Pneumolysin: A Double-Edged Sword During the Host-Pathogen Interaction

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Abstract: The cholesterol-dependent cytolysins are pore-forming toxins. Pneumolysin is the cytolysin produced by *Streptococcus pneumoniae* and is a key virulence factor. The protein contains 471 amino acids and four structural domains. Binding to cholesterol is followed by oligomerization and membrane pore formation. Pneumolysin also activates the classical pathway of complement. Mutational analysis of the toxin and knowledge of sequence variation in outbreak strains suggests that additional activities of biologic importance exist. Pneumolysin activates a large number of genes, some by epigenetic modification, in eukaryotic cells and multiple signal transduction pathways. Cytolytic effects contribute to lung injury and neuronal damage while proinflammatory effects compound tissue damage. Nevertheless pneumolysin is a focal point of the immune response to pneumococci. Toll-like receptor 4-mediated recognition, osmosensing and T-cell responses to pneumolysin have been identified. In some animal models mutants that lack pneumolysin are associated with impaired bacterial clearance. Pneumolysin, which itself may induce apoptosis in neurones and other cells can activate host-mediated apoptosis in macrophages enhancing clearance. Disease pathogenesis, which has traditionally focused on the harmful effects of the toxin, increasingly recognises that a precarious balance between limited host responses to pneumolysin and either excessive immune responses or toxin-mediated subversion of host immunity exists.

Keywords: *Streptococcus pneumoniae*, pneumolysin, pore formation, disease pathogenesis, immune response.

INTRODUCTION

Cholesterol-dependent cytolysins are a family of pore -forming toxins expressed by a wide range of Gram-positive organisms (reviewed in [1]). Over twenty species of Gram-positive bacteria produce these toxins, which produce large pores in cholesterol-containing membranes. Representative examples include pneumolysin expressed by *Streptococcus pneumoniae*, streptolysin O expressed by *Streptococcus pyogenes*, intermedilysin by *Streptococcus intermedius*, listeriolysin O by *Listeria monocytogenes* and perfringolysin O by *Clostridium perfringens*. *S. pneumoniae,* also known as the pneumococcus, is a leading cause of community-acquired pneumonia, meningitis, otitis media, sinusitis and a common cause of infection-related mortality at the extremes of life [2, 3]. Pneumolysin represents an important virulence factor for this organism [4]. The principal activities of pneumolysin have been viewed as the capacity to induce pores in cholesterol rich membranes, which equates with hemolytic activity, and the ability to activate complement, both of which play a role in virulence in an animal model of pneumonia [4]. However, additional biological activities are suggested by the observation that mutants lacking both of these functions have greater virulence than mutants in which the gene is deleted [5].

STRUCTURE FUNCTION CORRELATES OF PORE FORMATION

Pneumolysin is a 53 kDa protein composed of 471 amino acids [6-8]. The protein is an asymmetric four domain protein which is slightly curved not globular [9]. In solution it commonly exists as a monomer but self assembly into oligomers can occur and is dependent on the single cysteine residue C428 [10]. The unadecapeptide cysteine-containing region (CCR; residues 427-37 of pneumolysin) is highly conserved amongst cholesterol-dependent cytolysins [11]. C428 is required for optimal hemolytic activity and is important to the overall structure [12], though presence of a cysteine residue at this position per se is not essential for function since certain mutations such as C428A do not effect hemolytic activity [13]. C428 mutations that alter function probably do so by changing structure at a key conformational residue not by removing a thiol group. The thiol group is not essential to cholesterol binding or plasma membrane insertion of pneumolysin. Hence the description of these toxins as thiol-activated toxins may be a misnomer, at least for purified toxin *in vitro*. Attachment to the cell membrane is cholesterol-dependent and cholesterol binding is rapid, pH-dependent but temperature-independent [14]. The C-terminal domain (domain 4) is critical to binding to cholesterol-containing membranes [15, 16] and self-interaction [10, 17]. There are two models to describe pore formation by the CDCs. In one monomeric toxin inserts into the membrane bilayer and then assembles to form a pore. Once the toxin is membrane bound ring and arc shaped oligomers form [9]. In the second, most favoured model, toxin binds to the cell membrane and

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assembles to form a 'pre-pore' which then inserts into the lipid bilayer [21], see Fig. (**1**). The N-terminal domain (domain 1) contains a negative charge [18]. On insertion this negative charge contributes to the orientation of the oligomers in the membrane. H367 is important for oligomerization [19]. Oligomerization is associated with domain 4 being pushed upwards and domain 3 moving towards the inner surface of the developing ring, allowing an α-helix close to domain 2 to insert into the membrane [20]. Further conformational changes allow pore formation during which domain 3 is packed against domain 2 and parts of domains 3 and 4 interact with the plasma membrane. Cryoelectron microscopy studies have recently refined the model providing evidence that pore formation is associated with buckling of domain 2 and unfolding and insertion of helical regions of domain 3 into the membrane [21]. Tryptophan residues in domain 4 of the protein, contained within the conserved CCR at positions W433, W435 and W436 are important for the formation of large pores. This process is inhibited by bivalent cations [22]. W433 is the most important of these tryptophan residues in determining channel formation. Once formed pores contain up to 44 sub-units Fig. (**2**) and contain a central channel of 230-260Å in diameter flanked by 176 β strands [21]. Pore formation leads to the removal of phospholipids from phospholipid bilayers into structures which contain oligomers of pneumolysin complexed to lipid but lack the normal lipid bilayer structure of eukaryotic membranes, [23]. This results in destabilization of the eukaryotic lipid bilayer and formation of small vesicles in a liposome model.

COMPLEMENT ACTIVATION

Another major activity of pneumolysin is activation of complement *via* the classical pathway [24]. Pneumolysin shows sequence homology with the acute phase protein C-reactive protein (CRP) and can bind the Fc portion of immunoglobulin and also C1q, thus activating the classical pathway of complement [24, 25]. Domain 4 has a similar fold structure to the Fc portion of immunoglobulin and the ability of pneumolysin to self-interact or bind the Fc portion of immunoglobulin may be related functions [10]. Complement activation can however also occur in the absence of antibody, is mediated by sites distinct from those required for pore formation and is not cholesterol-dependent [24]. D385, located in one of the regions showing homology to CRP, is critical to immunoglobulin binding and complement activation [25]. *In vivo* instillation of pneumolysin into the lung induces features of pneumonia but this is less marked when D385 is mutated [26]. Pneumolysin decreases opsonization of pneumococci with C3 *in vitro* and in bacteria isolated from mice in models of pneumococcal infection [27]. The importance of inhibiting complement activation to the pneumococcus is demonstrated by the observation that complement activation makes an independent contribution, in addition to that provided by hemolytic activity, to pulmonary survival [28]. In the lung it influences bacterial persistence but also facilitates replication in the blood. The ability of pneumolysin to inhibit the classical pathway of complement activation may be particularly noteworthy in circumstances where the availability of complement to opsonize bacteria is limited as occurs in the lung, or in the blood or lung of rats who have decreased levels of complement

