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REVIEW

Subversion of host immune responses by otopathogens during otitis media

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Abstract
Otitis media (OM) is one of the most common ear diseases affecting humans. Children are at greater risk and suffer most frequently from OM, which can cause serious deterioration in the quality of life. OM is generally classified into two main types: acute and chronic OM (AOM and COM). AOM is characterized by tympanic membrane swelling or otorrhea and is accompanied by signs or symptoms of ear infection. In COM, there is a tympanic membrane perforation and purulent discharge. The most common pathogens that cause AOM are Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis whereas Pseudomonas aeruginosa and Staphylococcus aureus are commonly associated with COM. Innate and adaptive immune responses provide protection against OM. However, pathogens employ a wide arsenal of weapons to evade potent immune responses and these mechanisms likely contribute to AOM and COM. Immunologic evasion is multifactorial, and involves damage to host mucociliary tract, genetic polymorphisms within otopathogens, the number and variety of different otopathogens in the nasopharynx as well as the interaction between the host’s innate and adaptive immune responses. Otopathogens utilize host mucin production, phase variation, biofilm production, glycans, as well as neutrophil and eosinophil extracellular traps to induce OM. The objective of this review article is to discuss our current understanding about the mechanisms through which otopathogens escape host immunity to induce OM. A better knowledge about the molecular mechanisms leading to subversion of host immune responses will provide novel clues to develop effective treatment modalities for OM.

KEYWORDS
Otitis media, immune responses, otopathogens, chronic suppurative otitis media, Pseudomonas aeruginosa, defensins, phase variation

1 INTRODUCTION

Otitis media (OM) is a serious healthcare problem in both developed and developing countries. OM refers to the inflammation of the area behind the tympanic membrane called the middle ear (Fig. 1A). The inflammatory mediators generated during OM can penetrate from middle to inner ear potentially leading to hearing loss (Fig. 1A). OM-mediated hearing loss can have serious consequences especially during childhood, including delayed language development and impaired communication. OM accounts for more than 25 million visits to physician’s offices and is associated with significant healthcare costs. OM can be broadly classified into acute and chronic types. Acute otitis media (AOM) refers to any type of swelling of the tympanic membrane or otorrhea that is not attributable to otitis externa (Fig. 1B). Typically, the presentation includes inflammation of the middle ear or symptoms of infection (Fig. 1B). The most common pathogens associated with AOM include Streptococcus pneumoniae, nontypeable Haemophilus influenzae (NTHi), and Moraxella catarrhalis. In some cases, despite antimicrobial therapy, AOM progresses to chronic OM (COM). The most frequent type of COM is chronic suppurative OM (CSOM), characterized by tympanic membrane perforation and purulent discharge (Fig. 1B).

Host immune responses can play a crucial role in providing protection against infections including OM. Broadly, the two general types of immune defense mechanisms are innate and adaptive immune
FIGURE 1  Schematic representation of human ear, different types of otitis media (OM), and mucin production. A) The ear is composed of three main sections: outer, middle and inner. OM refers to inflammation and/or infection of the middle ear composed of stapes, incus, and malleus as well as lined by mucosal epithelium. B) OM presentation: 1) Under normal physiologic conditions, the middle ear is clear without effusion and intact tympanic membrane; 2) However, there exists mucoid effusion and inflammation of Eustachian tube during acute OM (AOM); 3) In chronic suppurative OM (CSOM), there is perforation of tympanic membrane and purulent discharge (adapted from Bhutta et al., [148]). C) The middle ear is lined with ciliated and secretory cells, covered with a thin layer of mucus (adapted from Bhutta et al., [148])
responses. Each are composed of unique but interrelated cellular and secretory components. In addition, mechanical, chemical, and microbiologic barriers, including mucin production in the middle ear (Fig. 1C), provide protection against invading pathogens. If an otopathogen manages to cross the epithelial barrier, it is then subject to recognition by neutrophils and macrophages. At times, however, the innate immune system may not be able to clear otopathogens and hence it may require lymphocytes and the adaptive immune system to halt invasion. The lymphocytes of the adaptive immune system give a more versatile defense that can provide lasting protection against subsequent reinfection from the same pathogen. The aim of this review article is to summarize the immune responses present in the middle ear and how otopathogens evade these responses to induce OM.

