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***Streptococcus pneumoniae*: Invasion and Inflammation**

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INTRODUCTION

Streptococcus pneumoniae (the pneumococcus) is a leading cause of otitis media, community-acquired pneumonia, bacteremia and meningitis. The pneumococcus is a human-specific pathogen which colonizes the nasopharynx and spreads between hosts through aerosol and potentially through the contamination of objects with mucosal secretions if the bacteria is living within a biofilm (1–3). Rates of carriage vary from 5–10% of healthy adults to 20–40% of healthy children. However, these numbers can vary widely based on where the samples are collected from (4–7). Risk factors associated with higher rates of carriage include race (particularly Australian Aboriginals and Native Americans) (8–12), infancy (13, 14), season with higher carriage during winter months (13), and crowded areas such as childcare centers with estimates suggesting 40–60% of children who attend childcare are colonized (15). Duration of colonization decreases with age and varies from 2 weeks to 4 months (14, 16, 17). The introduction of pneumococcal conjugate vaccines has reduced carriage rates for serotypes covered by the vaccine while non-vaccine serotypes have emerged to occupy this empty niche (18). Nasopharyngeal colonization is usually asymptomatic (19).

Invasive pneumococcal disease (IPD) occurs as a result of the spread of bacteria from the nasopharynx to other parts of the body including the lungs, blood, and brain. Infants, the elderly, and immunocompromised individuals are at an increased risk for developing IPD (20–22). Pneumococcal models of invasive disease must account for not only the commensal nature of the bacteria, but also the wide spectrum of disease the pneumococcus is capable of causing. Colonization is a prerequisite for IPD and while the incidence of infection is relatively low, high rates of colonization result in extensive morbidity and mortality that is a global concern. Worldwide, it is estimated that *S. pneumoniae* is responsible for 15 cases of IPD per 100,000 persons per year (23), and over a million deaths annually. As of 2004, in the United States, it is estimated that the pneumococcus was responsible for greater than 1.5 million cases of otitis media and 800,000 cases of pneumonia (24). Direct medical costs resulting from infections totaled \$3.5 billion (24). The World Health Organization estimates that close to half a million children under the age of 5 years die annually as a result of

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Streptococcus pneumoniae infection (<https://www.cdc.gov/pneumococcal/global.html>). Pneumococcal bacteremia and meningitis are also responsible for significant mortality particularly in the elderly where rates may be as high as 60% and 80% respectively (25).

In this chapter, we will review colonization and spread of *Streptococcus pneumoniae* from one anatomical site to another. We will also discuss the mechanistic basis of inflammation and cytotoxicity resulting from invasive pneumococcal infection.

TRAFFICKING OF PNEUMOCOCCI THROUGH THE RESPIRATORY TRACT

Interactions with epithelial cells of the nasopharynx

For over a century, *S. pneumoniae* has been categorized by serology with distinct serotypes identified on the basis of the greater than 90 immunologically and chemically distinct polysaccharide capsules that surround and protect the bacteria from phagocytosis (26). The capsular polysaccharide is also the basis of the current pneumococcal vaccines. Prior to the introduction of the 13-valent pneumococcal conjugate vaccine in 2010, studies found that only a small subset of the many capsular types was responsible for the majority of IPD isolates (27). The vast majority of pneumococci colonize the nasopharynx for up to 6 weeks and are then cleared with no systemic symptoms in the host (1, 28). IPD is thought to occur most frequently early after the acquisition of a new capsular serotype as evidenced by shift in the strains most commonly isolated from IPD patients after vaccine introduction (22, 29–31). Furthermore, attack rates are higher for serotypes that are carried for shorter periods of time versus those that colonize for extended periods (28).

S. pneumoniae undergoes spontaneous phase variation alternating between a transparent and opaque colony phenotype which can be visualized microscopically by oblique transmitted light (32). The different phenotypes occupy different niches based on selection with the transparent phenotype being the predominant phase in the nasopharynx (32), while the opaque phase is isolated from blood samples (33). The transparent phenotype expresses increased amounts of phosphorylcholine (34) and choline binding protein A (CbpA) on the surface (35), both of which function as adhesins and contribute to the ability of the bacteria to colonize the nasopharynx. The opaque phenotype expresses increased levels of capsule and pneumococcal surface protein A (PspA), which are important factors for survival in the blood. Phase-variation is one of the mechanisms by which the pneumococcus alternates between an adhesive phenotype best suited for the nasopharynx and a phagocytosis-resistant phenotype that can survive in the blood.