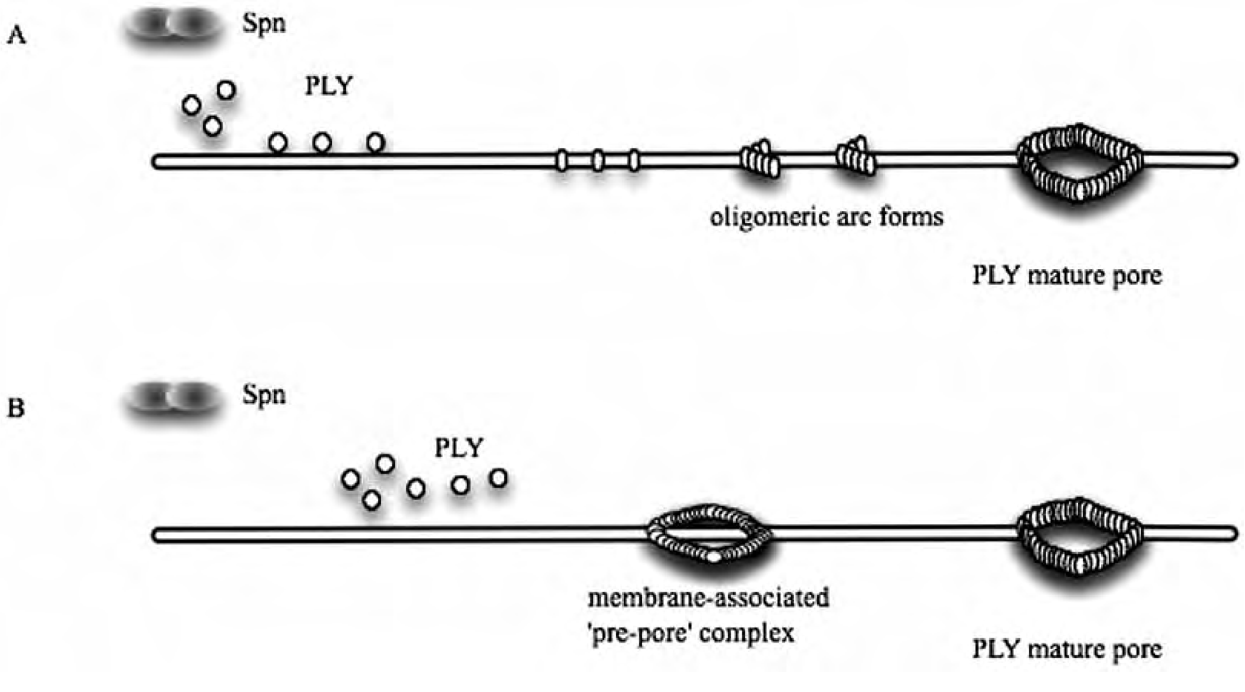

Fig. (1). The two potential models proposed for the development of the pneumolysin pore. In the first model (**A**) it is suggested that single units come together in the host cell membrane to form oligomeric complexes and ultimately mature pores. In the second model (**B**) it is proposed that the sub-units undergo pre-assembly after binding to the host cell membrane and form a pre-pore complex prior to insertion into the membrane.

