is often regarded as maladaptive (i.e., allergy) or relevant only to parasitic diseases, it has been shown that enhanced CCL8 production and neutrophil recruitment associated with type 2 responses augment clearance of *K. pneumoniae*.[105] Type 1 and type 17 inflammation are more commonly regarded as protective against respiratory pathogens, and lung epithelial cells are analogously capable of deviating immune responses to these patterns, as well. For example, lung cells express IFN-ϒ in response to mycobacterial and fungal infections, promoting protective type 1 inflammatory deviation[106,107] Similarly, not only do lung epithelial cells produce brisk cytokine responses when exposed to IL-17A and IL-17F but also epithelial

production of IL-6, TGFβ, and IL-21 (also possibly IL-23) can enhance differentiation of Th17 cell differentiation[108–111] sculpting local adaptive responses in response to PAMP exposure.

Tolerance and downregulation of inflammation

Host survival of pathogen challenges depends on two fundamental strategies: resistance and tolerance. Resistance relies upon reductions in the microbial burden to limit injury and preserve fitness. Alternately, tolerance promotes survival through host adaptations to limit the pathogen's ability to inflict damage or to reduce noxious and/or maladaptive responses associated with host defenses (immunopathology), rather than eliminating the pathogen.[112] Literature describing infection tolerance in the lung remains limited, and studies describing therapeutic manipulation are rarer still. However, induction of host-protective responses, rather than pathogen-toxic elements, holds promise as a strategy that could be applicable in many infectious or inflammatory conditions.

For example, recent studies indicate that alveolar epithelial cells communicate with alveolar macrophages through connexins via calcium spikes during LPS-induced inflammation.[113] This communication activates Akt-dependent signal propagation through adjacent cells, reducing neutrophil chemotaxis and proinflammatory cytokine release. These counterbalance proinflammatory responses in the lung, attenuating immunopathology. Similarly, the mucin MUC1 is induced by epithelial exposure to TNF and IL-8,[114] and can regulate inflammation during bacterial infections through inhibition of TLR signaling.[115,116] While MUC1-deficient mice show enhanced *P. aeruginosa* clearance following intranasal challenge, they also exhibit higher inflammatory chemokine expression, and greater neutrophil influx, resulting in greater immunopathology.[117]

The lungs also mitigate immunopathology via inhibitory feedback from the antimicrobial effector molecules described above. For example, epithelium-derived LL-37 can sequester LPS[72] and decrease cytokine release[118] to dampen immune responses. Similarly, epithelial α-defensin 1 can actively attenuate phagocyte ROS burst during influenza infections without impairing viral resistance.[119] Further preventing immunopathology, epithelial cells produce mediators that promote resolution of inflammation, induce apoptosis, and decrease neutrophil chemotaxis.[120,121] Polyunsaturated fatty acids present in epithelial cell membranes can be enzymatically converted to resolvins, maresins, protectins, and lipoxins. These molecules decrease proinflammatory cytokine expression, attenuate neutrophil oxidative burst, and decrease leukocyte chemotaxis, although their therapeutic inducibility remains largely untested.

THERAPEUTIC MANIPULATION OF EPITHELIAL IMMUNITY

The inducibility of the aforementioned epithelial defenses has prompted several groups to investigate exploitation of these mechanisms to protect against pneumonia. This approach offers an appealing complement to conventional antibiotic treatment, due to the lack of documented antibiotic resistance and broad protection that generally does not require prior pathogen identification. The most commonly applied strategies are exposure of lung cells to synthetic PRR ligands and the exogenous administration of AMPs (**Table 3**).

Targeting of PRRs

Early attempts to therapeutically harness inducible lung epithelial antimicrobial responses often centered on efforts to broadly stimulate PRRs by simulating native infections prior to experimental challenges.[3] Typical of this nontargeted approach to PRR stimulation was intrapulmonary treatment of mice or isolated lung cells with noncognate bacterial lysates before pneumonia challenge. Exposing mice to the myriad PAMPs in a bacterial lysate induces robust responses that were shown to be protective against a wide range of otherwise lethal pathogens, including *B. anthracis*, *Yersinia pestis*, *Francisella tularensis*, *S. pneumoniae*, *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, influenza A, and *A. fumigatus*.[62,122] The protection was uniformly associated with pathogen killing in the lungs, and noted to correlate with enrichment of numerous antimicrobial molecules such as cathelicidin, defensins, lipocalin 2, surfactant proteins, orosomucoids, and calprotectins,[62] suggesting activation of many of the above described processes. While treatment with crude lysates neither answered the questions of which PRRs were required for protection nor leant itself particularly well to clinical development, the finding that inducible protection was completely lost in MyD88-deficient mice[123] suggested that focused targeting of specific PRR population might be successful.

