

REVIEW

Inhaled innate immune ligands to prevent pneumonia

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Epithelial surfaces throughout the body continuously sample and respond to environmental stimuli. The accessibility of lung epithelium to inhaled therapies makes it possible to stimulate local antimicrobial defences with aerosolized innate immune ligands. This strategy has been shown to be effective in preclinical models, as delivery of innate immune ligands to the lungs of laboratory animals results in protection from subsequent challenge with microbial pathogens. Survival of the animal host in this setting correlates directly with killing of pathogens within the lungs, indicating the induction of a resistance mechanism. Resistance appears to be mediated primarily by activated epithelial cells rather than recruited leucocytes. Resistance reaches a peak within hours and persists for several days. Innate immune ligands can interact synergistically under some circumstances, and synergistic combinations of innate ligands delivered by aerosol are capable of inducing a high level of broad host resistance to bacteria, fungi and viruses. The induction of innate antimicrobial resistance within the lungs could have clinical applications in the prevention of lower respiratory tract infection in subjects transiently at high risk. These include cancer patients undergoing myeloablative chemotherapy, intubated patients being mechanically ventilated, vulnerable individuals during seasonal influenza epidemics, asthmatic subjects experiencing a respiratory viral infection, and healthy subjects exposed to virulent pathogens from a bioterror attack or emergent pandemic. In summary, stimulation of the lung epithelium to induce localized resistance to infection is a novel strategy whose clinical utility will be assessed in the near future.

LINKED ARTICLES

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Abbreviations

DAMP, danger-associated molecular pattern; IL, interleukin; LRR, leucine-rich repeat; NLR, NOD-like receptor; ODN, oligodeoxynucleotide, which refers here to a Class C unmethylated CpG oligodeoxynucleotide; Pam2, S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine; Pam3, N-palmitoyl-S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine (Pam₃CSK₄); PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; RLR, retinoic acid induced gene-I-like receptor; TIR, Toll/interleukin-1 receptor; TLR, Toll-like receptor

Inducible epithelial defences

Epithelia have traditionally been viewed as passive mechanical barriers to pathogen invasion, whereas leucocytes have been viewed as mediators of active antimicrobial defences. Increased appreciation of the importance of innate immunity in recent decades has led to recognition of the active defences of epithelial tissues. These active epithelial defences are carried out by barrier epithelia such as those of the respira-

tory, digestive, and reproductive tracts and skin in local innate immunity, and by internal epithelial organs such as the vertebrate liver and insect fat body in systemic innate immunity. Besides producing antimicrobial products that mediate baseline resistance to microbial infections, these barrier and internal epithelial tissues are now known to be capable of greatly increased antimicrobial function after stimulation. The high degree of inducibility of antimicrobial epithelial defences suggests that these may be manipulated