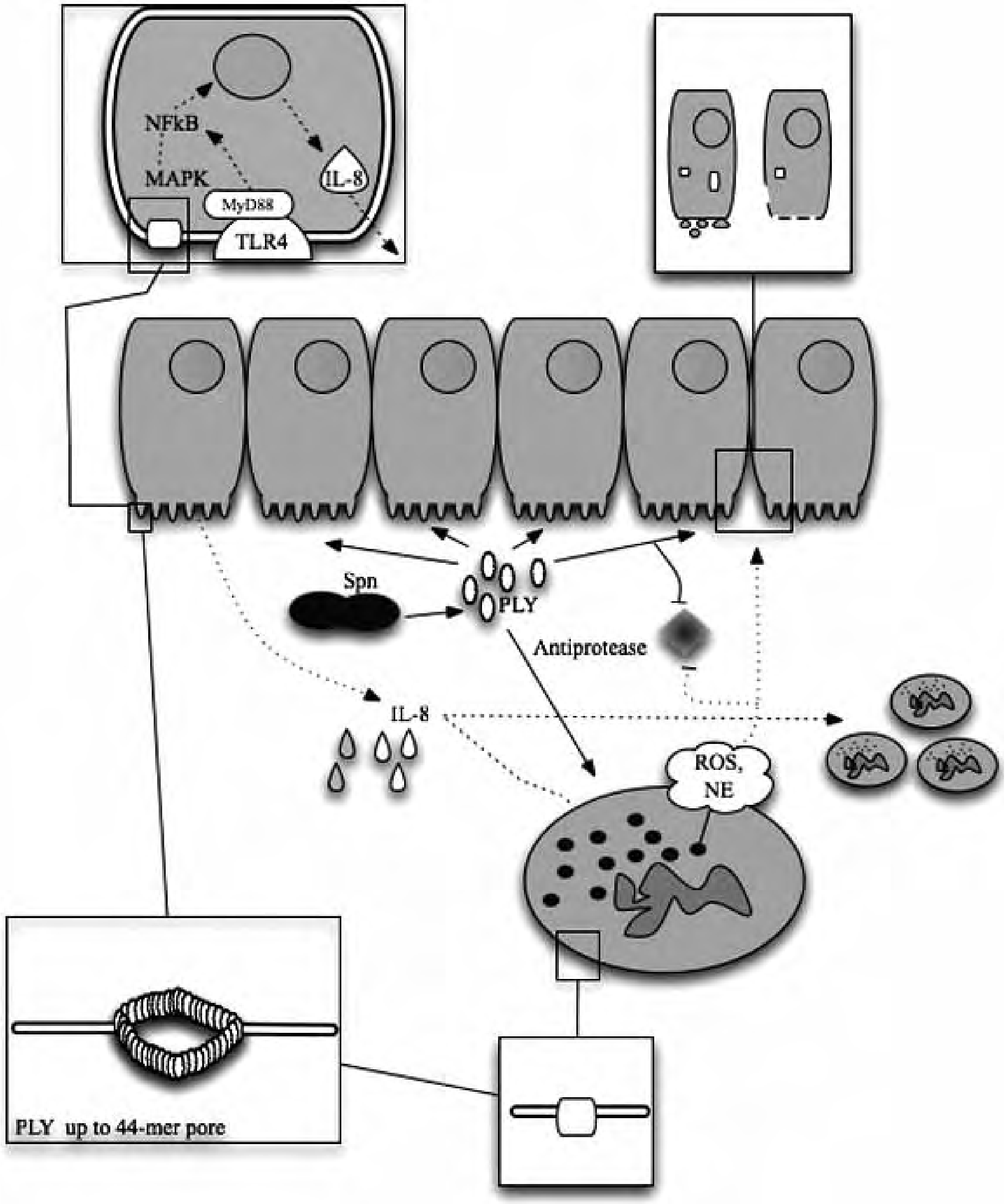

Fig. (2). Pneumolysin interaction with cells in the respiratory system. Pneumolysin (PLY) is released from the *Streptococcus pneumoniae* (Spn) and interacts with epithelial cells where it can form pores that range in size up to 44-mer pores in the cell membrane. This results in influx of large molecular weight osmoles into the cell and signal transduction. In epithelial cells pores may induce 'osmosensing', which is not dependent on intracellular Ca^{++}, but results in downstream activation of p38 mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NFκB). Alternatively interaction with Toll-like receptor 4 (TLR4) and its adaptor protein myeloid differentiation factor 88 (MyD88) may activate NFκB. This activates cytokine and chemokine production, including factors such as IL-8 that contribute to the recruitment of neutrophils. Neutrophil recruitment will enhance bacterial killing but factors released by neutrophils such as reactive oxygen species (ROS) and proteases including neutrophil elastase (NE) may contribute to tissue injury. Pneumolysin accentuates the release of these factors from neutrophils and may enhance release of IL-8 further increasing lung inflammation. The combined effect of pneumolysin and ROS leads to downregulation of antiproteases with the result that the protease-antiprotease balance is disturbed and activated proteases can combine with the cytolytic functions of pneumolysin to accentuate tissue injury and inflammation. Epithelial cells demonstrate surface blebbing, membrane permeabilisation, loss of tight junctions and mitochondria demonstrate dysmorphic features with swelling and loss of normal membrane structure.

in a model of liver cirrhosis [29, 30]. In other models, such as a peritoneal infection model, no effect on virulence was noted by loss of complement-activating function [31]. Although animal models confirm that hemolytic activity, and in many models complement-activating activity, contribute to virulence some studies have demonstrated that mutants that lack both activities still have greater virulence than pneumolysin deficient mutants suggesting other functions make additional contributions to virulence [5].

GENETIC VARIATION

The cholesterol-dependent cytolysins show significant variation. The pneumolysin gene shows 20% genetic sequence homology, 42% amino acid sequence homology and 60% structural homology, when amino acids of similar structure are regarded as equivalent, with that of streptolysin O [32]. However many key functional features are conserved, such as the unadecapeptide sequence that includes C428. Variation in the amino acid sequence of pneumolysin leads to some important differences in comparison to other cholesterol-dependent cytolysins, such as the lack of an N-terminal secretion signal sequence, reviewed in [33]. In contrast, there is much more limited genetic variation in the pneumolysin gene between strains of *S. pneumoniae*. Only six of 1650 base pairs differ between a type 1 and the D39 type 2 strain [34]. Many strains of *S. pneumoniae* produce similar levels of pneumolysin in late log-phase growth despite differences in growth characteristics *in vivo* [35]. Overall there is only 3.3% sequence variation in pneumolysin but at least 15 distinct alleles have been identified to date [36]. Serotype 7F and 8 strains producing pneumolysin with altered SDS-PAGE mobility and reduced hemolytic activity have a number of substitutions/deletions in amino acid sequence but the key alteration is a T172I mutation which alters function [37]. More recently serotype 1 clinical strains of the sequence type (ST) 306 have been shown to produce a nonhemolytic pneumolysin [38]. This strain contains a number of mutations similar to the serotype 7F and 8 strains described by Lock, including the T172I mutation. This may modify insertion of domain 3 into the plasma membrane since an aromatic acid at approximately this position has been demonstrated to be key to membrane insertion for perfringolysin O [39]. However, compared to the serotype 7F and 8 strains, ST306 has no residual hemolytic activity. This may be the result of an additional mutation Y150H, which further compromises the function of the mutant toxin, as evidenced by the reduced hemolytic effect of a mutant containing Y150A [38]. Another mutation of serotype 1 pneumolysin is addition of the insertion sequence 1515, which abolishes pneumolysin expression [40]. Since the ST306 variant pneumolysin allele and the related allele in ST53 lineage serotype 8 are found in dominant strains causing outbreaks of disease this further emphasises that functions independent of hemolytic activity contribute to virulence. There may be specific consequences to having these nonhemolytic pneumolysin alleles which contribute to the pathogenesis of out-breaks [36].

RELEASE OF PNEUMOLYSIN

Unlike other cholesterol-dependent cytolysins, pneumolysin lacks an N-terminal secretion signal sequence. In addition, its negative charge favours sequestration of the toxin in the cytoplasm rather than surface expression on the bacteria. The traditional paradigm was that pneumolysin was released by bacteria in stationary phase growth by the action of autolysin. Autolysin activation induces bacterial cell wall breakdown during cell division or following treatment with various antimicrobials or detergents, reviewed in Jedrzejas [33]. A number of antimicrobials are implicated in pneumolysin release, in particular β-lactam antibiotics, glycopeptides, fluoroquinolones and aminoglycosides, although other antimicrobials notably macrolides at sub-MIC levels, clindamycin and rifampicin induce less pneumolysin synthesis and/or release [41-43]. The view that autolysin was the key to pneumolysin release was challenged by a number of observations. Pneumolysin is released during earlier stages of log-phase growth in some strains, before autolysin-mediated lysis which was thought to be active at late-log or stationary phase growth [35]. Mutants that lack autolysin still release pneumolysin and, unlike the attenuated mutants that result from pneumolysin deletion, the phenotype of autolysin deficient mutants more closely resembles wild-type bacteria in murine models [44]. Resident bacteriophages may harbour autolysin like genes [45]. Pneumolysin release can occur in the absence of these bacteriophages in addition to the absence of autolysin [44]. ClpC a heat shock protein and molecular chaperone can also contribute to autolysis [41] but the effect of this is strain dependent [46]. The exact factors influencing pneumolysin release in the absence of autolysin, however, remain unclear. More recently it has been proposed that pneumolysin could be released by noncompetent cells during the process of genetic transformation [47]. In this model competent cells release a bacteriocin CibAB from which they, but not noncompetent cells, are protected by production of the specific immunity factor CibC. CibAB acts as the trigger for lysis but does not induce lysis itself. The combined action of autolysin, lysozyme and the amidase choline-binding protein D result in break-down of the cell wall, allowing not only the release of single-stranded DNA for uptake by the competent cell, but also pneumolysin. This not only allows the competent cell to take-up DNA but also allows release of pneumolysin. Finally the effect of bacterial killing by host cells has been comparatively little studied as a contributor to pneumolysin release in *in vivo* models.