Phosphorylcholine (ChoP) decorates the cell wall (Figure 1.) and serves as a docking group for a set of 15 secreted proteins, termed choline-binding proteins (CBPs) (36). Among CBPs, CbpA is a major pneumococcal adhesin (37) expressed predominantly in the transparent phenotype (35). Pneumococci lacking CbpA are not only largely unable to bind to the nasopharynx, but also have a diminished capacity to colonize the lower respiratory tract and cause pneumonia (35, 38). In vitro, CbpA mutants show decreased binding to both nasopharyngeal epithelial cells and activated type II human lung cell lines (37). Similarly, the CBPs LytB, LytC, CbpD, CbpE and CbpG also contribute to nasopharyngeal colonization when tested in rat pups. LytB functions as a glucosaminidase (39), whereas

LytC is a lysozyme (40), and CbpE is a choline esterase; all three enzymes are active on the bacterial cell wall (37). CbpG is a serine protease that in addition to contributing to nasopharyngeal colonization is required for development of high-grade bacteremia (41).

Other virulence factors affecting the capacity of the pneumococcus to colonize the nasopharynx include sIgA1 proteases (42). IgA antibodies are the predominant type found at mucosal surfaces and serve to aggregate and opsonize pathogens. sIgA1 proteases neutralize the activity of sIgA by cleaving the Fc portion of human IgA1 (43). Hydrolyzed sIgA1 also contributes to pneumococcal adhesion. Fab fragments remaining on the surface of the bacteria after sIgA proteolysis neutralize the negative charge of the capsule and enhance adherence (42).

The two-component system CiaR/H is required for efficient colonization and regulates gene expression in response to oxidative stress (44). Among the many genes regulated by CiaR/H is *htrA*. HtrA, or high temperature requirement A, is a heat-inducible serine protease that has both proteolytic and chaperone activities. HtrA assists in the survival against environmental factors such as oxidative stress, osmotic stress, and elevated temperatures (45). Deletion of the CiaR/H operon results in a 25-fold decrease in levels of *htrA* expression and a 1000-fold decrease in the number of bacteria colonizing the nasopharynx. Deletion of *htrA* alone results in a 100-fold decrease in nasopharyngeal colonization indicating that CiaR/H regulates other factors that contribute to colonization (44). Microarray analysis during bacterial adhesion to epithelial cells in vitro has revealed a number of genes with enhanced expression including CbpA and HtrA (46).

A second regulator important in nasopharyngeal colonization is RlrA which regulates the transcription of the pilus-1 structural subunit genes *rrg(A-C)* (47, 48). Pili are found in only 20% of *Streptococcus pneumoniae* strains, but are common in strains belonging to antibiotic resistant clones (49, 50). Piliated strains show stronger adherence to lung epithelial cells and out compete non-piliated strains in mixed infection models (47). Furthermore, introduction of the pilus-1 locus into a non-piliated strain increased adherence to the lung epithelial cells (47). Pilus-1 expression levels have been shown to change during the different stages of infection with higher expression during early colonization and lower expression as the disease progresses (51).

Other pneumococcal factors that may aid adherence and colonization of the nasopharynx include PspK, SlrA and PmpA. PspK, pneumococcal surface protein K, has been suggested to help in the colonization of the nasopharynx in unencapsulated strains by mediating adherence to epithelial cells (52). PspK has also been shown to bind the sIgA, however does not appear to be involved in invasion (discussed in further detail below) (53). The two surface associated lipoproteins SlrA and PpmA have both being shown in mutagenesis studies to be important for nasopharynx colonization but they do not appear to play a significant role in invasive disease (54, 55).

Another strategy employed by the bacteria to colonize and remain in the nasopharynx is formation of a biofilm. Whilst the specific role of biofilm formation in pneumococcal infection is not well understood, it confers an increased resistance to antimicrobial peptides

and may promote sharing of genetic information between the bacteria as a result of proximity (56). Although bacteria in a biofilm are often metabolically dormant, when they are dispersed they may be more virulent. Bacterial release from a biofilm can be triggered by changes in the microflora, inflammation or viral infection (57). These released pneumococci were found to be phenotypically different from planktonic or biofilm bacteria and had an increased ability to disseminate and cause infection (58, 59). These release events represent a potential switch between asymptomatic colonization and invasive disease.

Ascension into the middle ear

Otitis media (OM) is a highly prevalent pediatric disease and the primary cause of physician visits by small children. As many as 80% of children have presented with at least one case of OM (60). Along with *Moraxella catarrhalis* and *Haemophilus influenzae*, the pneumococcus is a primary cause of OM and is isolated in 30–40% of culture positive middle ear infusions (61–63). OM is thought to result when pneumococci in the nasopharynx ascend the Eustachian tube and gain access to the middle ear (Figure 2.). Alternatively, it has also been suggested that OM is caused by the blocking of the Eustachian tube resulting in a decrease of oxygen and increased dampness on the middle ear surface. While the mechanism is disputed, the transition from colonization of the nasopharynx to OM strongly correlates with accompanying viral infection (64). Typically, OM presents with earache, fever, nasal congestion, a feeling of fullness in the ear, and muffled hearing.