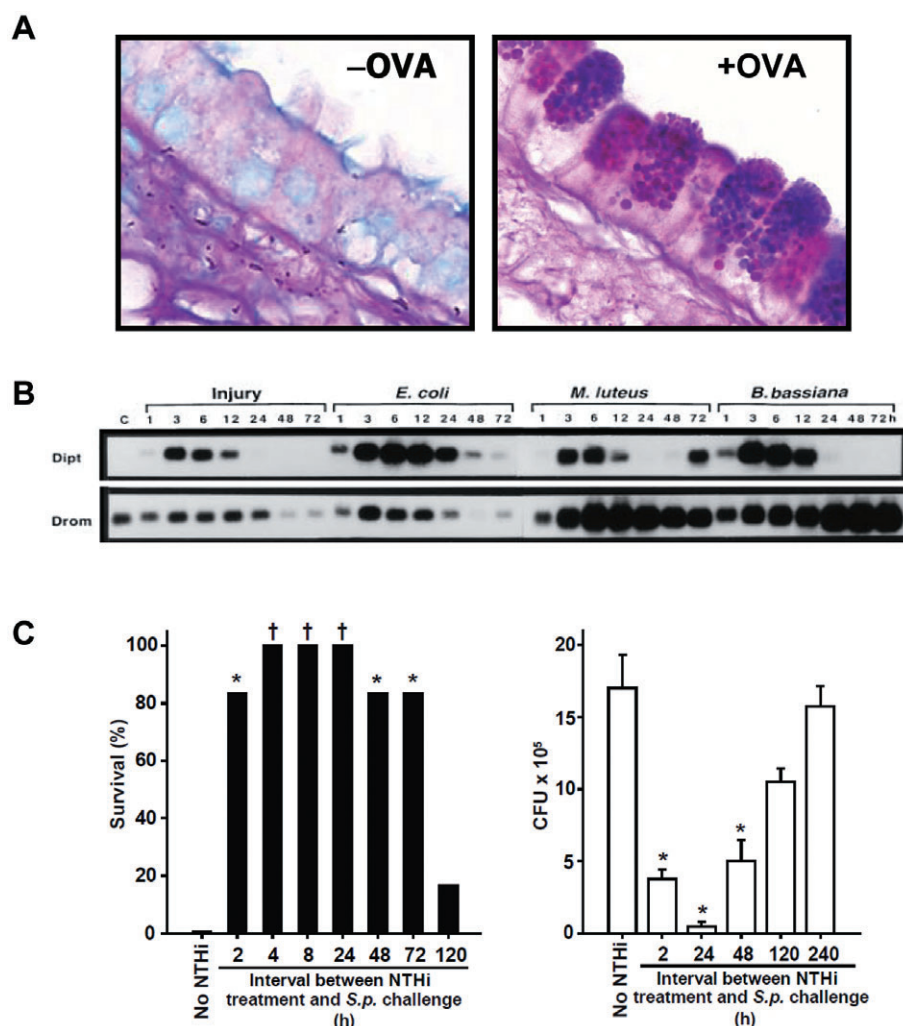


Figure 1

Structural, molecular and functional plasticity of epithelial cells. (A) Mucous metaplasia of mouse airways. Mice were sensitized to ovalbumin (OVA) by three intraperitoneal injections with alum as an adjuvant, then challenged with a single ovalbumin aerosol exposure. Three days after the ovalbumin aerosol, mice were killed and their lungs stained with Alcian blue and periodic acid Schiff's reagent to stain mucins. The airway of a healthy mouse at baseline is shown on the left, with alternating ciliated and secretory cells and no stainable intracellular mucin. The airway of a mouse with mucous metaplasia is shown on the right, with secretory cells filled with large mucin-containing granules (from Evans *et al.*, 2004). (B) Time course and specificity of the induction of antimicrobial peptides in *Drosophila*. The thorax was pricked with a needle (Injury), or a needle dipped into a culture of the Gram-negative bacterium *E. coli*, the Gram-positive bacterium *M. luteus* or the fungus *B. bassiana* (top row). At the designated times after challenge, total RNA was extracted and probed by Northern blot analysis for the expression of dipterin (Dipt) and drosocin (Drom) (from Lemaitre *et al.*, 1997). (C) Time course of the induction of resistance in mouse lungs. On the left is shown the survival of mice pretreated or not with a sterile aerosolized lysate of nontypeable *H. influenza* (NTHi), then challenged with live aerosolized *S. pneumoniae* (*S.p.*) (**P* = 0.015, †*P* = 0.002, treated vs. untreated). On the right is shown bacterial counts in the lungs of mice immediately after aerosolization of *S. pneumoniae* (from Clement *et al.*, 2008) (**P* < 0.05, treated vs. untreated).

therapeutically to promote resistance to infection. This article focuses on the therapeutic induction of innate defences of the lungs to prevent pneumonia.

Barrier epithelia and 'perimeter defence'

Our interest in inducible antimicrobial defences arose from work by us and others on airway mucous metaplasia during

allergic inflammation. We were struck by the remarkable structural plasticity of the epithelium (Figure 1A), indicating that it is highly responsive to its environment (Evans *et al.*, 2004; 2009; Williams *et al.*, 2006). Accompanying such structural changes, work by others had shown extensive changes in lung epithelial cell gene expression (Figure 1B) in response to inflammatory stimuli (Travis *et al.*, 2001; Knowles and Boucher, 2002; Martin and Frevert, 2005; Rogan *et al.*, 2006; Woodruff *et al.*, 2007; Zhen *et al.*, 2007). Since infection has been a powerful force shaping metazoan evolution, and these structural and molecular changes occur in response to inflam-

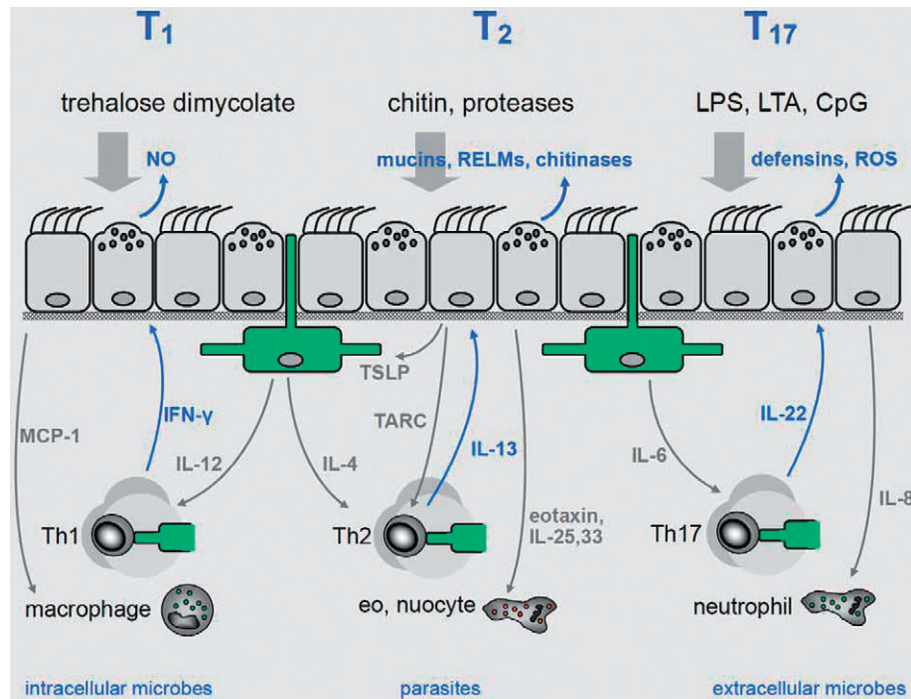


Figure 2

Interactions of airway epithelium with leucocytes and the external environment. The airway epithelium is comprised of a mosaic of ciliated and secretory cells (shown), overlaid by a mucus gel (not shown). Inhaled molecules contact the epithelium and induce quasi-specific innate immune and inflammatory responses. The inhaled stimuli include biological products such as trehalose dimycolate from mycobacteria, chitin and proteases from dust mites or parasites, and lipopolysaccharide (LPS), lipoteichoic acid (LTA) and CpG-rich nucleotides (CpG) from bacteria. These stimuli induce responses from the epithelium such as the production of nitric oxide (NO), the antihelminthic effectors Muc5ac, resistin-like molecule and chitinase, and the antimicrobial effectors defensins and reactive oxygen species (ROS). At the same time as it responds directly, the epithelium recruits innate leucocytes such as macrophages, eosinophils (eo), neutrophils and neutrophils through chemokines such as MCP-1, eotaxin, and IL-8, 25 and 33. The epithelium also recruits adaptive immune cells by elaborating chemoattractants such as thymus and activation regulated chemokine (TARC or CCL-17). Dendritic cells intercalated in the epithelium sense environmental stimuli as well as signals released from the epithelium such as thymic stromal lymphopoietin (TSLP), then travel to local lymphoid tissue to interact with recruited T cells to induce their differentiation into T helper subsets tailored to the perceived pathogen (Th1, Th2, Th17). These T cells release cytokines such as interferons (IFN), IL-13 and IL-22 that further promote epithelial resistance mechanisms. Thus, epithelial cells directly sense and respond to environmental stimuli, while also signalling to leucocytes that in turn reinforce epithelial defences and add defensive functions of their own such as phagocytosis.