2 | MECHANICAL, CHEMICAL, AND MICROBIOLOGIC BARRIERS

2.1 | Eustachian tube epithelium

The Eustachian tube epithelium is versatile in its function as a mechanical, chemical, and microbiologic barrier to infection. Beyond the epithelium’s basic function as a physical barrier by virtue of tight junctions, epithelial cells combine the mechanical action of mucociliary transport with the production of other chemical barriers. Some epithelial cells function to produce lysozyme, and this combines with mucoid and serous mucus from adjacent goblet cells. This mixture is antimicrobial in nature and prevents epithelial cell invasion by otopathogens. The mucociliary transport apparatus traps glycoproteins and mucus, ultimately propelling the fluid downward through the Eustachian tube and into the nasopharynx. Other innate defense mechanisms that prevent bacterial and viral pathogenicity on the epithelial surface can be described in terms of their secretory and nonsecretory nature. Importantly, secretory cells create mucins, lactotransferrin, lysozyme, defensins, and surfactants. These secretions foster a balanced, clean environment in the middle ear through both chemical and microbiologic mechanisms of action.7–9 Hence, both flow and antimicrobial proteins provide protection against potential invasive otopathogens.10 The middle ear also maintains inherently protective constituents including defensins, SPLUNC1, mucin, lysozymes, and TLRs.9

2.2 | Defensins

Defensins are members of a subset of antimicrobial peptides (AMPs). Defensins within the middle ear play a crucial role in nonenzymatic inhibition of bacteria, fungi, protozoa, and viruses.11 Additional associations of defensins include the inhibition of bacterial toxins, proinflammatory activity that stimulates cytokine and chemokine production, as well as the creation of pores on the membranes of pathogens.11 Three different types of defensins have been discovered, including α-, β-, and θ-defensins. Humans only express α- and β-defensins.11

α-Defensins are expressed in granulocytes and intestinal Paneth cells.12 When first discovered, α-defensins were named after the former function, that is, human neutrophil peptide (HNP). Six human α-defensins have been identified; they are abbreviated as HNP1, HNP2, HNP3, HNP4, HDS, and HD6. HNPs 1–4 are expressed in granules of neutrophils, entailing an importance in immune reactions associated with OM inflammation.13 The most potent of the α-defensins is HNP1, which appears to have activity against several strains of phagocytized NTHi.14

Human β-defensins (HBDs) are expressed within epithelial cells of various organs, including middle ear making them perhaps more intrinsically related to the Eustachian tube and OM. Although 11 HBDs have been discovered, only HBDs 1–4 have been extensively studied. While defensins have not been studied in large patient samples, in vitro and in vivo studies have demonstrated their induction in association with bacterial infections of the middle ear. Mouse β-defensins (mBD) 2, 3, and 4 were first noted to be up-regulated in the tubotympanums in experimental OM.15 Of the HBDs, HBD2 is most extensively demonstrated to play a role in middle ear host immune defense. HBD1 and HBD2 are AMPs that are capable of killing NTHi, S. pneumoniae, and M. catarrhalis.16 Indeed, the presence of such bacteria within the middle ear is a primary source of HBD2 up-regulation. Increased levels of HBD2 have also been associated with the increased production of cytokines.17,18 NTHi can trigger the up-regulation of IL-1α that can act synergistically with bacteria to enhance the expression of HBD2 in middle ear epithelial cells through the p38 MAP kinase pathway.18 The increased expression of HBD-2, both at the mRNA and protein levels, has been demonstrated in inflamed middle ear mucosa from OM patients in comparison to that obtained from normal subjects.19 In addition, the proinflammatory cytokine, IL-1α, up-regulates HBD-2 expression via the activation of an Src-dependent Raf-MEK1/2-ERK signaling pathway in human middle ear epithelial cell line.19 HBD2 has also shown to be up-regulated by other proinflammatory mediators such TNF-α, and LPS.20 Finally, the recombinant human β-defensin 3 (rhBD-3) has been observed as an integral part in NTHi eradication that can be compromised through biofilm formation within the middle ear.20