PNEUMOLYSIN EFFECTS ON EUKARYOTIC CELLS

Pneumolysin has multiple over-lapping actions on eukaryotic cells, see Table **1**. *In vivo* pneumolysin expression is unregulated [48]. Pneumolysin activates a large number of genes in eukaryotic cells [49]. In the THP-1 monocyte cell line cDNA microarray identified 142 genes upregulated in the presence of pneumolysin that were not upregulated by a pneumolysin deficient mutant in the first three hours after infection. In particular genes for chemokines/cytokines were upregulated including those contributing to the recruitment of inflammatory cells such as IL-8, macrophage inflammatory protein (MIP)-1β and monocyte chemotactic protein (MCP) - 3. Components of the receptors for IL-2, IL-15 and interferons, as well as molecules such as lysozyme, mannose binding lectin (MBL), caspases 4 and 6

Table 1. Major Functions and Consequences of Pneumolysin's Interactions with Eukaryotic Cells

Function	Mechanism of Alteration	Consequence	Ref
Membrane integrity	Pore formation, lytic concentration	Cell cytotoxicity, mitochondrial swelling, membrane blebbing. Cell-type and concentration dependent	[60]
	Pore formation, sub-lytic concentration	Osmosensing, activation of p38 mitogen-activated protein kinase and innate responses in epithelial cells	[54]
Cell signalling	Direct and indirect activation of GTPases, protein kinases and phospholipases	Roles in host responses, inflammatory responses and tissue injury.	[52-54]
Gene transcription	Multiple mechanisms, activation/repression of transcription factors	Altered transcription of a large number of genes involved in host responses, cell adherence and cell survival	[49]
	Epigenetic regulation *via* Histone H3 dephosphorylation	Downregulation of genes involved in host defence	[51]
Cytokine production	TLR4-stimulated or TLR-independent (*e.g.* by osmosensing) induction of cytokines	Increased production of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 enhancing innate responses but also inflammation.	[111, 113]
Generation of microbicidal molecules	Enhanced expression of reactive oxygen species, nitric oxide and proteases by neutrophils and macrophages	Role in microbial killing but also tissue injury.	[69, 85]
Cell adhesion	Transcriptional upregulation of adhesion molecules such as intercellular adhesion molecule 1. Cited example dependent on cytolytic activity	Potential enhancement of inflammatory response	[50]
Apoptosis	Induction of cell death in a range of cell types. Some likely due cytototoxic effects of toxin others may be host mediated responses	Tissue damage or host responses that enhance bacterial killing depending on cell type.	[99, 112, 145]

Different functions of pneumolysin overlap. For example pore formation influences interactions with cell signalling pathways and gene transcription and each of these functions may influence functions such as cytokine expression, generation of adhesion molecules or generation of factors which regulate susceptibility to apoptosis.

and cathepsin E were upregulated. These studies were conducted using a wild-type and pneumolysin deficient mutant and they do not allow assessment of whether the transcriptional effects result from direct toxicity or from host responses to infection. The study did however identify increased transcription of genes for adhesion molecules. A further study, which identified that intercellular adhesion molecule (ICAM)-1 is upregulated in response to pneumolysin, has identified that this is dependent on pore formation since a W433F mutation abolishes the upregulation of ICAM-1 mRNA, suggesting that at least some transcriptional events result from cytotoxicity [50]. Recently our understanding of how cholesterol-dependent cytolysins modify gene transcription has been refined by illustration that these toxins, including pneumolysin, modify epigenetic regulation [51]. One of these changes observed for pneumolysin is histone H3 dephosphorylation and this change is independent of pore formation. The combined effects of these epigenetic modifications are the downregulation of a subset of immunity genes early after infection. This illustrates transcriptional events can occur independent of cell cytotoxicity.

Pneumolysin exerts important effects on cell signalling. At sublytic concentrations cholesterol-dependent binding induces early membrane depolarization and micropores form in the plasma membrane leading to calcium influx and activation of rac and rho GTPases, including rac-1 and rho-associated kinase (ROCK) [52]. These changes induce a series of cytoskeletal rearrangements within the target cells. Formation of mature membrane pores results in calcium-dependent phospholipase A activation in endothelial cells with broad substrate specificity amongst plasma membrane phospholipids [53]. Sublytic concentrations of pneumolysin activate p38 mitogen-activated protein kinase (MAPK) by inducing calcium-dependent pores and osmotic stress in epithelial cells [54]. That a process of osmosensing in the cell represents a novel way of activating immune responses such as p38-MAPK was suggested by showing that solutes such as high molecular weight dextran prevent p38-MAPK activation. In macrophages phosphoinositide 3-kinase (PI3K) γ activation, in response to pneumolysin challenge, is required for recruitment of inflammatory macrophages to sights of infection [55]. Thus a diverse set of protein kinases are activated in signalling in response to pneumolysin and contribute to cell-specific responses. As with transcriptional regulation these likely result from both direct toxin mediated effects on membranes (ranging from gross cytotoxicity to the more subtle effects of sublytic concentrations of toxin altering membrane charge or osmotic stress) and responses induced by the interaction of pneumolysin with eukaryotic receptors or components of cell signalling pathways.

PNEUMOLYSIN EFFECTS ON THE RESPIRATORY TRACT

A large number of publications, most using animal models of pneumococcal pneumonia testify to the critical role of pneumolysin in contributing to the virulence of *S. pneumoniae*. Mice infected with pneumolysin deficient mutants show decreased levels of bacteria colonizing the nasopharynx, increased bacterial clearance from the lung and prolonged survival following intranasal challenge [26, 56]. Both hemolytic activity, which influences early bacterial replication, and complement activation, which seems to be more important in regulating bacterial replication at a later stage after infection and determining the level of bacteremia, influence pulmonary virulence [4, 57]. In the Jounblat study, however, both mutants with attenuated activity had enhanced bacterial replication at some early time points [57]. In particular the mutant lacking complement activating activity had enhanced bacterial replication at 12 hours after infection [57]. This suggests that an associated change in phenotype in this mutant impedes host defence, either directly due to decreased complement activation or due to an associated alteration in conformation or function that compromises some other aspect of the immune response to pneumolysin. Hemolytic activity contributes to neutrophil recruitment while complement activating activity appears to contribute to T-cell recruitment [57]. Since T-cells may play important early roles in host defence against *S. pneumoniae* and pneumolysin may contribute to T-cell recruitment it may be that the impaired clearance of the mutant lacking complement activating activity could be related to impaired T-cell function in the early stages after infection [58].