mation that evolved for pathogen defence, we inferred that mammalian airway epithelial cells likely possess strong defensive functions. This inference was supported by work with insects during the past three decades showing that epithelial cells are capable of sensing microbial products and responding with the induction of highly effective antimicrobial defences (Lemaître *et al.*, 1997; Medzhitov *et al.*, 1997; Lehrer and Ganz, 1999; Hoffmann, 2003; Ferrandon *et al.*, 2007). Furthermore, the insect epithelial response is partially specific for the class of infecting pathogen (Figure 1C), indicating that an effective inflammatory stimulus to the lung epithelium should be appropriate for the desired response. Specifically, Type 2 allergic inflammation signals a defensive response to parasitic worms (Anthony *et al.*, 2007), whereas Type 1 inflammation signals a defensive response to intracellular pathogens, and Type 17 inflammation to extracellular pathogens (Aujla *et al.*, 2007a,b; Dubin and Kolls, 2008) (Figure 2). In order to strongly stimulate antimicrobial defences, we administered a bacterial lysate by aerosol, reasoning that this would expose lung epithelial cells to microbial products in proportions they are present in a natural

infection (Figure 1D). This resulted in a high level of protection against subsequent respiratory challenge with a broad array of bacterial, fungal and viral pathogens (Clement *et al.*, 2008; 2009; Tuvim *et al.*, 2009; Evans *et al.*, 2010a,b). In general, hosts utilize two contrasting strategies to survive infection: active reduction of pathogen burden (resistance) or host adaptation to pathogen virulence (tolerance). As our experiment showed that treatment-enhanced host survival was accompanied by killing of the infecting pathogens within the lungs (Figure 1C), a resistance mechanism was indicated. The induction of resistance does not depend upon leucocytes (Clement *et al.*, 2008; Evans *et al.*, 2010a), and can be recapitulated *in vitro* by lung epithelial cells stimulated with a bacterial lysate (Clement *et al.*, 2008; Evans *et al.*, 2010a), indicating that epithelial cells are primarily responsible both for sensing the innate stimuli and generating the antimicrobial effector response.

These data and related work by others (see below) indicate that rather than outsourcing all active antimicrobial defences to leucocytes during metazoan evolution, epithelia have retained their capability for active defence while additionally

acquiring the ability to collaborate with leucocytes through mediators such as cytokines. Barrier epithelia can be viewed as akin to military perimeter defences that include both passive mechanical and active killing components to prevent invasion. Advantages of passive biological defences include the exclusion of a high proportion of potential invaders through mechanisms such as a cornified epithelium or a mucus layer with little expenditure of energy or damage to the host. Conversely, active defences can incur a substantial cost to the host such as injury from reactive oxygen or nitrogen radicals, and promotion of carcinogenesis by induction of proliferation or inhibition of apoptosis. Nonetheless, a reduction of microbial burden at the epithelial perimeter reduces the chances of pathogen invasion leading to host death, and concentrating active defences in barrier epithelia where pathogens occur in the highest numbers maximizes benefit while limiting the costs of spreading such defences evenly throughout the host.

Innate immune signalling in the lungs

While the induction of a high level of resistance to microbial infection by an aerosolized bacterial lysate provided proof-of-principle of the potential value of therapeutically manipulating innate immune defences within the lungs, this is not itself a practical therapeutic agent. Therefore, a greater understanding of the mechanisms of inducible resistance is required to develop this technology for clinical translation.

Jawed vertebrates have evolved two distinct pathogen sensing mechanisms that are distinguished by the nature of their receptors. Adaptive immunity utilizes antigen receptors expressed by T and B lymphocytes that are encoded by somatically recombined genes, resulting in an immense library of epitope specificities. Clonal proliferation of cells expressing these precise receptors allows for pathogen-appropriate lymphocyte responses and for immunological memory, but limits the number of pathogens that can be detected by individual cells and requires prior exposure to the pathogen (Medzhitov, 2007). In contrast, innate immune receptors detect conserved molecular features common to multiple microorganisms known as pathogen-associated molecular patterns (PAMPs). Germline-encoded pattern recognition receptors (PRRs) bind PAMPs, allowing recognition of a large number of different microorganisms (Boldrick *et al.*, 2002; Barton and Medzhitov, 2003; Kopp and Medzhitov, 2003; Kato *et al.*, 2006; Kapetanovic and Cavaillon, 2007). Some PRRs also identify host molecules that are expressed in response to infection or host molecules that have been modified in the course of infection. These host molecules are known as 'danger signals' or danger-associated molecular patterns (DAMPs). Recognition of PAMPs and DAMPs by PRRs leads to the expression of effector molecules involved in microbial defence, inflammation and modulation of adaptive immunity (Zhang and Ghosh, 2001).

Toll-like receptors (TLRs) were the first PRRs identified, and remain the best characterized (Kawai and Akira, 2010). TLRs are highly conserved transmembrane proteins comprised of an oligospecific pattern-recognition ectodomain with multiple leucine-rich repeats (LRRs), a membrane-

spanning α -helix, and a Toll/interleukin-1 receptor (TIR) domain for intracellular signalling. (Kato *et al.*, 2006; Gay and Gangloff, 2007; O'Neill, 2008; Beutler, 2009). As shown in Figure 3, PAMP detection by TLRs results in receptor-specific recruitment of cytosolic TIR adaptor protein combinations required for signal transduction. In concert with one or more of the four others, MyD88 is involved in more TLR signalling than any other TIR adaptor (Kopp and Medzhitov, 2003; Yamamoto *et al.*, 2003; Kawai and Akira, 2006; Kapetanovic and Cavaillon, 2007; O'Neill and Bowie, 2007). The MyD88-independent signalling from TLR3 and some TLR4 events utilize the TIR adaptor TRIF, with or without TRAM (Yamamoto *et al.*, 2003; Kawai and Akira, 2006; O'Neill and Bowie, 2007). Ten functional TLRs (TLR1–TLR10) have been described in humans. Mice express nine orthologous TLRs (TLR1–TLR9), a non-functional TLR10 and TLR11–TLR13 that are not found in humans (Gay and Gangloff, 2007; Beutler, 2009; Kawai and Akira, 2010). PCR investigations of primary cells and immortalized cell lines indicate that TLR1–TLR9 are all expressed by human and mouse lung epithelial cells (Muir *et al.*, 2004; Schleimer, 2004; Sha *et al.*, 2004; Gay and Gangloff, 2007; Bartlett *et al.*, 2008).

The primary ligand for TLR4 and its co-receptor CD14 is a complex of the host protein MD2 with bacterial lipopolysaccharide (LPS), facilitating detection of Gram-negative pathogens. TLR2-dependent detection of lipopeptides, such as peptidoglycan, lipoteichoic acid and atypical LPS, promotes recognition of Gram-positive bacteria, parasites and some Gram-negative bacteria. TLR2 functions as a heterodimer with TLR1 or TLR6, with TLR2/1 recognizing triacylated lipopeptides (e.g. Pam3CSK₄), and TLR2/6 recognizing diacylated lipopeptides (e.g. Pam2CSK₄; 'Pam2', hereafter) and fungal zymosan. A highly conserved motif of flagellin found in many bacterial species is recognized by TLR5. Four TLRs recognize microbial nucleic acids. TLR3 recognizes double-stranded RNA, and can be stimulated by synthetic copolymers, such as poly inosine : poly cytosine (poly I : C). TLRs 7 and 8 detect microbial single-stranded RNA containing polyuridine (poly-U) or GU-rich sequences, as well as imidazoquinolones (Diebold *et al.*, 2004; Heil *et al.*, 2004). TLR9 recognizes DNA with unmethylated CpG motifs. A number of host danger signals (DAMPs), such as heat shock proteins, are also ligands for TLRs (Kawai and Akira, 2006; Gay and Gangloff, 2007; O'Neill, 2008; Beutler, 2009).