The ability of *S. pneumoniae* to ascend the Eustachian tubes involves neuraminidases (61, 65). *S. pneumoniae* encodes two neuraminidases NanA and NanB, with NanA being the major neuraminidase (66, 67). Neuraminidases cleave N-acetylneuraminic acid from glycoproteins and glycolipids on the eukaryotic cell surface. Neuraminidases can also cleave mucin reducing the viscosity of this barrier and permitting the bacteria to access the epithelium. At the epithelium, neuraminidase cleaves oligosaccharides on the host cell surface exposing cryptic receptors and enhancing bacterial adherence (66, 67). Support for this mechanism is seen in chinchilla infection models whereby clearance of a neuraminidase deficient mutant occurs twice as quickly as wild type *S. pneumoniae*. Furthermore, structural changes in the cell surface carbohydrates of Eustachian tube epithelial cells occurs following infection with pneumococci compared to its isogenic *nanA* mutant (68, 69).

Once the pneumococcus is in the middle ear, inflammation is triggered by pneumolysin and cell wall components (70). Pneumolysin is a potent pore forming toxin that directly kills host cells and activates complement. In addition, cell wall components activate TLR (Toll like receptor) signaling and the alternative pathway of complement. Pneumolysin strongly contributes to hearing loss and cochlear damage during OM (71, 72). Guinea pigs infected with wildtype *S. pneumoniae* or a mutant deficient in neuraminidase demonstrated significant damage to the reticular lamina, sensory hair cells and supporting cells of the organ Corti. Guinea pigs infected with a pneumolysin deficient mutant had no visible damage. The contribution of pneumolysin and cell wall to inflammation will be discussed in more detail at a later point in this chapter.

CbpA/pIgR mediated invasion in the upper respiratory tract.

Once attached to epithelial cells, the pneumococcus is able to translocate across the mucosal barrier by co-opting the polymeric immunoglobulin receptor (pIgR) (73). Mucosal epithelial cells transport IgA and IgM in a vesicle moving from the basolateral to the apical surface by binding to pIgR. On the apical surface, pIgR is cleaved and immunoglobulins are secreted into the lumen. Cleaved pIgR is subsequently shuttled back to the basolateral surface (74). *S. pneumoniae* translocates through epithelial cells by hijacking pIgR on the apical side of the host cell resulting in transport as the receptor is endocytosed and recycled to the basolateral surface (Figure 3.). The pneumococcus binds to pIgR by ligating the motif YRNYPT of CbpA (75). *In vitro* CbpA alone is sufficient to mediate translocation across a cell, as latex beads coated with CbpA are endocytosed (73). *In vivo* this interaction is manifest as decreased nasopharyngeal colonization in mice lacking pIgR and the reduced capacity for *S. pneumoniae* mutants lacking CbpA to enter the bloodstream (76).

Accessing the Lower Respiratory Tract

Development of pneumonia is contingent on the ability of *S. pneumoniae* to establish a lower respiratory tract infection despite host defenses that either kill or clear the aspirated bacteria. The first barrier is the mucociliary escalator which mechanically works to keep aspirated particles and microorganisms out of the lungs. As is true for many respiratory viruses, neuraminidase plays an important role in initiating bacterial pneumonia. As indicated, neuraminidase deficient pneumococci do not cleave mucin efficiently and have a diminished capacity to adhere. Mutants deficient in *nanA* have a reduced capacity to bind to chinchilla tracheas *ex vivo* (77) and are attenuated in their ability to cause a lower respiratory tract infection following intranasal challenge (38).

The second step in adjusting to the environment of the lung involves changes to the bacterial surface that promote bacterial adherence to epithelium. The respiratory epithelium produces copious antibacterial peptides that kill bacteria on contact. Recent evidence indicates that pneumococci counteract this defense by shedding capsule which results in relative resistance to the killing by antimicrobial peptides (78). This process is driven by the bacterial autolytic enzyme, LytA. Although LytA has long been known to promote autolysis in response to antibiotics, this new role of the enzyme drive capsule loss without bacterial lysis. Loss of capsule permits a close interaction of the bacteria with host cells leading to successful initiation of infection.

Pneumococcal adhesion to eukaryotic lung cells is a two-step process that initially entails a loose interaction with host cell surface glycoconjugates followed by a tighter, more secure interaction with host cell protein receptors that promote internalization. During the initial stages of infection, *S. pneumoniae* and other respiratory tract pathogens such as *P. aeruginosa* and *H. influenzae*, bind to N-acetylgalactosamine β 1–3 galactose (79). Neuraminidases cleave the sialic acid and expose N-acetylgalactosamine β 1–3 galactose and other ligands on the host cell surface (69, 80). The efficient removal of sialic acid by neuraminidases can be reduced by the amount and position of acetylation on sialic acid. It is thought that the major pneumococcal esterase, EstA, deacetylates the sialic acid and increases the release of sialic acid by NanA (81). *In vitro*, pneumococci adhere more

efficiently to tissue culture cells treated with neuraminidase (77). The synergism observed in a pneumococcus and influenza co-infection may be the result of enhanced adherence mediated by neuraminidase activity (82). It has long been known that influenza primes the lungs for the development of a secondary bacterial infection. This synergism has been successfully modeled in mice, where pretreatment with influenza readily enhances severe pneumococcal pneumonia leading to death despite challenge with a dose that is usually insufficient to infect an otherwise healthy mouse (83) (84, 85). Mechanistically, superinfection likely occurs as a result of neuraminidase expressed by influenza stripping sialic acid from the mucosa and exposing bacterial receptors. Oseltamivir, a neuraminidase inhibitor, has been shown to prevent pneumococcal superinfection in this post-influenza pneumococcal challenge model (86).