Instillation of pneumolysin directly into the lung can mimic many of the features of pneumococcal pneumonia, Fig. (**3**) with features of neutrophilic alveolitis and lung injury in rat lungs [59] That hemolytic activity is a major contributor to the lung manifestations is shown by attenuation of the phenotype when a mutant that lacked hemolytic activity was instilled. Direct cytotoxic effects of pneumolysin on respiratory epithelial cells include cytoplasmic blebbing, mitochondrial swelling and cell death [60]. Pneumolysin induces separation of epithelial tight junctions and alters alveolar permeability [61, 62]. *S. pneumoniae* produced hydrogen peroxide and hyaluronidase adds to the pneumolysin-mediated epithelial cell toxicity [63, 64]. Ciliary function is impaired by pneumolysin, an effect dependent on hemolytic activity since the W433F mutation abrogated the effect [60, 65]. Pneumolysin combines with bacterial produced hydrogen peroxide to impair ciliary function [66]. Apart from the direct effects of pneumolysin on epithelial cells the contribution of interactions with others cells in the pathogenesis of pneumolysin-mediated lung injury is less clear. Endothelial cells are also susceptible to direct cytotoxicity which contributes to pulmonary hemorrhage and edema [67].

Neutrophil recruitment is a marked feature of pneumolysin instillation, however, the acute lung injury in this model may be neutrophil-independent since inhibiting neutrophil migration did not ameliorate injury [68]. Despite these observations activated neutrophils have

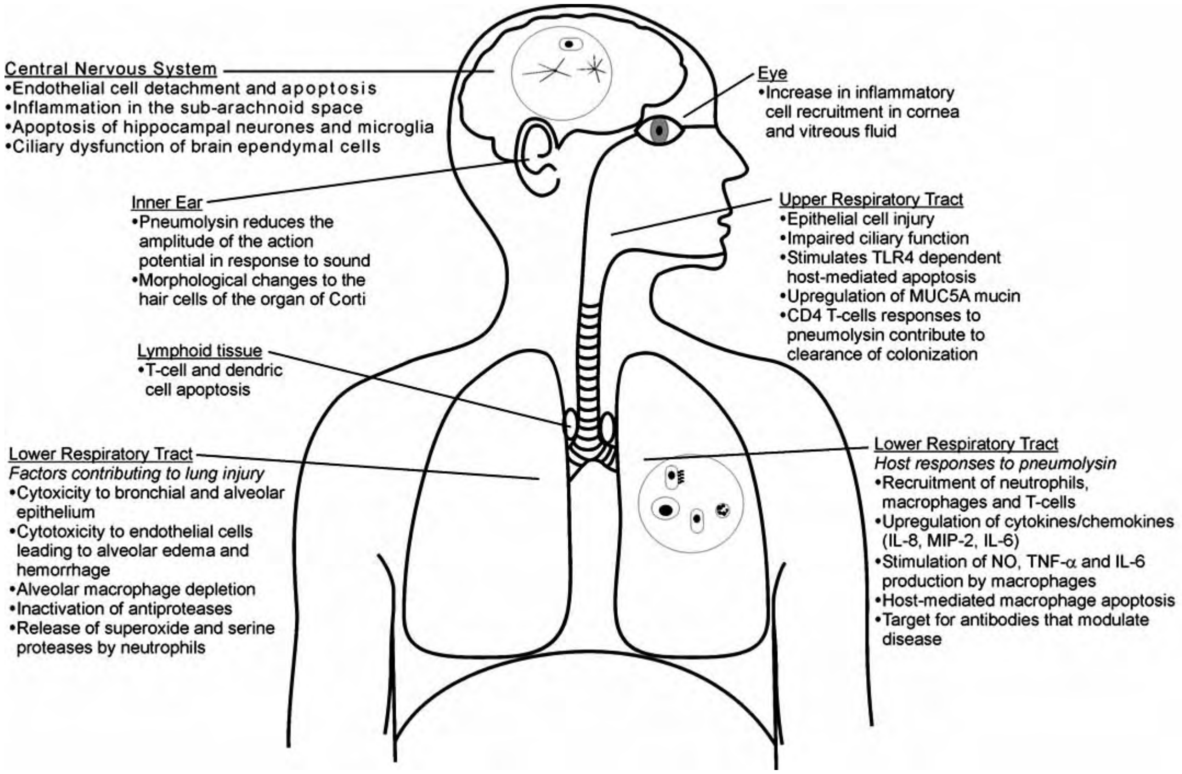

Fig. (3). Anatomical sites of action of pneumolysin. Major sites of action of pneumolysin are depicted along with the major consequences from the perspective of tissue injury and the host responses that pneumolysin stimulates.

the potential to exacerbate lung injury. Neutrophils exposed to pneumolysin have enhanced production of superoxide and neutrophil elastase [69]. Pneumolysin enhances oxidative inactivation of α-1 protease inhibitor by neutrophils *in vitro* thereby shifting the protease-antiprotease balance [70]. Recently a further mechanism altering the protease-antiprotease balance was delineated [71]. Pneumolysin induces expression of the deubiquitinating enzyme cylindromatosis which inhibits MKK3-p38 kinase and plasminogen activator inhibitor-1.

Resident alveolar macrophage do not contribute significantly to pneumolysin-induced lung injury and in fact are reduced in number following exposure to pneumolysin [68], although they may be less susceptible to direct cytotoxicity than epithelial cells [72]. Conversely, the loss of macrophages may contribute to the overall dysregulation of the inflammatory response by depleting a key cell involved in the resolution of the inflammatory response and one that contributes to the production of anti-inflammatory cytokines. High levels of cytokines may not be essential for significant lung injury, since in some models pneumolysin instillation and resultant lung injury are not associated with high-levels of cytokines [68]. However this may in part reflect the cytotoxic effects of pneumolysin on the lung parenchyma which depletes cytokine producing cells. NK cells may play a role in enhancing cytokine expression, since depletion of NK cells, in a severe combined immunodeficiency disease (SCID) mouse model, decreased pneumolysin-dependent pro-inflammatory cytokine production and lung inflammation [73].

Recruitment of inflammatory cells involves pneumolysin dependent cytokine production. A variety of cells including epithelial cells, neutrophils and monocytes secrete IL-8, or in murine models the related CXC chemokine KC, in response to pneumolysin [49, 54, 74, 75]. Pneumolysin-mediated neutrophil recruitment also involves MIP-2 and IL-6 [75]. In certain circumstances pneumolysin inhibits neutrophil chemotaxis and also blocks respiratory burst at sub-lytic concentrations [76]. Other studies have suggested low doses of pneumolysin could enhance neutrophil chemotaxis and release of factors such as lysozyme [77]. Pneumolysin-induced chemotaxis seems to require complex interactions between host and pathogen since the effects require whole bacteria and are not replicated by isolated pneumolysin alone [78]. However pneumolysin can enhance neutrophil production of reactive oxygen species (ROS) and release of neutrophil elastase in some studies [69] and may modulate inflammatory responses by enhancing prostaglandin E2 and leukotriene B4 production [79]. The variability in these findings in part reflect differences in experimental conditions but may also reflect the ability of pneumolysin to both accentuate and to inhibit neutrophil recruitment and degranulation under specific conditions.