Insight into the role of TLRs in defence against pneumonia is provided by experiments in TLR-deficient mice. Mice deficient in TLR4 show increased susceptibility to *Haemophilus influenzae*, *Escherichia coli* and Respiratory syncytial virus pneumonia (Kurt-Jones *et al.*, 2000; Wang *et al.*, 2002; Lee *et al.*, 2005). TLR5 deficient mice have increased susceptibility to *Legionella pneumophila* pneumonia (Gribar *et al.*, 2008). Interestingly, mice deficient in both TLR2 and TLR4 do not demonstrate hypersusceptibility to *Pseudomonas aeruginosa* (Ramphal *et al.*, 2005), although mutations of pseudomonal flagellin that prevent TLR5 binding impair bacterial clearance and host survival (Ramphal *et al.*, 2008). TLR3 deficiency may actually confer a survival advantage in influenza pneumonia (Le Goffic *et al.*, 2006), presumably by preventing an excessive host response. However, the finding that intranasal pretreatment with TLR3 agonists protects against influenza pneumonia highlights the requirement for precise regulation

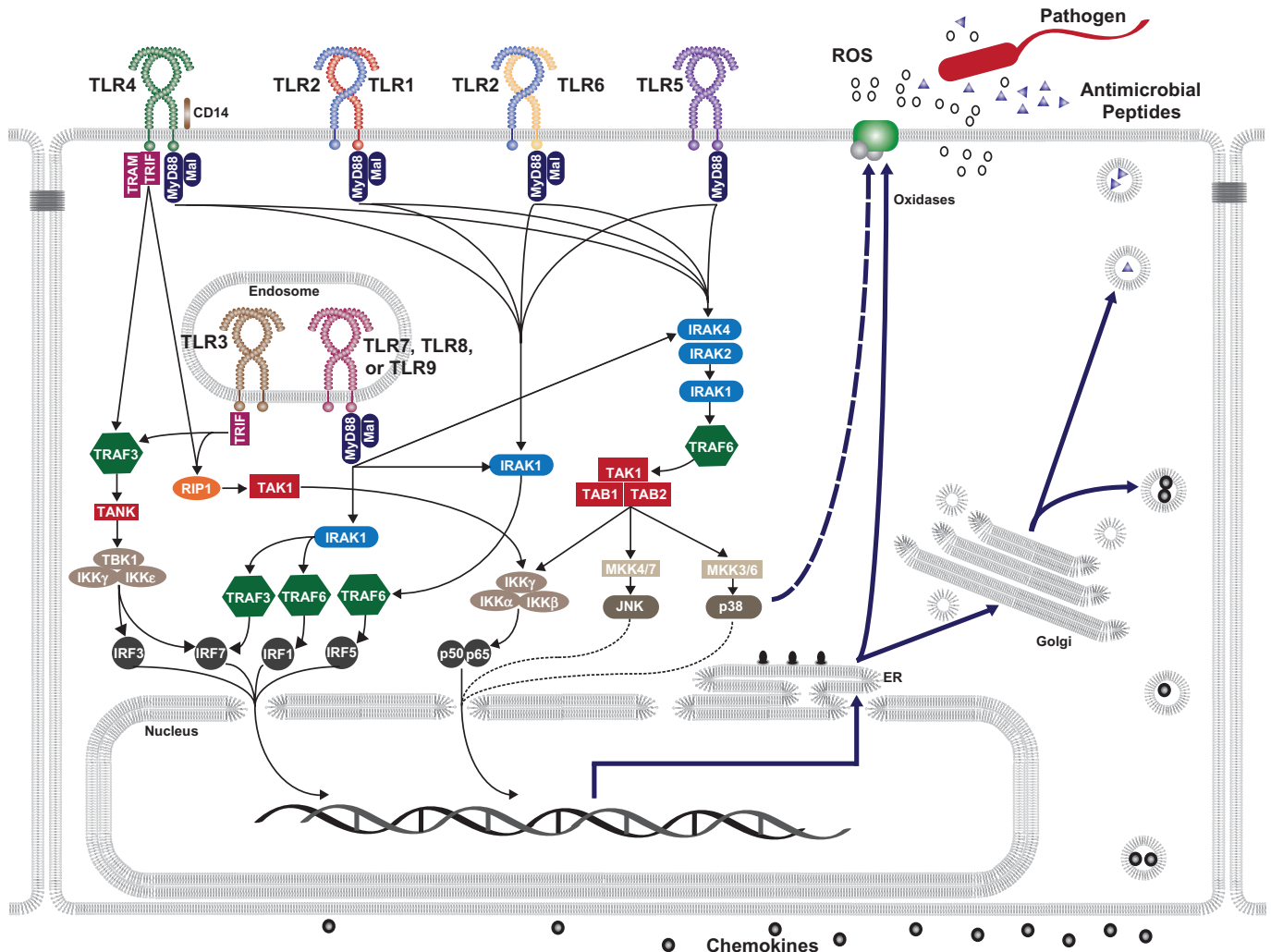


Figure 3

TLR-induced microbial resistance. Detection of microbial PAMPs, endogenous DAMPs or synthetic ligands by lung epithelial TLRs results in receptor-specific recruitment of the TIR adaptors MyD88, Mal, TRIF and/or TRAM. TRIF-dependent signalling from TLR3 and TLR4 activates TRAF3 signalling via TANK and TBK1-IKK complex, as well as RIP1-TAK1-dependent activation of the canonical IKK complex. Together, these TRIF-dependent pathways promote transcription of inflammatory mediators, type I interferons and antimicrobial effectors via IRF3, IRF7 and NF- κ B translocation to the nucleus. MyD88-dependent signalling from TLR 4, 2/1, 2/6, 5, 7, 8 and 9 proceeds by IRAK activation. All of the MyD88-utilizing TLRs can activate IRAK4 signalling (with or without IRAK2), leading to activation of IRAK1-TRAF6. In turn, TRAF6 activates the TAK1-TAB1/2 complex, which promotes IKK and MAPKK signalling. NF- κ B, JNK and p38 signalling then results in transcription of inflammatory and antimicrobial genes. Direct MyD88-dependent IRAK1 activation by TLR 7, 8 and 9 also results in activation of TRAF3-IRF7 and TRAF6-IRF1-dependent transcription of inflammatory and antimicrobial genes. Chemokines are secreted basolaterally to recruit leucocytes to participate in defence of the lungs. Microbicidal effectors are directed apically, such as antimicrobial polypeptides and oxidases that generate ROS. Further, the epithelium utilizes TLR-dependent, non-transcriptionally regulated events to kill pathogens, such as p38-dependent activation of oxidases. (DAMPs, danger-associated molecular patterns; ER, endoplasmic reticulum; IKK, I κ B kinase; IRAK, Interleukin-1 receptor-associated kinase; IRF, interferon regulatory factor; JNK, c-Jun N-terminal kinases pathway; Mal, MyD88-adaptor-like, also known as TIRAP for TIR domain containing adaptor protein; MKK, MAP kinase kinase; MyD88, myeloid differentiation primary response gene 88; p38, p38 mitogen-activated protein (MAP) kinase pathway; PAMPs, pathogen-associated molecular patterns; RIP1, receptor-interacting protein 1; ROS, reactive oxygen species; TAB, TGF-beta activated kinase 1/MAP3K7 binding protein; TANK, TRAF family member-associated NF- κ B activator; TBK1, TANK-binding kinase 1; TIR, Toll/interleukin-1 receptor; TLRs, Toll-like receptors; TRAF, TNF receptor associated factor; TRAM, TRIF-related adaptor molecule, also known as TICAM-2; TRIF, TIR-domain-containing adapter-inducing interferon- β , also known as TICAM-1 for Toll-like receptor adaptor molecule 1.)

of TLR-dependent responses in microbial defence (Wong *et al.*, 2009).

It has long been apparent that microbial products could stimulate inflammatory responses from the respiratory epi-

thelium, and it has been suspected that TLRs contributed to those responses from the time they were first identified in mammals (Diamond *et al.*, 2000). Cultured respiratory epithelial cells respond to stimulation with TLR agonists by