2.3 | Surfactant and other proteins

In addition to the defensins, surfactant proteins have been found to halt infection, albeit by different mechanisms. Some of these functions include opsonization, aggregation, and phagocytosis. One of the surfactant proteins is SPLUNC1, which is a constituent of liquid covering middle ear mucosal surfaces that delivers both mechanical and antimicrobial effects against invading pathogens.21 SPLUNC1 demonstrates broad-spectrum antimicrobial activity, while also preventing biofilm formation by organisms such as P. aeruginosa.22–24 Further, as a surfactant, SPLUNC1 reduces surface tension within the upper airway and the Eustachian tube. Within a chinchilla model, however, inhibition of SPLUNC1 did not alter NTHi proliferation, while still leading to dysfunction of the Eustachian tube. It is hypothesized this dysfunction occurred as a result of decreased mucociliary clearance through the diminished antimicrobial action.

Similarly, mucins aid in the propagation of mucus cells, which creates a protective barrier. However, overproduction of mucin can
result in delayed clearance of OM pathogens, which underscores the importance of homeostasis of the middle ear.25 Lysoyzymes have a slightly different defensive role. Their primary function is to destroy the bacteria’s peptidoglycan cell wall.26 Lysoyzymes, target bacteria by cleaving peptidoglycans at their polysaccharide backbone. As an essential piece of the bacterial cell wall, glycans are frequently targeted by the host immune system. Lastly, TLRs, which can be found on the surface of many epithelial cells, provide specific immunity against pathogen-associated molecular patterns (PAMPs). PAMPs are typically unique to individual microbes.10,27,28 As previously mentioned, should any of the potential invaders traverse through the epithelium, leukocytes, act the first line of defense after the epithelium.

3 | INNATE DEFENSES OF THE MIDDLE EAR

Besides mechanical, chemical, and microbiologic barriers, innate immunity plays a crucial role in providing protection against pathogens. Neutrophils and macrophages are an integral component of innate immunity in the middle ear and help in killing otopathogens.9 Other cells that function as part of the initial response to infection or to foreign bodies within the middle ear include fibroblasts, mast cells, and NK cells.9

Overall, the innate immune system is responsible for generating the initial protective responses by triggering TLRs leading to the production of cytokines in response to invasion of the middle ear by pathogens.29–31 The up-regulation of these cytokines can result in multiple outcomes including eradication of the infection, or can also lead to chronic otitis media (COM), which is often seen in organisms such as Pseudomonas aeruginosa and Staphylococcus aureus.22,23 Such organisms create biofilms, which paradoxically increase host inflammation and cytokine production that can result in COM.34 Specific cytokines that have been associated with COM include IL-8, TNF, IL-1β, IL-6, and IFN-γ.25,30 The inflammatory response is beneficial to a defending host because it up-regulates lymph flow, and this transports increasing quantities of antigen to the lymphoid tissue. As the antigen is transported to lymphoid tissue, it can be taken by dendritic cells that can activate adaptive immune responses.

4 | ADAPTIVE AND OTHER DEFENSES OF THE MIDDLE EAR

The activation of adaptive immune responses facilitates in clearance of pathogens from the middle ear.37 The nasopharyngeal tonsils and adenoids play an important inductive role as components of the mucosa-associated lymphoid tissue (MALT). These areas display similarities with lymph nodes because of their role in activating an adaptive response. An important implication of these adaptive immune system sites is that they can affect both the systemic and mucosal realms of adaptive immunity, making them a particularly versatile and specific defensive barrier for invasion against incoming viruses and bacteria.