In vivo, pneumolysin deficient mutants are associated with lower levels of neutrophil recruitment but this is more likely to reflect the lower levels of bacterial replication one observes with these strains [80]. Despite the capacity of pneumolysin to subvert the neutrophils role in anti-pneumococcal host defence neutrophils retain the capacity to inactivate pneumolysin [81]. The combination of ROS, an intact enzymatic system for the generation of ROS, such as myeloperoxidase and a source of halides to allow the generation of halide containing ROS are the necessary components needed to allow neutrophils to allow oxidative inactivation of pneumolysin. However pneumolysin if it is not inactivated can induce neutrophil death by necrosis, an effect that is further enhanced by bacterial produced hydrogen peroxide [82].

Monocyte/macrophage function is also altered by pneumolysin. Generation of ROS and monocyte degranulation is impaired [83]. However, as with neutrophils, other aspects of host defence may be enhanced by pneumolysin. TNF-α and IL-1β, two key cytokines to the early host response to pneumococci, are upregulated [84]. Nitric oxide (NO) production is enhanced which also contributes to upregulation of cytokines such as TNF-α and IL-6 [85]. NO production is stimulated by pneumolysin-induced interferon-γ production from lymphocytes, a pathway that does not require the cytolytic function of pneumolysin [86]. TNF-α and IL-1β, play important roles in pneumococcal host defence in the lung facilitating neutrophil recruitment and bacterial clearance [87-89], while IL-6-induced neutrophil recruitment in theory aids neutrophil-dependent bacterial clearance [75]. However excessive levels of any of these cytokines will facilitate excessive lung inflammation and potentially lung injury. Interferon-γ production in response to pneumolysin may not have a major impact on bacterial clearance from the lung but may improve the outcome of invasive disease [90, 91]. NO also contributes to bacterial clearance and downregulation of the inflammatory response [92-94]. This once again emphasises the double-edged effect of pneumolysin both as a factor that both stimulates and inhibits critical host responses and as a factor driving excessive pulmonary inflammation.

The overall effects of pneumolysin include cytotoxicity of lung parenchyma, enhanced production of pro-inflammatory cytokines by cells including epithelial cells and macrophages and recruitment of inflammatory cells including neutrophils. Since blocking neutrophil recruitment does not modify acute lung injury [68], neutrophils may be less important in the early stages of *in vivo* infection but they may become more important at the later stages of infection when production of ROS and unopposed protease action can further compound the direct cytotoxic effects of pneumolysin on the lung.

PNEUMOLYSIN AND THE NERVOUS SYSTEM

Pneumolysin is critical to the virulence of pneumolysin in the central nervous system [95]. In a murine model a pneumolysin deficient strain was associated with improved survival, lower morbidity and decreased numbers of bacteria in the blood. In this model there was a non-significant trend towards lower numbers of bacteria in the brain and lower numbers of leukocytes in the subarachnoid space. An early step in the pathogenesis of pneumococcal meningitis is transmigration of bacteria across the blood brain barrier. Culture of pneumococci with brain microvascular endothelial cells induces cell detachment [96]. Pneumolysin mediates this effect and requires protein synthesis and activation of tyrosine kinases. Loss of viability is associated with raised levels of intracellular calcium [97]. Death occurs relatively early (6 hours) after exposure and requires both pneumolysin and bacterial production of hydrogen peroxide [97]. Once bacteria have penetrated the blood brain barrier they seed the subarachnoid space where the recruitment of neutrophils is associated with neurone apoptosis in the dentate gyrus of the hippocampal region of the brain [98]. However, the same investigators have shown that production of pneumolysin and hydrogen peroxide also induces hippocampal neurone cell death which, as with brain microvascular epithelial cells, is dependent on raised levels of intracellular calcium [99]. Cell death requires the hemolytic activity of pneumolysin [99]. Activation of p38-MAPK is also required for neuronal death [100]. Microglia are susceptible to the same pathway of death which is accentuated by the host cell's generation of ROS [99]. Ciliated brain ependymal cells, a potential neuronal stem cell, which contribute to cerebrospinal fluid flow show ciliary dysfunction during pneumococcal infection [101]. Pneumolysin causes slowing of ciliary beating and also ciliary sloughing. Hemolytic activity contributes to dysfunction and nonhemolytic toxin has less effect, only inducing ciliary stasis at the highest dose tested. β-lactam antimicrobials enhance this process by enhancing pneumolysin release [102]. Bacterial production of hydrogen peroxide has an additive effect to that of pneumolysin and explains the residual effect on cilia observed in pneumolysin-deficient mutants [103].

One of the major sequelae of meningitis is sensineural deafness and pneumolysin contributes to the pathogenesis of this complication [104]. Infusion of pneumolysin into the guinea pig cochlea reduces the amplitude of the action potentials generated in response to sound while the hair cells of the organ of Corti show morphological changes including loss of sterocilia and break-down of the hair bundles, while cell contact is also disrupted. Similarly a guinea pig model of meningitis demonstrates that pneumolysin-deficient strains induce less diminution of the compound action potential generated in the auditory nerve in response to a tone pulse than do pneumolysin-sufficient strains [105]. Pneumolysin production was associated not only with damage to sensory hair cells, as above but also the reticular lamina and supporting cells. Sensory hair cells showed vacuolation, breaks in plasma membranes, fusion of steroecilial hair bundles and swollen dysmorphic mitochondria on electron microscopy [105]. The apical surface of the supporting inner sulcus cells developed craters. Nerve endings also showed dysmorphic vacuolated mitochondria. NO generation in response to N-methyl-D-Aspartate (NMDA) receptor signalling mediates cytotoxicity in the cochlea [106]. A nitric oxide synthase inhibitor protects against pneumolysin-mediated cytotoxicity and NMDA receptor antagonism, which is associated with decreased NO production, also inhibits pneumolysin-induced cytotoxicity [106]. Inner hair cells in the organ of Corti are especially susceptible to pneumolysin and die by a pathway of apoptosis similar to that described for neuronal cells and brain microvascular endothelial cells [107].

In rabbit models of endophthalmitis pneumolysin deficient mutants show attenuated pathogenesis with lower degrees of inflammation, as assessed by slit lamp examination [108]. Pneumolysin-deficient mutants show decreased inflammatory cell infiltration of the cornea and vitreous fluid [109]. Use of an isogenic mutant of the pneumococcus carrying a point mutation within the functional domains of pneumolysin showed that the ability of pneumolysin to activate complement plays an important role in the pathology of corneal infection [110].