expression of proinflammatory and antimicrobial mediators (Sha *et al.*, 2004; Uehara *et al.*, 2007; Koff *et al.*, 2008). *In vivo*, LPS has been administered intranasally and by aerosol to protect against bacterial and fungal lung infections, either by enhancing innate defences or by attenuating lung injury associated with infection (Jean *et al.*, 1998; Empey *et al.*, 2007; Clement *et al.*, 2008). Intratracheal and intraperitoneal administration of CpG oligodeoxynucleotides (ODNs) (TLR9 ligands) enhances survival of lung infection by a number of pathogens, including *Mycobacterium avium* and *Klebsiella pneumoniae* (Deng *et al.*, 2004; Standiford and Deng, 2004). Treatment of mice with the TLR2/6 agonist MALP-2 induces cytokine production, reduces the pathogen burden, and enhances host survival after challenge with *Streptococcus pneumoniae* (Reppe *et al.*, 2009). Mice pretreated with poly I : C or liposomal preparations of CpG ODNs display enhanced survival after challenge with influenza (Wong *et al.*, 2009). Mice pretreated with flagellin were protected from *P. aeruginosa* pneumonia, even in the absence of neutrophils (Yu *et al.*, 2010).

In addition to TLRs, the lung epithelium expresses many non-TLR PRRs (Sha *et al.*, 2004; Bartlett *et al.*, 2008; Evans *et al.*, 2010b). The nucleotide oligomerization domain (NOD)-like receptors (NLRs) family is defined by proteins with a PAMP-interacting C-terminal LRR domain, a central nucleotide NOD, and one of three N-terminal signalling domains (Shaw *et al.*, 2008). Humans express at least 23 of these proteins. While most are restricted to leucocytes, the best studied NLRs, NOD1 and NOD2, are both present in lung epithelial cells (Uehara *et al.*, 2007; Shaw *et al.*, 2008). Unlike TLRs, NLRs are cytosolic in distribution. NOD1 recognizes γ -D-glutamyl-meso-diaminopimelic acid present in the peptidoglycan of Gram-negative and some Gram-positive bacteria (Shaw *et al.*, 2008), whereas NOD2 binds the muramyl dipeptide present in bacterial peptidoglycan (Chamaillard *et al.*, 2003). Activation of signalling via NOD1 or NOD2 results in MAPK and NF- κ B-dependent production of proinflammatory mediators. NLRs are critical to the host response to *S. pneumoniae*, *P. aeruginosa*, *Moraxella catarrhalis*, *Chlamydomydia pneumoniae* and *Mycobacterium tuberculosis* in the lungs (Opitz *et al.*, 2004; Bartlett *et al.*, 2008; Divangahi *et al.*, 2008; Shimada *et al.*, 2009).

Additional NLRs, including NALP1, NALP2, NALP3, Ipaf and NAIP, induce activation of the inflammasome, thereby activating caspases to convert pro-interleukin (IL)-1 β , pro-IL-18 and pro-IL-33 into their mature forms (Martinon *et al.*, 2009). These proteins primarily recognize danger signals, including host inflammatory mediators and crystals, but also detect products of microbial pathogens (Miao *et al.*, 2007; Martinon *et al.*, 2009). The observation that IL-1 β mRNA increases in the lungs of mice by almost 100-fold after treatment with an aerosolized bacterial lysate to induce resistance suggests a role for inflammasome activation (Evans *et al.*, 2009). Furthermore, NLRs demonstrate synergistic signalling with TLRs (Bourhis and Werts, 2007).

Retinoic acid induced gene (RIG)-I-like receptors (RLRs) are cytosolic PRRs involved in TLR-independent sensing of viruses and the associated production of type I interferons (Kawai and Akira, 2008). The RLR family has two known members – RIG-I and melanoma differentiation associated gene 5 (MDA5). RIG-I detects noncapped 5'-triphosphate

RNA (Hornung *et al.*, 2006), allowing recognition of ssRNA viruses (Kawai and Akira, 2008). RIG-I-deficient mice demonstrate impaired antiviral responses (Kato *et al.*, 2006). The primary ligand for MDA5 is dsRNA (Hornung *et al.*, 2006), although it also likely detects poly I : C in a TLR-independent manner, and its deficiency increases susceptibility to several picornaviruses (Kato *et al.*, 2006).