4.1 | Antibody production

The primary defensive mechanism of mucosal secretions is the immunoglobulin IgA.38 IgA has demonstrated efficacy in protection against Streptococcus in human buccal epithelial cells as well as Escherichia coli in the urinary tract. Additionally, researchers have investigated IgA’s influence in combating adherence of S. pneumoniae and H. influenzae to the nasopharynx as this specifically applies to OM. IgA in the nasopharynx has been found to be protective against OM pathogens by inhibiting adherence of bacteria to the epithelial cells. This also explains why such barriers are less effective in patients with IgA deficiency, making these individuals more prone to OM infections.38

4.2 | Protection via lymphocytes

T and B cells can help in providing protection against infection. At the onset of an infection, naïve CD4+ T cells create memory CD4+ T cells.39–41 CD4+ T cells can further be subdivided into the cytokines they respond to and produce. CD4+ T cells that secrete IFN-γ are Th1 cells, and those that release IL-4 are Th2 cells.42 In children, CD4+ T cells have been demonstrated to reduce in the inhabitance of the nasopharynx by S. pneumoniae and H. influenzae.43–46 In terms of B cell response, the primary antibodies found in the middle ear are IgA and IgG.10 Children with fewer memory B cells have been found to be more likely to develop OM, further demonstrating the importance of B cells in immunologic protection.47

4.3 | Other mechanisms

Besides immunologic responses, the ear has additional protection mechanisms against microbial colonization such as defensins and other biomolecules. Commensal organisms can also provide protection against ear infections. It has been observed that although some commensal organisms, which function to preserve the synchronous organism, can perpetuate OM, others may even be protective against contracting post-upper respiratory tract infection (URTI) OM.48 For example, S. aureus combats otopathogens by preventing nasopharyngeal colonization and thus may inhibit the development of OM. Similarly, Sphingobium may be protective against OM whereas Bifidobacterium has limited influence on URTI or OM.48

5 | IMMUNOLOGIC EVASION

Despite the presence of potential antimicrobial defense mechanisms, otopathogens can cause OM. Pathogens employ a wide arsenal of weapons to evade potent immune responses and induce OM. Examples of such evasion mechanisms include coexisting viral infections resulting in a negative pressure buildup in the Eustachian tube, exaggerated cytokine production leading to increased inflammation, and a build-up of mucin, which decreases mucociliary clearance and hence results in OM infections that are less likely to be cleared (Table 1).49,50 Bacteria are even able to manipulate their gene expression through phase variation and interact with other pathogens, increasing their virulence and
**TABLE 1** A summary of evasion strategies employed by otopathogens to subvert host immune responses