The overall impact of pneumolysin on the central nervous system is a combination of direct cell cytotoxicity which in many cases induces death by apoptosis and the recruitment of inflammatory cells which further contribute to neuronal apoptosis. Induction of apoptosis contributes to the overall morbidity of meningitis including the development of sensineural deafness.

IMMUNE RESPONSES TO PNEUMOLYSIN

In contrast to the potent ability of pneumolysin to induce cytotoxicity, in particular to pulmonary epithelial cells and to neurones, pneumolysin also represents a major target of the immune response to *S. pneumoniae*. The innate immune response to *S. pneumoniae* includes recognition of pneumolysin by macrophages *via* Toll-like receptor (TLR) 4 [111]. Expression of TLR4 and the TLR adaptor myeloid differentiation factor 88 is required for macrophage expression of TNF-α and IL-6 in response to pneumolysin. The importance of this mechanism of host immunity is reinforced by the observation that TLR4 deficient mice are more susceptible to fatal infection after colonization with *S. pneumoniae* [111]. TLR4 binds to pneumolysin in a solid phase binding assay and mediates signalling in both epithelial cells and macrophages [112]. Binding of TLR4 to pneumolysin enhances host-mediated apoptosis in the upper respiratory tract, which favours bacterial clearance, in murine models. A recent study suggests that pneumolysin stimulates TLR4-dependent capsase-1 activation and production of caspase-1 dependent cytokines (IL-1α, IL-1β and IL-18) [113]. This study did not show any evidence of a role for pneumolysin in TNF-α production and the reason for the different findings are unclear but may reflect differences in the activation or differentiation state of the macrophages studied. In addition TLR4-mediated recognition of pneumolysin by epithelial cells leads to upregulation of MUC5AC mucin *via* ERK activation and a pathway that involves I kappa B kinase α and β [114]. However pneumolysin also triggers host responses in a TLR-independent fashion. Pore formation in epithelial cells following exposure to pneumolysin results in p38 MAPK activation and IL-8 release [54]. However the level of IL-8 produced is modest in comparison to the amount stimulated when the response to pneumolysin synergizes with responses to other bacterial components, such as those produced by *Haemophilus influenzae,* during colonization of the upper respiratory tract [115]. This pathway results in p38-MAPK phosphorylation and NF-κB activation. In the proposed model pneumolysin pores allow peptidoglycan from *H. influenzae* to gain access to the cells and stimulate host responses by binding to the cytoplasmic pattern recognition receptor Nod1 [116]. In theory it could also facilitate intracellular responses to other pneumococcal components.

Other studies have suggested that TLR4 mediated responses to pneumolysin may make only a minor contribution to the production of IL-8 by epithelial cells since the major pathway leading to NF-κB activation and IL-8 secretion seems to be principally mediated by TLR1/2 signalling [117]. In keeping with this observation animal model data suggest that TLR2, but not TLR4 mediated signalling, is essential for host responses to colonization by *S. pneumoniae* [118]. In this study the TLR2-mediated effect was delayed, only becoming apparent after the first two weeks, suggesting that it contributed to adaptive rather than earlier innate responses to colonization. Nevertheless the same study suggested TLR4-independent responses to pneumolysin contributed to bacterial clearance since pneumolysin deficient mutants demonstrated higher levels of bacteria in the upper airway. This finding fits with prior observations that in models of nasopharyngeal colonization, as opposed to pneumonia or invasive disease, pneumolysin deficiency impedes bacterial clearance [119]. It also fits with the early rises in bacterial numbers seen with pneumolysin mutants in the lung in the study by Jounblat [57], although these differences occurred in the first few hours after infection and were not delayed as in the van Rossum study. Thus, although models have differed in terms of the relative importance ascribed to TLR4-mediated signalling in protection against disease following colonisation, they have been relatively consistent in identifying a role for host responses against pneumolysin in host defence. The studies suggest that pneumolysin expression aids the host response during colonization, sub-clinical infection or the early stages of established infection. In contrast when disease is firmly established pneumolysin contributes to virulence by helping drive excessive inflammatory responses. There are likely to be multiple mechanisms by which the host recognises and responds to pneumolysin. The exact circumstances during which TLR4-mediated signalling contributes require further clarification. It may be that the TLR4 mediated contribution becomes most important when TLR2 or other key pathogen receptor responses are limited, since considerable redundancy exists, and this may otherwise mask the role TLR4 plays [120].

T-cells represent an important part of the immune response to pneumococci and depletion of T-cells reduces pneumococcal clearance in models of colonization [118]. T-cells, which contribute to both innate and adaptive immunity, demonstrate decreased levels of recruitment in the absence of pneumolysin [58, 80]. In addition recruited cells demonstrate increased expression of cytokines that contribute to the outcome of the host response to pneumococci, for example IFN-γ. That strains that lack complement fixing activity have altered recruitment of T-cells and impaired peribronchiolar localisation [57], suggests that classical pathway complement activation, which hinders host responses by limiting opsonization of bacteria [24, 121], may at the same time enhance T-cell responses. As discussed before for other mutants it also remains possible that the mutation that abrogates complement activation induces conformational changes that alter T-cell recruitment by quite separate mechanisms. Nevertheless although pneumolysin stimulates T-cell responses it also inhibits lymphocyte proliferation and cytokine production [122].

Pneumolysin represents a target for anti-pneumococcal antibodies. Natural infection leads to development of anti-pneumolysin IgG and IgA antibodies in children exposed to *S. pneumoniae* [123, 124]. Adult household contacts of young children respond to nasopharyngeal colonization with small but significant rises in anti-pneumolysin specific IgG [125]. In murine models anti-pneumolysin antibodies do not protect against colonization [126], but prolong survival when disease is established and reduce bacterial colony counts and the incidence of invasive disease [127-129]. Antibodies against pneumolysin enhance the degree of protection provided with immunization against the surface proteins choline binding protein A or pneumococcal surface protein A [130, 131].

In HIV-1 infection impaired production of anti-pneumolysin antibodies correlates with susceptibility to invasive disease [132]. Individuals with pneumococcal disease have lower specific anti-pneumolysin IgG levels than healthy controls [133]. Furthermore hospitalized patients with pneumococcal pneumonia who have non-bacteremic infection, as opposed to bacteremic infection, have higher titres of antibodies specific to pneumolysin. Pregnant women with high levels of anti-pneumolysin antibodies are less likely to have infants who become colonized with *S. pneumoniae* during infancy [134]. While, in contrast to some animal studies, children with higher levels of anti-pneumolysin IgG are less likely to be colonized with pneumococci [135]. However, in these studies antibodies to pneumolysin were associated with antibodies to choline binding protein A and other pneumococcal antibodies so it remains possible that the anti-pneumolysin antibodies are a marker of other protective responses rather than a key protective antibody itself. This study also demonstrated the importance of the adenoids as a lymphoid organ involved in the production of mucosal and systemic antibodies. In further studies the same group have demonstrated that peripheral blood mononuclear cells from children who are culture negative for *S. pneumoniae* have greater levels of TNF-α and IFN-γ production following *ex vivo* challenge with pneumolysin, than children who are culture positive, and that CD4 T-cell proliferation in response to pneumolysin is TLR4-dependent [136]. This further emphasises that anti-pneumolysin responses are at least tightly correlated with clearance of colonization and may be a key mediator of the protection observed.