Interactions in the alveoli

As pneumonia progresses, the respiratory epithelium is denuded, exposing the underlying extracellular matrix of the bronchioles and alveoli. Surface bound bacterial proteins such as PepO, PavA, PavB, PfbA, PclA and PsrP all contribute to attachment through interactions with the extracellular matrix (87–90). Once established in the alveoli, inflammation is particularly intense resulting in consolidation of the affected lobes. Consolidation progresses through stages of engorgement and red hepatization during which capillaries and epithelial cells become inflamed, and fluid, and erythrocytes accumulate in the alveoli in a fibrin mesh (red hepatization). Subsequently, the lungs darken (gray hepatization) as leukocytes enter the lesion and the bacteria are engulfed by macrophages. Resolution continues for several days as capsule-specific antibodies provide efficient opsonization and inflammatory mediators dissipate (91).

Pneumococcal cell wall, pneumolysin and hydrogen peroxide are the virulence determinants that mediate the greatest inflammation and cytotoxicity observed in the lungs (92–94). Challenge of mice with purified pneumolysin or cell wall TLR ligand products is sufficient to cause edema and influx of neutrophils that recapitulate pneumonia (95, 96). Multiple studies clearly demonstrate that deletion of the genes that encode autolysin, pneumolysin or the enzyme that produces hydrogen peroxide, greatly attenuates the ability of the bacteria to survive and replicate in the lungs (38, 92, 94, 96–98). The contribution of these virulence products is discussed in greater detail below.

INVASION OF PNEUMOCOCCI INTO THE BLOODSTREAM

Access to the bloodstream

During red hepatization, infected alveoli are overflowing with bacteria, edema fluid and erythrocytes enwrapped in a fibrin mesh. Fibrin strands pass through interalveolar connections, also known as the pores of Kohn, from one alveolus to the next and the lymphatics are dilated and filled with cells and fibrin. When infection reaches this stage, a mouse becomes bacteremic. Access to the bloodstream by *S. pneumoniae* may occur through several pathways including via the lymphatics, via cell damage to the epithelial and endothelial cells, and via direct invasion of endothelial cells. Most likely all three pathways contribute to bloodstream invasion in an infected animal.

Although the pneumococcus can directly invade cells, this occurs at a relatively low efficiency when compared to commonly thought of invasive bacterial pathogens such as *Salmonella* and *Shigella* species (99). Invasion is dependent on activation of the host cell by pneumococcal cell wall components and pneumolysin and results in *de novo* expression of host defenses such as platelet activating factor receptor (PAFr), C3 and other factors. PAFr, a receptor abundant on lung cells, recognizes phosphorylcholine (ChoP) on its natural ligand the chemokine PAF. The surface of the bacteria mimics this by decorating the cell wall with ChoP. COS cells expressing PAFr have been shown to bind more bacteria than COS cells not expressing PAFr (99). Studies have also colocalized PAFr with adherent bacteria on the surface of human cells (100). PAFr binding is not limited to the pneumococcus, other respiratory pathogens such as *Haemophilus spp.*, *Neisseria spp.*, and *Pseudomonas spp.*, also express ChoP on their surfaces in a phase variable manner (101, 102). The pneumococcus can also bind the PAFr on other tissues (103, 104). In defense against the widespread expression of ChoP on respiratory pathogens, the host deploys C-reactive protein (CRP), an acute phase reactant that activates complement and opsonizes the bacteria (105). Thus, phase variation of the amount of surface bound ChoP serves as a mechanism by which the pneumococcus switches from a more adherent (high ChoP) form suited for the mucosa to one that is more resistant to phagocytosis (low ChoP) and well adapted to the bloodstream.

Unlike PAF, the binding of pneumococcus to the PAFr does not result in the activation of a G-protein-mediated signal transduction pathway (100). Rather, pneumococcal uptake requires activation of ERK kinases consistent with activation by β -arrestin. Uptake of the pneumococcus into a vacuole involves clathrin followed by recruitment of β -arrestin scaffold, Rab5, then Rab7 and Rab11. Rab 5 is involved in early endocytosis, Rab 7 is found in the late endosome and Rab 11 is responsible for vacuole recycling (106). Over expression of arrestin in endothelial cells enhances colocalization of the bacteria with Rab 7 and Rab 11 and increases survival of the pneumococci normally killed by the lysosome. Thus it is currently thought that association of β -arrestin with the PAFr vacuole complex contributes to the successful translocation of the bacteria away from the lysosome (100).