| Evasion strategy                              | Mechanism                                                                 | References                                                                 |
|----------------------------------------------|---------------------------------------------------------------------------|----------------------------------------------------------------------------|
| **Viral Infection and Eustachian tube dysfunction** | Viral infection can serve as a catalyst for inflammation in the Eustachian tube. Viral inflammation prevents the normal function of mucociliary flow and lysozyme proteins that typically eliminate bacteria. The result is dysfunctional negative pressure in the middle ear, enabling previously colonized organisms to evade normal defenses. | Avadhani et al., 2006, Pittet, Hall-Stoodley, Rutkowski, 2010                |
| **Cytokines**                                | Increased viruses and bacteria in the middle ear are associated with inflammatory mediators such as histamine, leukotriene B4, and IL-8, all of which prevent the efficacy of antibiotics. Additional cytokines such as IL-2, IL-10, TGF-β, IL-4, IL-5, CCL3, and G-CSF released by viruses cause tissue damage and subsequent bacterial infection. | Avadhani et al., 2006, Bakaletz, 2010, Canafax et al., 1998, Chonmaitree et al., 1994, Chonmaitree et al., 1996, Josset et al., 1994, Smirnova, Birchall, and Pearson, 2004 |
| **Mucin**                                    | Mucus is the initial barrier in the middle ear for protection from viruses and bacteria. Mucins form the mucus layer, saturate the cilia, and facilitate the mucociliary transportation clearance system. However, exaggerated mucin lead to bacterial retention and hampers mucociliary clearance. Specific mucins (MUC2, MUC5AC, and MUC5B) have been correlated with the pathogenesis of OM and evasion of immune responses by otopathogens. | Precaiaido et al., 2010, Samuels et al., 2017                                |
| **Phase variation**                          | Some bacterial pathogens are also able to employ phase variation to regulate gene expression and evade host immune responses. NTHi phase variation creates a rearrangement of glycosyltransferase genes, allele on/off switching of N6-adenine DNA methyltransferase (ModA), and manipulation of the polythymidine (poly-T) tract in the hia promoter. Phase variation enables pathogens to modify their genetic makeup to both obtain nutrients from their environment, and to resist oxidative stress from the host immune system. Phase variation also affects biofilm formation. | Apicella et al., 2018, Borrelli et al., 1999, Brookman et al., 2016, Wren et al., 2014 |
| **Polymicrobial infections**                 | OM commonly infects the middle ear after a viral URI, resulting in diminished antibiotic response and penetration. Viruses also create a more viscous mucous, in addition to releasing cytokines that prolong the course of OM. | Giebink, 1989, Canafax et al., 1998, Chonmaitree et al., 1996, Bakaletz, 2010 |
| **Biofilms**                                 | Biofilms found on bacteria give pathogens increased resistance to being cleared, hence leading to chronic OM. Pathogens such as S. pneumoniae and H. influenzae have biofilms that enable avoidance of complement immunity and phagocytosis. | Pang and Swords, 2017, Andre et al., 2017, Cuevas et al., 2017, Das et al., 2017, Domenec et al., 2013, Martí et al., 2017, Tikhomirova and Kidd, 2013 |
| **Glycans**                                  | Glycans have unique evasion mechanisms. Not only do they prevent complement activation, but they also apply molecular mimicry and commensal interactions to evade host cell detection. | Comstock and Kasper, 2006                                                    |
| **Neutrophils**                              | Neutrophils are the first line of host defense against infections and form “neutrophil extracellular traps” (NETs). NETs and fibrin, which are often the primary mode of defense, are inhibited from being released by respiratory pathogens, such as S. pneumoniae and S. aureus. NETs can also contribute to extracellular DNA (eDNA) that can promote biofilm formation and subversion of host immune responses. NETs can also contribute to thicker effusion. | Schachern et al., 2017, Val et al., 2016                                   |
| **Eosinophils**                              | Eosinophilic extracellular traps (ETs) are more often seen with eosinophilic OM. Their function is to release eosinophilic granules and DNA traps to destroy pathogens. ETs can also contribute to thicker effusion. | Hurst and Venge, 2000, Ueki et al., 2016, Ueki et al., 2017                 |

5.1 **Viral infections and evasion through Eustachian tube dysfunction**

It is estimated that 94% of AOM cases are preceded by either the "common cold" or another URTI.54 In order of importance, such viral infections include: respiratory syncytial virus (RSV), rhinovirus, adenovirus, coronavirus, bocavirus, influenza virus, parainfluenza virus, enterovirus, and human metapneumovirus.10,55 Typically, children have asymptomatic bacterial colonization of the nasopharynx. However, a viral infection can initiate inflammation within the Eustachian tube. Although the epithelium’s normal mucociliary flow and lysozyme proteins readily eliminate bacteria under normal circumstances, this host protection can be stymied by new virally induced inflammation. This inflammation of the Eustachian tube precipitates a dysfunctional negative pressure within the middle ear that allows previously colonized organisms to evade the epithelium’s normal defenses. This negative pressure often occurs with greater severity in decreasing the chances of host clearance.51–53 Immunologic evasion is multifactorial, and often depends on damage to the host mucociliary tract, genetic regulatory changes within otopathogens, the number as well as a variety of different otopathogens that have colonized the nasopharynx, and finally, the delicate interplay between the host’s adaptive and innate immune response (Table 1).
children less than 24 months old in comparison to children from 25 to 47 months of age.