In view of its conserved structure throughout serotypes of pneumococci pneumolysin is an attractive candidate as a component of protein vaccines. The residual toxicity of some mutants has fuelled the search for mutants which retain immunogenicity but lack hemolytic activity. The W433F mutant often referred to as pneumolysin toxoid (PdB), although immunogenic, retains approximately 1% hemolytic activity and still forms pores in membranes [22]. A promising nonhemolytic mutant, that cannot form membrane pores and is highly immunogenic, is the ΔA146 mutant which contains a single amino acid deletion [137].

PNEUMOLYSIN AS A CAUSE OF CELL DEATH

A recurring theme of the investigation of pneumolysin has been its dual roles both as a virulence factor that contributes to disease pathogenesis and, as discussed above, a focal point of the immune response to pneumococci. This point is particularly well illustrated by studies on the role of cell death in pneumococcal pathogenesis. Programmed cell death or apoptosis has been particularly well characterised in the central nervous system during pneumococcal meningitis [97, 99]. In these example pneumolysin, acting in combination with hydrogen peroxide produced by the bacterium, induces apoptosis of neurones, endothelial cells and microglia. The pathway described involves calcium influx, p38-MAPK activation, increased ROS production and direct mitochondrial toxicity [100, 138]. There is no role for TLR4 or caspase activation [97, 138]. Apoptosis of cochlear hair cells shows similar features with calcium-mediated mitochondrial toxicity triggering apoptosis [107].

In these models death is rapid occurring a few hours after exposure and in the case of neurones a direct interaction between the toxin and the mitochondrial membrane has been observed [138]. This results in release of the pro-apoptotic factor apoptosis-inducing factor (AIF) from mitochondria and apoptosis induction [139]. That induction of apoptosis involving mitochondrial permeabilisation occurs without caspase activation has been explained as being due to a simultaneous upregulation of the caspase inhibitor X-linked inhibitor of apoptosis (XIAP) by pneumolysin [138]. A further factor in the apoptosis of neurones appears to be depletion of membrane phosphatidylcholine, a known pro-apoptotic stimulus [140]. However induction of apoptosis in the brain seems to be multifactorial and pneumococcal cell wall components and recruited leucocytes contribute to apoptosis induction *via* caspase-dependent pathways [97, 98]. These pathways involve more classic apoptotic cells while the direct pneumolysin-induced apoptotic death results in cleavage of DNA into large molecular weight fragments but does not induce the classic DNA ladder [97, 138]. That death is multifactorial is supported by the observation that inhibitors of apoptosis during pneumococcal meningitis, such as brain-derived neurotrophic factor (BDNF) have broad mechanisms of action [141].

Direct-pneumolysin induced apoptosis is not unique to the brain. Lymphocytes which are subject to apoptosis during pneumococcal sepsis demonstrate apoptosis when incubated with pneumolysin [142]. Pneumolysin can synergize with granzyme B to induce apoptosis in lymphocytes, although in this model pneumolysin induced only low levels of apoptosis in its own right [143]. Dendritic cells also seem to be susceptible to a rapid direct pneumolysin-mediated apoptosis [144]. However, just as with brain microvascular endothelial cells [97], the pathway coexists with a TLR-dependent caspase-mediated delayed pathway of apoptosis [144].

In contrast to the situation with the central nervous system and the example with dendritic cells apoptosis during pneumococcal infection of the respiratory tract has been associated with improved outcomes during infection [112, 145]. Macrophage apoptosis is observed during pneumococcal infection [145]. Pneumolysin-deficient mutants induce less macrophage apoptosis than pneumolysin sufficient strains [92, 146]. In this case it is a host response to pneumolysin released by killed bacteria which triggers the apoptotic pathway. Exogenous pneumolysin does not induce apoptosis but live bacteria expressing pneumolysin do [92]. Apoptosis is enhanced by phagocytosis of bacteria and mutant bacteria of attenuated virulence that are more readily internalized induce greater levels of apoptosis than do wild-type bacteria [147]. Apoptosis of some macrophage phenotypes and of epithelial cells in the lung involves recognition of pneumolysin by TLR4 further emphasising that it is a host response to pneumolysin that triggers cell death [112]. These pathways benefit the host and enhance bacterial clearance in models of colonization and sub-clinical infection [112, 145, 148] while in established infection they may aid downregulation of the inflammatory response [94].

Pneumolysin-induced cell death may therefore be both driven by the pathogen and by the host. Since pore formation and calcium influx or pattern recognition receptor-mediated detection of pneumolysin may trigger host responses [54, 111] it remains possible that the cytotoxicity directly attributable to pneumolysin could represent a conserved host response that links the detection of micro-organisms to a programme of apoptosis to remove potentially infected cells. The pathogenesis that results could result from the high sensitivity of the sensing mechanism combined with the potential of the toxin to perturb key elements in cell homeostasis such as cell membrane function or mitochondrial integrity. In the case of the pulmonary responses they may be more obviously biased towards a host benefit but also run the risk of being excessive and compromising host defence.

CONCLUSIONS

Cholesterol-dependent cytolysins are a diverse group of proteins and differences between the proteins expressed by each bacterium have informed our understanding of the function of these molecules. Significant progress has been made in determining structure function relationships of pneumolysin. In addition recognition of the subtle genetic variation that occurs between different strains of *S. pneumoniae* has informed our understanding of how pneumolysin contributes to disease pathogenesis. The major activities of pneumolysin are the ability to form pores in cell membranes and to activate compliment *via* the classical pathway. These functions and potentially other unrecognised activities contribute to the pathogenesis of pneumococcal disease. The host response to pneumolysin targets this protein and although recent work high-lights the role of TLR4-mediated recognition of pneumolysin it is likely that other mechanisms may also contribute to the host response to pneumolysin. Pneumolysin therefore represents a critical fulcrum around which the host response and disease pathogenesis rotate. Host responses aimed at pneumolysin may variously benefit the host or contribute to pathology if they are excessive or subverted by the pathogen. Bacterial clearance and clinical disease represent points on the continuum of the host response to pneumolysin. The identification of outbreak strains which lack hemolytic activity emphasises we have still much to learn about how this toxin contributes to disease pathogenesis.

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