The pilus-1 system may also be employed in the escape from the lungs and peritoneal cavity into the bloodstream. RrgA, the major adhesive determinant of the pilus, has been shown to facilitate the spread of the bacteria to the bloodstream (107). RrgA binds the complement receptor 3 (CR3) on macrophages and increases intracellular survival (107). The severity and progression of disease was shown to be affected in mice lacking CR3 and may suggest that phagocytosis by macrophages may facilitate the spread of infection to distal sites (107). This theory may be further supported by the fact that ubiquitously expressed pneumococcal protein PepO may lead to an increase in macrophage phagocytosis (108).

Recently a new macropinocytosis pathway has been described that is independent of the PAFr and does not require ChoP (109). This pathway relies on actin interactions with the uptake vesicle independent of dynamin, clathrin and caveolin (109). This pathway represents a potential alternative pathway for bacterial translocation, however without the requirement of ChoP it could be used by a wider group of bacterial pathogens. In model systems, roughly half of adherent pneumococci enter epithelial cells via micropinocytosis and half via PAFr.

Thus while the pneumococcus is the prototypical extracellular pathogen, intracellular translocation is an important part of its pathogenesis.

Survival in the bloodstream

Once the bacteria escape into the bloodstream, capsular polysaccharide (CPS) becomes the most important virulence determinant and is responsible for inhibiting phagocytosis. The chemical structure (serotype) and amount of CPS present on the surface of the bacteria contribute to the differential ability of different serotypes to survive in the blood (110–113). Mutants lacking capsule are essentially avirulent (111), requiring 10,000 to 100,000-fold more bacteria to kill a mouse than the encapsulated parent strain following intraperitoneal injection. It is believed that CPS inhibits phagocytosis by preventing phagocytes from physically reaching opsonizing serum components, such as complement, CRP, mannose-binding proteins, and antibodies that are deposited on the cell wall and by giving the bacteria a negative charge which repels a close association with leukocytes (32, 114, 115). Formation of antibody to the serotype specific CPS marks the initiation of clearance of the infection, as antibodies to CPS are highly opsonic and are protective against subsequent pneumococcal challenge with the same serotype (116). The fact that capsular antibodies are so effective forms the basis of current effective pneumococcal vaccines.

Pneumococcal proteins also contribute to resistance to defenses in the serum. PspA has been demonstrated to inhibit complement activation mediated via the classical pathway on the bacteria surface (117, 118). Mutants deficient in PspA are cleared more rapidly from the bloodstream of XID mice compared to wildtype mice. XID mice lack the ability to form an antibody response to polysaccharides and thus clearance correlates with the amount of complement on the bacterial surface (119). PspA also protects bacteria from the bactericidal peptides of apolactoferrin, blocking PspA with antibodies enhances apolactoferrin killing (120). CbpA also has the ability to inhibit complement deposition by binding to C3 without cleavage and binding Factor H (121, 122). PspA allows the bacteria to reduce C3 deposition, whereas Factor H, a negative regulator of the alternative pathway, leads to interference of the formation of the C3 convertase. Factor H can also be bound by the pneumococcal surface protein Tuf (123). Furthermore, resistance to opsonophagocytic activity of neutrophils is mediated through the action of the exoglycosidases NanA, BgaA and StrH that decrease the amount of C3 being deposited onto the pneumococcal surface (124).

TRAFFICKING OF PNEUMOCOCCI INTO HEART, BRAIN AND FETUS

Interactions with the heart

Once in the bloodstream, pneumococci disseminate widely into many organs by the binding of bacterial CbpA to endothelial laminin receptor (LR) and ChoP to the PAFr. For instance, a common complication of severe bacterial pneumonia is cardiac dysfunction. Pneumonia and cardiac events may be closely associated with heart disease predisposing people to pneumonia or potentially pneumonia increasing the risk of heart disease, further studies are needed (125). Bacteremia delivers pneumococci to the cardiac vascular endothelium where CbpA/LR mediated translocation occurs into the cardiac tissue. In mice and non-human primates, cardiac damage ensues in the form of microscopic lesions in the myocardium (126,

127) caused by the release of pneumolysin and H₂O₂ with added pathology resulting from the influx of immune cells (126, 128). Immunization of mice with the CbpA protects the animals from cardiac damage (126). Importantly pneumococcal cell wall products are also inhibitory to cardiac contractility (129), as is pneumolysin which disrupts Ca⁺⁺ signaling due to pore-formation even if cells are not immediately killed (130). Once in the heart of the mice, the bacteria use clathrin-mediated endocytosis to invade cardiomyocytes (131).

Interactions between the pneumococcus and the heart is an emerging field.

Interactions at the blood brain barrier.