5.2 | Up-regulation of cytokines by otopathogens

Although cytokine production is often explained as a directed action by a host immune system, several examples suggest that the secretion of chemokines and cytokines can be manipulated by otopathogens for enhanced survival. For example, viral infections are associated with Eustachian tube dysfunction, which is at least partially due to their up-regulation of cytokine production and through their mediation of the inflammatory response. More recently it has been hypothesized that inflammatory mediators could facilitate bacterial adherence and colonization. With rising levels of inflammation, a number of epithelial cell surface antigens increase, and many of these are known to serve as sites for bacterial receptors.49,56

Increased levels of live viruses and bacteria in the middle ear are associated with mediators of the inflammatory response, including histamine, leukotriene B4 and IL-8, all of which are known to hinder the delivery and ultimate penetration of antibiotics.57–60 The cytokines such as IL-2, IL-10, TGF-β, IL-4, IL-5, and G-CSF induced by viral infections can cause tissue damage making a fertile ground for bacterial infection.42

Although many of these cytokines appear to be induced by viral infections, and potentially later manipulated by bacterial pathogens, this is not always the case. CCL3, for example, is one cytokine that is known to be a potent OM inflammation effector and it also appears to have a predominantly protective role in mice models. CCL3 knockout mice have been shown to have higher pathogenic colonization rates and a defect in host macrophages, resulting in reduced ability of the host to combat P. aeruginosa. Such knowledge of the pervasive role of CCL3 could help in developing therapeutic strategies to treat persistent OM infections.62

5.3 | Mucin

Damage to the host mucociliary transport system of the middle ear is thought to be the initial event that facilitates evasion of Eustachian tube defenses by otopathogens. Mucus is the first barrier that protects the epithelium from viruses and bacteria. Secreted by goblet cells within the middle ear epithelium, mucins form a mucus layer that saturates the cilia and enable the mucociliary transportation clearance system of Eustachian tube (Fig. 1C). The production of mucins is normally limited to the orifice of the Eustachian tube and the areas immediately surrounding the area. The normal physiologic levels of mucin promote the clearance of microbes. However, exaggerated production of mucins leads to entrapment of bacteria preventing their clearance and enhances the retention of bacteria leading to OM. Specific mucins have also been correlated with various types of OM. For example, MUC5B has been found to be the most common mucin involved in COM.63 OM with effusion has demonstrated the presence of mucin 2 (MUC2), mucin 5AC (MUC5AC), and mucin 5B (MUC5B).64

When left untreated, AOM may progress to chronic OM with effusion (COME). Specifically, NTHI is a common bacterial pathogen that contributes to this pathology. Although researchers continue to investigate NTHI’s pathogenic mechanism, it is known that NTHI induces MUC5AC mucin transcription once the bacterial cell has been disturbed.65 The initial step required to stimulate MUC5AC transcription is the triggering of p38 mitogen-activated protein kinase. Conversely, a negative feedback mechanism exists in which phosphoinositide 3-kinase-Akt pathway leads to inactivation of NTHI-influenced MUC5AC transcription by communicating with p38 mitogen-activated protein kinase pathway.66 Ultimately, the activation of this pathway can lead to an overabundance of mucin, thereby contributing to conductive hearing loss in COME, decreased mucociliary clearance, increasing the bacterial retention in the middle ear and persistent infection.65 Further, it has recently been discovered that curcumin, the principal curcuminoid of turmeric (Curcuma longa), is an inhibitor of NTHI-associated MUC5AC production.66 The molecular mechanisms by which curcumin down-regulates MUC5AC transcription is through the manipulation of AP-1, a transcription factor in the MAPK pathway. Curcumin has the ability to impede NTHI-MUC5A expression by down-regulating MKK3/6 activation of p38 MAPK and by up-regulating MKP-1.66 Further studies are warranted to explore the therapeutic potential of curcumin for OM.