Besides these innate receptor families, additional cellular macromolecules participate in microbial recognition. For example, lactosylceramide, a glycosphingolipid found on the apical surface of lung epithelial cells, detects fungal β -glucans (Hahn *et al.*, 2003; Evans *et al.*, 2005). Other PRRs, such as class A scavenger receptors (e.g. MARCO and SR-AI/II) appear to participate in lung defence, but their expression and function in lung epithelium are unresolved. Adaptive immune signalling through cytokines such as IL-17, IL-22, and interferons also induces epithelial resistance (Kolls *et al.*, 2008; Ank and Paludan, 2009; Billiau and Matthys, 2009).

Synergistic combination of TLR ligands in lung defence

To identify critical pathways in inducible resistance that might be exploited pharmacologically, we tested mice lacking TIR adaptors that mediate signalling between TLRs and downstream effectors. The absence of MyD88, but not of TRIF, resulted in a complete loss of the induction of resistance by an aerosolized bacterial lysate, pointing to a dominant role of a subset of TLRs in the response to this stimulus (Duggan *et al.*, 2011). We then systematically screened available TLR ligands for the induction of resistance (Duggan *et al.*, 2011). To determine an efficacious dose, the elicitation of neutrophil influx into bronchoalveolar lavage fluid was used as evidence of epithelial stimulation because even though neutrophils are not required for inducible resistance, inflammation characterized by elaboration of neutrophil chemotactins accompanies the induction of antibacterial responses (Clement *et al.*, 2008; 2009). No TLR ligand alone elicited a resistance response equal in magnitude to that elicited by an aerosolized bacterial lysate (Duggan *et al.*, 2011). However, prior literature suggested that innate immune ligands can interact synergistically to activate effector responses (Powell *et al.*, 2004; Merlo *et al.*, 2007; Trinchieri and Sher, 2007), so the TLR ligands were also screened in combination. The combination of Pam2 and a class C oligodeoxynucleotide (ODN) induced a high level of resistance to bacterial (Duggan *et al.*, 2011) and viral (You *et al.*, 2010) pathogens (Figure 4). The magnitude, time course and breadth of protection induced by the combination of Pam2 and ODN (Pam2/ODN) were comparable to that of a bacterial lysate, suggesting that this combination might serve as a clinically useful drug.

The initial dose-formulation of Pam2/ODN was identified based upon the recruitment of neutrophils by each component alone. To optimize the dose-formulation to achieve maximal host protection with minimal drug exposure, the concentrations of each component were systematically varied to determine the optimal dose-ratio and dose-strength using a Bayesian approach (Figure 5A,B). This demonstrated an optimal molar dose-ratio of Pam2 : ODN between 3:1 and

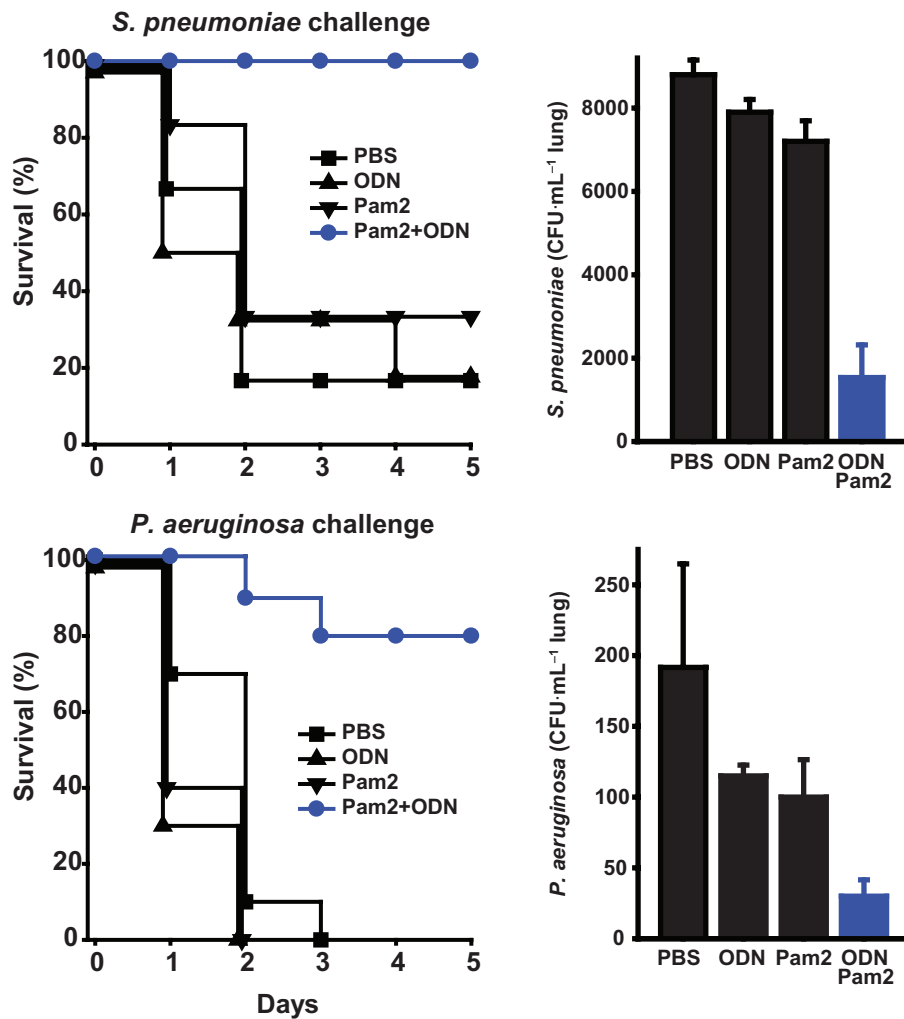


Figure 4

Synergistic combination of Toll-like receptor ligands. Either Pam2 (20 mg·mL⁻¹) or oligodeoxynucleotide (ODN) (10 mg·mL⁻¹) alone, delivered to the lungs by aerosol (8 mL), induces little protection of mice (left) or pathogen killing within the lungs (right) when the mice are challenged 24 h later with *S. pneumoniae* (top) or *P. aeruginosa* (bottom). However, combined treatment with Pam2 and ODN (blue) induces greater-than-additive host protection and pathogen killing. PBS, phosphate buffered saline.