Pneumococcal meningitis is by far the most devastating complication of IPD. Some estimates suggest approximately one third of affected individuals die (132–134) and half of the survivors suffer from permanent neurological sequelae (135–137). Severe damage occurs in the hippocampus, particularly in the dentate gyrus, with survivors suffering from hippocampal atrophy and defects in learning and memory (138). Neuronal damage is, in part, mediated by the response of the host defenses to bacterial products (139), for example cell wall components are detected by host cells, and the influx of leukocytes leads to extensive inflammation (140, 141). Inflammation in the cerebrospinal fluid (CSF) exacerbates neuronal damage by releasing matrix metalloproteases such as MMP-9 (142), neurotoxic free radicals such as peroxynitrate (143), and pro-inflammatory cytokines that recruit more leukocytes (141). Ultimately the over exuberant host-response triggers caspase-dependent and independent apoptosis of neurons (139). Blocking leukocyte entry into the CSF during meningitis decreases damage by approximately 50% (144, 145) and as such is the basis for treating individuals with pneumococcal meningitis with dexamethasone prior to antibiotic therapy. The other half of neuronal damage is due directly to cytotoxic compounds such as pneumolysin and hydrogen peroxide that damage the mitochondria and initiate apoptosis (146). These factors are reviewed later in the text. The extent of the host response to damage can be seen by the fact that introduction of purified cell wall alone into the CSF can lead to signs and symptoms of meningitis and ultimately to neuronal damage (147).

Pneumococcal invasion from blood into the CSF is thought to occur either in the choroid plexus or by crossing the blood brain barrier in the cerebral capillaries that traverse the subarachnoid space. The pneumococcus is thought to initially bind to the blood brain barrier endothelium through interactions of the NEEK motif of CbpA with the laminin receptor (148). Such binding to laminin receptor is a common strategy of meningeal pathogens, including *H. influenzae*, meningococcus, prions, and several viruses. Studies in mice have determined that once bound to the endothelium, actual translocation across the barrier is dependent on the interaction between ChoP and PAFr (148–151). PAFr knockout mice were resistant to development of meningitis (100). Likewise CbpA mutants were unable to cross the blood brain barrier despite bacterial titers in the blood of 10⁸ CFU/ml (38). Thus, the two-step process of recognition of laminin receptor on the cerebrovascular endothelium by homologs of CbpA followed by translocation across the barrier using surface ChoP binding to PAFr is a conserved process of invasion of the central nervous system that is shared by the most successful meningeal pathogens.

Interactions with the placenta and fetus

PAFr is found on a number of tissues including the placenta during pregnancy. Infection and inflammation during pregnancy has been shown to lead to postnatal cognitive deficiencies in a number of different studies reviewed in Loughran et al 2016 (103). The pneumococcus itself does not cross the placenta to the fetus. However, fragments of cell wall released during antibiotic treatment cross the placenta in a PAFr dependent manner and accumulate in the developing fetal cortex in mice. Rather than neuronal death, as seen in postnatal meningitis in mice, the fetal brain responds with an increase in neuroproliferation (104). Neuroproliferation is increased by interfering with the levels of the cytostatic transcription factor FoxG1 as a result of cell wall interaction with TLR2. The abnormal brain architecture is associated with behavioral abnormalities in the postnatal period in mouse models. This leads to the possibility that bacterial products encountered during pregnancy may be associated with cognitive disorders in children.

INFLAMMATION AND CYTOTOXICITY

Intense inflammation is a hallmark of pneumococcal disease and the pneumococcus serves as a prototype for understanding the molecular mechanisms of inflammation in response to gram-positive bacteria (152, 153). Inflammatory components released by the pneumococcus include peptidoglycan, teichoic acid, pneumolysin, hydrogen peroxide, and a number of other secreted proteins. Alone, several of these factors have been shown to trigger inflammation and are cytotoxic (see above). In concert, these factors trigger inflammation through multiple inflammatory cascades including the TLR pathway, chemokine/cytokine cascade, the complement cascade, and the coagulation cascade. In a naïve host lacking serotype-specific antibodies, these cascades hasten the accumulation of leukocytes, which are ineffective in phagocytosing pneumococci coated in capsule. The increased white blood cells at the site of infection lead to an even greater release of pro-inflammatory mediators without efficient clearance of the bacteria.

Inflammation

The key surface component recognized by the innate immune system is the cell wall (Figure 4.). Pneumococcal cell wall is composed of the peptidoglycan network with teichoic acid attached to roughly every third *N*-acetylmuramic acid residue (154). When tested in animals, peptidoglycan and teichoic acid can elicit inflammation and re-capitulate many of the symptoms of pneumonia, otitis media, and meningitis (147, 152, 155). Although CPS is effective in protecting the bacteria, the CPS itself is not inflammatory (156). Components of the bacterial cell wall can mediate inflammation through multiple pathways. At the cellular level, peptidoglycan and teichoic acid bind to pattern recognition molecules such as lipopolysaccharide binding protein and peptidoglycan recognition proteins. These complexes in turn bind to Toll-like receptor 2 (TLR-2) on the surface of epithelial and endothelial cells, monocytes and macrophages (157, 158). Cross linking of TLR-2 triggers intracellular signaling that activates transcriptional regulators such as NF- κ B. NF- κ B expression then results in production of pro-inflammatory cytokines such as IL-1 β , IL-6, IL-8, and TNF (159). Nod receptors in the cytoplasm may also modulate inflammation by binding to intracellular peptidoglycan (160, 161). The importance of Nod receptor activation

for resolution of the infection is supported by the need for the expression of the chemokine receptor CCR2 to efficiently recruit macrophages to the site of infection for bacterial clearance (162). Activation of epithelial and endothelial cells results in recruitment of effector cells such as neutrophils and macrophages, altered vascular permeability and creation of a serous exudate.