TLR stimulation

A substantial majority of studies investigating targeted PRR stimulation in the lungs have focused on manipulation of TLRs. The broad array of TLRs expressed by airway cells makes intrapulmonary administration of TLR agonists a conceptually attractive means of inducing resistance. Since shortly after the discovery of mammalian TLRs, investigators have delivered TLR agonists systemically to animals to initiate or modify leukocyte-mediated immunity, including efforts attempting to elicit secondary antimicrobial responses from intestinal epithelial cells. However, the presence of TLRs at the accessible environmental interface of the lungs allows for direct action on epithelial cells, which is a fundamentally different strategy than eliciting responses from circulating or intraepithelial leukocytes that then act on epithelial cells. In many cases, TLR ligands previously used to study signaling pathways have been repurposed for intrapulmonary delivery, with some ligands modified and/or conjugated to improve bioavailability and pharmacokinetics. For instance, intranasal delivery of a purified TLR5 agonist (flagellin) to the lungs protected mice against otherwise lethal *P. aeruginosa* pneumonia. This protection was partially dependent upon cathelicidin induction and persisted when neutrophils were depleted.[124] A related strategy delivering intranasal pretreatment with an albumin-conjugated TLR7 agonist (UC-1V150) protected mice against pneumonia caused by *B. anthracis* or H1N1 influenza. This

Table 3 Targeted therapies for innate immunity in lung infections

Component	Receptor/ ligand	Route	Protects against	Presumed mechanism	Reference
Pattern-recognition receptors					
UC-1V150	TLR7	I.T.	*B. anthracis*, influenza H1N1, VEE	IFN induction	126
3M-011	TLR7 and TLR8	I.N.	Influenza virus	IFNα induction	129
PO R10-60	TLR9	I.N.	*B. anthracis*		130
Poly (I:C)	TLR3	I.N.	*P. aeruginosa, F. tularensis*, influenza A	Chemokine induction	127
Pam2CSK4 + ODNM62	TLR2/6 and TLR9	Nebulized	*S. pneumoniae, P. aeruginosa, B. anthracis*, influenza A, *A. fumigatus*	AMP and ROS generation	123,132–134,155
Flagellin	TLR5	I.N.	*S. pneumoniae* and influenza A	Neutrophil recruitment	156
5'pppRNA	RIG-I	I.V.	VSV, Dengue, Vaccinia, HIV, H1N1 influenza	Type 1 IFN induction	135,139
Eritoran (E5564)	TLR4 antagonist	I.V.	Influenza A	Proinflammatory cytokine blockade	136
AGP	TLR4	I.N.	*F. novicida*	IFNγ induction	131
CpG ODN	TLR9	I.T.	*K. pneumonie*	IFNγ and chemokine induction	157
Antimicrobial Peptides					
LL-37		I.T.	*P. aeruginosa*, MRSA, RSV, *M. tuberculosis*	Membrane disruption	142,158
CAP18		I.T.	*P. aeruginosa*	Chemokine induction	118
L-isoleucine		I.T.	Multidrug-resistant *M. tuberculosis*	HBD-2 induction	145
Novospirin G10		I.T.	*P. aeruginosa*	Direct microbicidal	146
β-defensin 2		I.T.	*P. aeruginosa*	HBD-2 upregulation	144

AGP, amynoalkyl-glucosamine phosphate; AMP, antimicrobial peptide; HBD, human beta defensin; IFN, interferon; I.N., intranasal; I.T., intratracheal; I.V., intravenous; MRSA, methicillin-resistant *Staphylococcus aureus*; ROS, reactive oxygen species; RSV, respiratory syncytial virus; TLR, Toll-like receptor; VEE, venezuelan equine encephalitis virus; VSV, vesicular stomatitis virus.