4:1, and a plateau of effect of dose-strength ~3 μ M Pam2 and ~0.75 μ M ODN (Figure 5C). A dose-formulation of 6 mL of a nebulized solution of 4 μ M Pam2 and 1 μ M ODN is being used in further toxicity and efficacy studies.

Properties of inducible epithelial resistance

Magnitude

The maximal extent of epithelial defences has probably been uncovered by the dose-response relationship to aerosolized bacterial lysates. It remains a formal possibility that greater resistance could be induced with the addition of an innate immune ligand not present in the lysates tested, or that substances which suppress resistance are present in the lysates so that fractionation of a lysate or substitution with

purified innate immune agonists would result in greater resistance. However, no greater response has been reported to any combination of innate immune agonists, so the plateau of benefit observed with increasing doses of bacterial lysates or the synergistic combination of Pam2/ODN more likely reflects maximal activation of epithelial effector mechanisms.

Time-course

The induction of increased resistance to infection is clearly apparent 2 h after stimulation (Clement *et al.*, 2008; Duggan *et al.*, 2011; Yu *et al.*, 2010). It is likely that resistance begins to increase by post-translational mechanisms within minutes after stimulation, although such early time points have not yet been carefully analysed. Resistance reaches a maximum 4–24 h after stimulation and is accompanied by extensive changes in gene expression; however, the causal roles of changes in expression of specific genes have not yet been

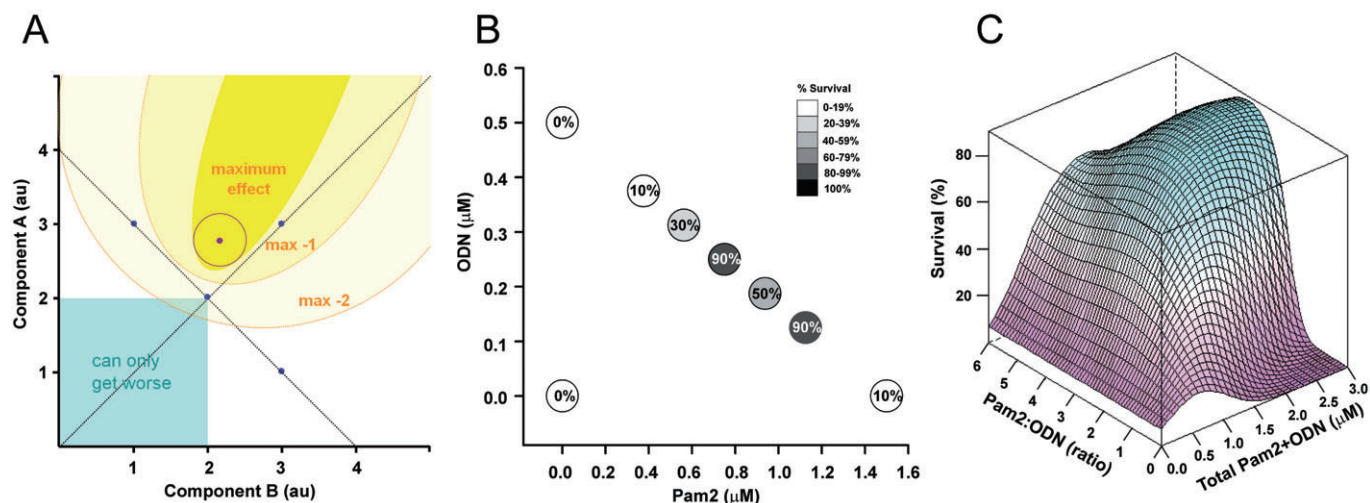


Figure 5

Optimization of the dose-formulation of synergistically interacting TLR ligands. (A) Scheme for optimizing the dose-formulation of synergistically interacting TLR ligands. The previously determined optimum is placed at the intersection (central blue dot) of perpendicular lines that plot an increase in dose-strength while maintaining constant dose-ratio versus maximal deviation in dose-ratio while maintaining constant dose-strength. Additional blue dots indicate concentrations of the two compounds that bracket the current optimum in all directions except a balanced decrease. This process is repeated iteratively until a neighbourhood of equally efficacious doses is determined, defined as no measureable difference by raising or lowering any component by 25%. The therapeutic formulation is selected as the centre of a circle with a diameter of 25% of the efficacious concentration of either compound (violet), resting in the apex of the parabola defining the neighbourhood of maximally efficacious formulations (dark yellow), with the parabola open in the direction of higher concentrations due to a plateau of efficacy. Neighbourhoods of less efficacious formulations can be similarly defined [maximum (max) –1 and –2]. (B) Representative experiment varying the dose-ratio of Pam2/oligodeoxynucleotide (ODN). Mice in groups of ten were exposed to aerosolized Pam2/ODN in the concentrations indicated, then challenged 24 h later with a dose of *P. aeruginosa* that resulted in survival of no untreated mice. The percentages of mice in each group surviving the infectious challenge are indicated. (C) Estimated dependence of the survival probability on the ratio and total dose of the components. A total of 15 experiments as in (B) that met the criterion of survival of $\leq 20\%$ of the mice in the untreated group were analysed using a generalized mixed model implemented in R function 'glmer' (R version 2.11.1, <http://www.r-project.org/>). The logit of the probability of survival as a function of the ratio and total dose of Pam2/ODN was described by a power-4 polynomial function of these two variables, retaining only significant terms.

determined. A small decline in the magnitude of resistance is detectable 48 h after stimulation if a virulent microbial challenge is used (Duggan *et al.*, 2011). The detection of such small differences in host survival with strong but not weak challenges is akin to uncovering small differences in agonist efficacy or in tissue tachyphylaxis through the use of functional antagonism when biochemical or physiological endpoints are used in traditional pharmacological studies (Johnson, 1995; Booth *et al.*, 1996; Dryden *et al.*, 2010). Induced resistance continues to slowly decline with time after stimulation, such that it is not consistently detectable after 7 days.

Tachyphylaxis

There has been no detectable decrease in the magnitude of inducible resistance with repetitive dosing every 3 days up to seven doses (Tuvim *et al.*, 2009; Evans *et al.*, 2010a). This makes teleological sense in that an epithelial barrier should not lessen its defences as long as signals indicate that a microbial threat persists, but is surprising in that there is tachyphylaxis of the inflammation that accompanies the induction of resistance (Clement *et al.*, 2008; Moghaddam *et al.*, 2008). However, these results are consistent with work in macrophages that found tachyphylaxis of inflammatory but not of antimicrobial gene expression changes with repeti-

tive exposure to innate immune ligands (Medzhitov, 2007). Together, these findings are exciting because they point to the prospect that resistance and inflammation can be resolved at least partially, raising the possibility of therapeutic benefit with minimal side effects.