Cell wall components not only cause inflammation through interaction with cells but also activate several complement pathways. Antibodies specific to bacterial proteins on the cell surface activate the classical pathway (117). Similarly, CPS and cell walls bind to hydrolyzed C3 and activate the alternative complement pathway (156, 163, 164). The classical and alternative pathways result in release of C3a and C5a both of which are chemoattractants and potent anaphylactic molecules leading to inflammation. Cell wall also activates complement via the lectin-binding pathway. Mannose binding lectin (MBL), a member of the collectin family, binds to carbohydrates such as *N*-acetyl-glucosamine, a constituent of peptidoglycan (165). The binding of MBL to the bacterial cell results in formation of a C3 convertase on the surface of the bacteria and deposition of C3b. MBL deficiency is associated with an increased risk of invasive pneumococcal infection (166). Finally CRP bound to cell wall ChoP also activates the classical complement cascade by binding C1q (167). This results in additional opsonization and further release of the C3a and C5a. Pneumococcal PepO can disrupt this process by binding to C1q increasing bacterial survival (168). However, PepO expression in the lungs has been suggested to trigger release of the chemoattractant IL-8 and IP-10 which leads to neutrophil recruitment and contributes to the host response pathology (169).

Complement activation is not limited to the surface of the bacteria; cell wall fragments released by the bacteria following lysis and during cell wall turnover are also capable of activating complement (170). Likewise, pneumolysin released into the milieu also activates complement (171). Pneumolysin binds to the Fc portion of immunoglobulins and activates the classical pathway. It has been suggested that cell wall components, pneumolysin and other proteins such as PepO are released by the bacteria to deplete complement (168, 172, 173). This would have the most direct impact during blood stream infections and in the lungs. Release of cell wall components is mediated by the murein hydrolase, LytA. LytA is responsible for pneumococcal lysis in stationary phase as well as in the presence of antibiotics (174). Furthermore, it has been shown that LytA is important for the release of capsule in response to antimicrobial peptides found on the epithelial surface (78). Autolysin-mediated lysis is responsible for the spike in inflammation observed immediately following antibiotic treatment of meningitis. Autolysin-negative mutants may have reduced virulence as compared to wild type, as a result of the inability of the mutant to release CW or due to the inability to shed the capsule leaving the bacterium sensitized to the antimicrobial peptides (78, 175).

Although complement is crucial for the opsonization of the bacteria, it also leads to the formation of the membrane attack complex (MAC) formed through the action of C5, C7 and C9 which form a hole in cellular membranes in a fashion similar to pneumolysin. The pneumococcal glycolytic enzyme phosphoglycerate kinase (PGK) has been shown to inhibit MAC formation by blocking C9 polymerization (176). Gram positive bacteria, such as

Streptococcus, are also in general more resistance to MAC killing than their Gram negative counterparts since they lack an outer membrane leaving MAC to form on the thick peptidoglycan wall.

Cytotoxicity

In addition to the pathology derived from inflammation, the pneumococcus can directly damage eukaryotic cells. The principal mediators of cytotoxicity are pneumolysin and hydrogen peroxide (72, 92, 93, 146, 177). Pneumolysin has long been recognized as a principal virulence factor of the pneumococcus (Figure 5.). Studies using a variety of challenge routes and animal models have convincingly demonstrated that pneumolysin deficient mutants are drastically attenuated (38, 92, 96, 177). The mechanism responsible for pneumolysin secretion from the bacteria is poorly understood. While it was initially thought that pneumolysin was only released as a result of autolysis (178), it has also been shown to have an independent route as mutants lacking the autolytic enzyme, *LytA*, show the same pattern of release as the wild-type bacteria (179) (180). Pneumolysin is a pore forming toxin that kills cells via necroptosis. At high concentrations, pneumolysin, like other pore-forming toxins triggers necroptosis a programmed mode of necrosis, due to ion dysregulation (181, 182). Critically, pneumolysin mediated cell death has been shown to be preventable, using drugs that block RIPK1, RIPK3 or MLKL, the signaling pathway involved. The toxin binds to cholesterol on the surface of the host cell and oligomerizes to form pores as large as 30nm in diameter (183). This results in Ca^{++} and K^{+} dysregulation. At lower concentrations, the toxin has a variety of effects on different cell types. Pneumolysin has been demonstrated to slow ciliary beating of epithelial cells (184), disrupt tight junctions (185), and inhibits the capacity of neutrophils and macrophages to kill by inhibiting oxidative burst (186, 187). Disruption of the alveoli-capillary barrier contributes to the leakage that allows serous exudates to enter the lungs and the bacteria to cross into the blood stream (188). In the middle ear, pneumolysin is responsible for damage to the cochlea and hair cells contributing to hearing loss (72). During meningitis, pneumolysin causes neuronal damage mediated by an influx of extracellular calcium triggering apoptosis (146). Chelating extracellular calcium inhibits the release of AIF and protects cells from pneumolysin-induced apoptosis in vitro.