enhanced survival correlated with increased airway cytokine and chemokine production.[125,126] Similarly, intranasal administration of a TLR3 agonist (poly I:C) during a cecal ligation and puncture sepsis model enhanced survival and decreased lung bacterial burdens following a secondary *P. aeruginosa* pneumonia challenge.[127] Prophylactic or post-exposure intranasal poly I:C treatment also enhanced survival of *F. tularensis* pneumonia in a manner that was associated with increased local cytokine secretion and neutrophil influx to the airway, along with decreased lung pathogen burden.[128] Another study of intranasal pretreatment of the lungs with a TLR7/8 agonist (3M-011) enhanced H3N2 influenza clearance in a rat model of pneumonia, noting the viral titer reductions to correlate with increases in TNF, IL-12 p40/70, and type I IFNs.[129] Intranasal prophylactic TLR9 stimulation with a phosphodiester-oligodeoxynucleotide (PO R10-60) also protected mice against *B. anthracis* pneumonia, and increased inflammatory cytokines and type I interferons in lavage fluid.[130] In a model of pneumonic tularemia, mice treated with the synthetic TLR4 ligand aminoalkyl glucosaminide phosphate before and after infection displayed increased survival. This protection was dependent on neutrophil influx and cytokine production in the airway and was lost in IFNγ knockout mice.[131]

The broad protection induced by nontargeted stimuli (e.g., bacterial lysates), the robust responses generated by intrapulmonary application of specific ligands, and the positive microbicidal interactions observed when combining antimicrobial effector molecules together suggest that combinations of TLR stimuli might confer greater pneumonia protection than achieved with a single ligand. This hypothesis is supported by studies such as those testing concurrent inhalational administration of a TLR2/6 ligand (Pam2CSK4) and a TLR9 ligand (ODN M362) that synergistically interact to robustly protect mice against viral, bacterial and fungal pneumonia.[132,133] Protection induced by the synergistic inhaled TLR ligands is dependent upon lung epithelial TLR signaling and is associated with production of both ROS and AMPs.[134]

Notably, all of the TLR agonist treatments described above initiate local inflammatory responses, and the reported survival benefits are all associated with reduced lung pathogen burdens, suggesting generation of a microbicidal environment. Further, all of the foregoing TLR stimulation strategies are presumed to predominantly engage lung epithelial TLRs, based on their delivery to the airways via intranasal, intratracheal or inhalational exposure. While epithelial cells comprise the vast majority of the treated surface area, TLRs on other cells (particularly, leukocytes) also encounter the ligands and likely generate relevant responses, as well. Thus, although the epithelial TLR requirement is established in these various treatments by loss of protection when TLR signaling is

conditionally disrupted in the lung epithelium and/or by recapitulation of the antimicrobial phenomena when epithelial cells are studied in isolation,[122,125,127,130,134,135] *in vivo* sufficiency of the epithelium to effect the complete phenotype is seldom established.

TLR inhibition

The immunopathology caused by excessive inflammation induced by certain infections may exceed the injury caused by pathogen virulence factors. In these cases, induction of tolerance may be a better strategy to survive infection. Mice lacking TLR4 seem to have an increased tolerance to influenza and inhibition of TLR4 signaling with eritoran 2 days after established infection decreased viral titers, alveolar inflammation, cytokine secretion, and overall survival.[136] Although this strategy has been investigated in the clinical context of sepsis, it has not been demonstrated to improve overall mortality and it risks rendering the host susceptible to death by secondary bacterial pneumonia,[137] potentially limiting its current applicability.

RIG-I stimulation

A synthetic 5'-triphosphate RNA RIG-I ligand (M8) has been shown to initiate protective responses against viral infections. Similar to several synthetic TLR agonists, M8 induces antiviral responses from isolated A549 lung epithelial cells against both RNA and DNA viruses. However, although *in vitro* epithelial responses have been demonstrated and intravenous injection of M8 before challenge with H1N1 influenza increased survival and decreased viral titers in the lung,[138,139] there remain no *in vivo* data following direct intrapulmonary administration.