Inflammation

Stimulation of lung epithelium with innate immune ligands that induce antimicrobial resistance is accompanied by a vigorous inflammatory response characterized by the elaboration of numerous chemokines and cytokines and the infiltration of lung tissue with neutrophils and other leucocytes. However, no leucocyte or extracellular signalling molecule has been shown to be necessary for the induction of resistance. Furthermore, dissociations between inflammatory signalling and the induction of resistance are indicated by the fall in cytokine levels back towards baseline before resistance has even reached its maximum (Clement *et al.*, 2008; Moghaddam *et al.*, 2008; Tuvim *et al.*, 2009) and the tachyphylaxis that occurs to inflammation but not to resistance (above). Thus, while resistance and inflammation are often associated, and the recruitment of mediators and leucocytes from the systemic circulation clearly helps in the clearance of infection, resistance is not synonymous with inflammation.

Side effects

The induction of resistance within the lungs has been accompanied by inflammation, airway fibrosis, and the promotion of epithelial carcinogenesis (Moghaddam *et al.*, 2008; 2009). However, effects that cannot be dissociated from the resistance phenomenon must be distinguished from those that are specific to a particular ligand or delivery system. For example, it may be possible to identify ligands that maximize the induction of resistance while minimizing the induction of inflammation, or to administer a drug that suppresses inflammation together with one that induces resistance. Importantly, inflammation is mostly confined to the lung lumen with little systemic inflammation (Clement *et al.*, 2008; Tuvim *et al.*, 2009; Evans *et al.*, 2010a). Therefore, inflammation may not be a serious side effect of aerosolized innate immune ligands as it can be with systemic administration of immunomodulatory drugs (Suntharalingam *et al.*, 2006), although this will require careful clinical study. Similarly, the airway fibrosis that results from prolonged repetitive exposure to a bacterial lysate (25 weeks or more) probably requires an adaptive immune response to the protein components of the lysate that could be avoided with the use of synthetic innate immune ligands (Moghaddam *et al.*, 2008), but this will also require further investigation. Much remains to be learned about inhaled pharmaceuticals in general and of inhaled immunomodulatory compounds in particular (Patton *et al.*, 2010).

In contrast to side effects that might be dissociated from resistance, the promotion of lung carcinogenesis is likely an intrinsic property of the induction of resistance because of the activation of anti-apoptotic and epithelial repair pathways in parallel to antimicrobial pathways (Houghton *et al.*, 2008; Moghaddam *et al.*, 2008). Therefore, this side effect may limit the duration of treatment to induce microbial resistance, particularly among subjects prone to harbour oncogenic epithelial mutations such as chronic cigarette smokers. It is not yet known whether stimulation of alveolar epithelial cells is required together with stimulation of airway epithelial cells to effectively induce resistance within the lungs, but targeted delivery of innate immune ligands to the conducting airways alone through the control of aerosol droplet size could minimize alveolar inflammation that might compromise gas exchange. Hyperresponsiveness to bronchoconstrictor stimuli is a feature of allergic inflammation so is unlikely to accompany the induction of antimicrobial resistance, but this possibility has not been assessed to our knowledge.

Prospects for the future

Mechanism of action

While innate immune ligands and receptors that effectively induce resistance within the lungs have been identified, and adaptor proteins and transcription factors involved in signalling pathways downstream from those receptors have been implicated (Bals and Hiemstra, 2004; Bartlett *et al.*, 2008; Beutler, 2009), necessary and sufficient antimicrobial effector responses are not yet known with certainty. As detailed above, the effectors are likely to be combinations of

oxidants and antimicrobial peptides that act synergistically to induce microbial killing. Additional efficacious ligands and receptors will probably be identified that may involve other pathways and effectors, and the roles of subsets of lung epithelial cells need to be delineated. These will continue to be active areas of basic investigation that could lead to improved pharmacological properties of therapies introduced into the clinic.

Potential therapeutic uses

It is possible to envision several scenarios in which the delivery of innate immune ligands to the lungs to induce resistance to microbial infection could be useful. Cancer patients undergoing myeloablative chemotherapy are highly susceptible to bacterial and fungal pneumonia during the 2 to 3 week period of profound neutropenia (Lyman and Delgado, 2003; Lyman *et al.*, 2003). Lung epithelial cells turn over very slowly, in contrast to gut epithelial cells or neutrophils, so are less susceptible to the acute effects of most cytotoxic chemotherapy (Bowden, 1983; Rawlins *et al.*, 2007; Rawlins and Hogan, 2008; Rock *et al.*, 2009; 2010). Stimulation of the lung epithelium with aerosolized innate immune ligands every few days during this period of vulnerability could reduce the incidence of pneumonia and might allow more intensive chemotherapy to increase the chance of a cure of the underlying malignancy. Organ transplantation patients and other subjects undergoing immunosuppression might similarly benefit from the transient induction of innate antimicrobial resistance within the lungs. Intubated patients being mechanically ventilated in intensive care units are at high risk of nosocomial pneumonia, and the rate of ventilator-associated pneumonia might be reduced by treatment with aerosolized innate immune ligands. The principal cause of asthma exacerbations is respiratory viral infection (Rosenthal *et al.*, 2010). The degree to which this reflects impaired viral clearance or an excessive and Type 2-deviated response to the virus is not yet known. Nonetheless, limitation of viral spread within the lower respiratory tract by treatment with aerosolized innate immune ligands could be beneficial, and there is the additional possibility of therapeutic deviation of the immune response away from an excessive Type 2 response. Normal people could benefit if they are exposed to pathogens of high virulence in the setting of a bioterror attack or an emergent infection such as with avian influenza or the severe acute respiratory syndrome virus before vaccines become available.

Summary

The airway epithelium is capable both of directly sensing microbial products and of indirectly sensing pathogens through cytokines and other mediators released by other host cells. These stimuli induce powerful epithelial antimicrobial responses that result in a high level of resistance to microbial infection. Pharmacological manipulation of these pathways through the inhalational delivery of agonists may be useful therapeutically to protect vulnerable populations against pneumonia.

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Conflicts of interest

Evans, Tuvim and Dickey have ownership interests in Pulmo-protect, Inc., which is commercializing the use of aerosolized innate immune ligands for the induction of resistance to pneumonia, and have received grant support from the US National Institutes of Health for studies on inducible resistance.

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