Hydrogen peroxide (H_2O_2) is a major product of pneumococcal metabolism and also damages host tissues. H_2O_2 is the result of the activity of the enzyme pyruvate oxidase (*SpxB*) which decarboxylates pyruvate to produce acetyl phosphate, H_2O_2 and CO_2 (38, 97). Mutation of *spxB* dramatically attenuates virulence in the respiratory tract but not in the blood stream (38). Studies looking at the cytotoxic effects of H_2O_2 are not as comprehensive as those for pneumolysin. Nonetheless, H_2O_2 also contributes to mitochondrial damage of neurons resulting in apoptosis (146), and inhibits beating of ciliated ependymal cells lining the ventricular system of the brain and cerebral aqueducts (189, 190). It is also required for cardiac damage (131).

CONCLUDING REMARKS

The ability to invade and cause pathology in such varied organ systems while also avoiding killing by the immune system makes *Streptococcus pneumoniae* a highly successful

pathogen. Asymptomatic colonization allows the pneumococcus to persist in the population and extensive serotype diversity complicates the development of effective vaccines. The successful invasion strategy of ChoP/PAFr and CbpA/LR shared by a number of major respiratory pathogens coupled with cytotoxic pneumolysin makes the pneumococcus a prototypic pathogen to study host pathogen interactions.

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Figure 1. Immunohistochemical and schematic depiction of the choline biology of the pneumococcal surface. Immunogold labelling of pneumococci with A) TEPC-15 antibody recognizing free choline and B) anti-autolysin antibody. These two images contrast free (A) versus CBP-bound (B) choline. C) Schematic view of the capsule (blue), cell wall (green) and membrane (red). The teichoic and lipoteichoic acids are indicated as dark blue lines bearing choline (circles). A proportion of these are capped by choline binding proteins. Courtesy of Dr. K.G. Murti, St Jude Electron Microscopy Core Facility.

Figure 2.

Schematic depiction of the spread and progression of *Streptococcus pneumoniae* infection. Carriage of the pneumococcus occurs in the nasopharynx and is usually asymptomatic in healthy individuals. The bacteria is spread by aerosol from the nasopharynx of carriers. The pneumococcus can spread from the nasopharynx to a number of different tissues. In children the bacteria most commonly causes otitis media. Invasive diseases generally start in the lungs and spread to the blood with the most serious complication being meningitis. The switch from asymptomatic colonization to invasive disease in healthy individuals usually occurs when there is a disruption in the innate immune defenses. (This figure contains some artwork produced by Servier Medical Art, (<http://smart.servier.com/>) under creative commons license 3.0).

Figure 3.

Schematic representation of the pneumococcus hijacking the pIgR/IgA system to cross the mucosal epithelia into the blood. A) Mucosal epithelial cells transport IgA (Black) from the basolateral to the apical surface using the receptor pIgR (Green), this receptor is then endocytosed and recycled back to the basolateral surface to transport more IgA. B) To protect itself from IgA the pneumococcus produces the protease sIgA1 (yellow) which cleaves the host IgA into Fab fragments. C) The choline binding protein, CbpA (Red), is able to bind to the empty pIgR and shuttle the pneumococcus from the apical side to the basolateral side of the epithelial cells. (This figure contains some artwork produced by Servier Medical Art, (<http://smart.servier.com/>) under creative commons license 3.0).

Figure 4.

Structure of the pneumococcal cell wall and its relationship to inflammation. (A) Penicillin induces cell wall degradation by the autolysin releasing cell wall fragments such as lipoteichoic acid, glycan polymers with and without teichoic acid, and small stem peptides. All teichoicated species contain phosphorylcholine (ChoP), a key component increasing inflammatory activity. All of these components interact with a variety of human cells, which in turn produce inflammatory mediators. Particularly important in this response is the platelet activating factor (PAFr). These mediators combine to produce the symptomatology of pneumococcal infection including changes in blood flow, fluid balance in the tissue, and leukocytosis. Glc: glucose; TDH: trideoxyhexose; NAcGaln: N-acetylgalactosamine; Galn:

galactosamine; L-Ala: L-alanine; D-Glu: D-glucose; L-Lys: L-lysine. TNF: tumor necrosis factor; NO: nitric oxide; PGE2: prostaglandin E2; IC pressure: intracranial pressure.

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Figure 5.

Domain structure of pneumolysin. Pneumolysin has three functionally separate domains: one activating complement, one causing hemolysis, and the other binding to cholesterol. Site specific mutations alter these properties individually (191, 192).