EFFECTOR MOLECULE SUPPLEMENTATION

Antimicrobial peptides

In vitro investigations using synthetic small cationic AMPs have long offered promise as microbicidal interventions, although the ion charges and concentrations used in pathogen killing experiments may be meaningfully different from *in vivo* conditions. In fact, survival benefit following *in vivo* administration of cationic peptides appears to largely rely on their ability to modulate inflammatory responses in the lungs, with rather inconsistent antimicrobial effects.[140,141] For example, while it was found that synthetic human LL-37 instilled intranasally during *P. aeruginosa* infection decreased the pulmonary pathogen burden at 24 h, there was no bacterial difference at earlier time points and enhanced survival appeared to correlate more strongly with neutrophil chemotactic properties of LL-37.[142] This is concordant with other reports that LL-37 instillation promotes recruitment of neutrophils and monocytes.[143] Intratracheal delivery of a related rabbit-derived molecule cationic antimicrobial peptide 18 (CAP18) also enhanced mouse survival of *P. aeruginosa* pneumonia. Similar to LL-37, the survival advantage was not associated with differences in bacterial burdens, but there was a significant decrease in proinflammatory interleukins in lavage fluid.[118] Human beta-defensin-2 is a highly inducible airway epithelial AMP. Overexpression of human beta-defensin-2 before intratracheal *P. aeruginosa* infection, attenuates lung inflammation and a significantly increases survival.[144] Similarly, in a pulmonary multidrug-resistant *M. tuberculosis* model, intratracheal administration of L-isoleucine 60 days after infection significantly increased mBD-3 (the murine ortholog of human beta-defensin-2). This increase was associated with a decreased bacterial burdens and pneumonia extent,[145] as well as increased levels of lung TNF and IFNγ. Novispirin G10, a cationic protein isolated from sheep neutrophils, delivered to rats intratracheally immediately after infection with *P. aeruginosa* significantly decreased bacterial burdens and lung damage compared to sham treatment, although no survival difference was noted.[146] IL-22 which can upregulate lipocalin-2 has been shown to rescue IL-23 deficient mice in the setting of acute *K. pneumoniae* infection.[88] The lung epithelium also expresses members of the regenerating islet-derived proteins which can bind peptidoglycan on gram-positive bacteria.[147] These proteins are STAT3-regulated and undergo post-translational modification in the lung to exert bactericidal activity against MRSA.[148] Collectively, these examples demonstrate that therapeutically supplemented AMPs frequently elicit effects by both microbicidal and immunomodulatory mechanisms, just as observed with native AMP induction.

Relatedly, cytokine supplementation has been undertaken with the intent of modulating immune responses, but as discussed above, several of these molecules also possess antimicrobial activity. Thus, their supplementation may directly reduce pathogen burden, as well.

ALTERNATE STRATEGIES

Although induction of epithelial ROS can kill pathogens, direct supplementation of ROS or other volatiles to the lung is generally not feasible. However, dietary supplementation or nebulized delivery of halide and pseudohalide substrates to the lung epithelium can enhance peroxidase-catalyzed production of highly microbicidal, ROS-dependent molecules (e.g., hypothiocyanate, hypoiodous acid, hypochlorous acid). To date, these studies have been associated with increases in the investigated antimicrobial molecules and reductions in pathogen burdens,[99,149,150] but have not demonstrated enhanced pneumonia survival.

Another epithelium-targeted strategy to protect against pneumonia is the potentiation of antimicrobial and mucociliary function through manipulation of periciliary pH. In piglets with cystic fibrosis, inhalation of bicarbonate or tromethamine increases periciliary pH, enhancing the bactericidal function of AMPs.[151] This may explain the decrease in *P. aeruginosa* colonization in patients treated with the CFTR potentiator ivacaftor.[152,153] Reports describing successful manipulation of barrier function or induction of pneumonia tolerance remain lacking. However, TLR5 stimulation has been shown to reduce immunopathology in other conditions, such as radiation injury,[154] so targeting these elements of epithelial defense may be feasible.

CONCLUSIONS

Taken together, the lung epithelium is capable of significant antimicrobial responses and actively participates in different threat-reduction strategies against pathogen load and noxious stimuli. It is possible to take advantage of these mechanisms to prevent infection during peak susceptibility periods despite leukocyte dysfunction or depletion. Further research in mucosal innate immunity will provide insight into potential targeted therapies for induction of epithelial antimicrobial responses and prevention of lung injury in humans.

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DISCLOSURE

M.M.L.-J. declares no conflicts of interest.J.K.K. declares no conflicts of interest. S.E.E. is an author on US patent 8,883,174 entitled 'Stimulation of Innate Resistance of the Lungs to Infection with Synthetic Ligands' and owns stock in Pulmotect, Inc., which holds the commercial options on these patent disclosures.

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