5.4 | Pathogen phase variation

Besides mucin, many bacterial pathogens are also able to use phase variation to regulate gene expression and evade potent host immune responses (Fig. 2), though researchers are still attempting to better elucidate this phenomenon.52,67–69 NTHI, in particular, has various forms of phase variation such as rearrangement and modification of glycosyltransferase genes, allele on/off switching of N6-adenine DNA methyltransferase (ModA), and manipulation of the polythymidine (poly-T) tract in the hia promoter.51,52,67,70 NTHI ModA allele phaseversion was studied in animal models that specifically investigated OM. It has been demonstrated that animals with the “on” modA2 phaseversion had a higher burden of disease.51–53 Tetranucleotide repeats that influence phase variation in lipo-oligosaccharide genes have also been studied. It was observed that an “on” to “off” switch of the oafA gene can provide an overall benefit in the middle ear.71

NTHI is also known for hia transcription in its promoter region, and Hia protein, which is another form of phase variation.70 As previously alluded to, hia is able to modify its poly-T tract. The overall objective of poly-T tract variation is to escape opsonophagocytic killing by the host immune system. It has been demonstrated that strains of NTHI with less hia expression are more successful at evasion of the host.70 Phase variation also allows species of pathogens to manipulate their genetic makeup to obtain nutrients from their environment as well as to resist oxidative stress from host immune system.52 Phase variation affects biofilm formation and can prolong its formation for a longer period of time leading to immune system evasion.52 Findings also suggest increased biofilm formation under alkaline conditions at pH of 9. Further, biofilms formed in alkaline environments have an associated increase in HMW adhesins in the modA2 ON phase version group.52 ModA2 ON is also associated with greater susceptibility to oxidative stress and less resistance to neutrophil-directed killing.72
**A.** Example of outer membrane protein that is phase variable

Simple sequence repeat\(_{(n)}\)

Gene  
Target expressed, antibody effective  
Protein  
Expression of **single** gene is altered  
Target not expressed, antibody ineffective

Simple sequence repeat\(_{(n\pm 1)}\)

STOP

**B.** Example of phasevarion genome wide changes

Simple sequence repeat\(_{(n)}\)

Gene  
Expression of **multiple** genes are altered  
Multiple targets expressed, antibodies effective

Simple sequence repeat\(_{(n\pm 1)}\)

STOP

Multiple genes

Multiple proteins

Multiple targets not expressed, antibodies ineffective or less effective

STOP

**FIGURE 2**  Phase variation in bacteria. **A)** The presence of simple sequence repeats (SSR) in outer membrane proteins of otropathogens leads to simple strand mispairing during genome replication. This causes alteration in DNA sequence and consequently “OFF/ON” expression of selected proteins. Due to unavailability of selected protein during “OFF” expression, antibody against the target is not able to recognize it leading to evasion of potent immune responses. **B)** Otropathogens can employ phasevariome genome variation leading to alteration of multiple genes and proteins. Antibodies are no longer able to recognize or bind with very low affinity to altered proteins leading to subversion of host immune responses.

Protection against oxidative stress is also seen in other OM pathogens, such as *S. pneumoniae* by manipulation of the thiol-specific antioxidant (TlpA/TSA), which is an additional example of host immune evasion.\(^{73}\)

Such phase variation can also be seen amongst other OM pathogens such as *M. cararrhalis*.\(^{74-80}\) Three DNA methyltransferase (ModM) alleles, modM1-3, have been found in *M. catarrhalis* that can affect pathogenesis and recovery.\(^{75}\) Phase variation in *S. pneumoniae* (specifically transparent [T] variants) demonstrate differences in host evasion based specifically on avoidance of complement-mediated destruction. It has been hypothesized that the transparent T variant specifically enables better adhesion to the environment of the nasopharynx.\(^{74,76,81,82}\) In human experimental models with tympanostomy tube placement, there was increased expression of NanA, HylA, and PspA in transparent T variants. T variants also had increased levels of NanA and HylA at baseline, suggesting increased virulence in these variants.\(^{77}\) These findings suggest that the T variant of the pneumococcal pathogen is responsible for the pathogenesis of OM.\(^{81}\)

### 5.5 Polymicrobial infections

In addition to phase variation, otropathogens take advantage of other existing infections to induce OM. This phenomenon typically involves
viral coinfections and often makes OM a polymicrobial disease. The most common viruses associated with OM are influenza virus, parainfluenza virus, rhinovirus, coronavirus, and RSV. Most frequently, OM occurs 2–5 day after a viral URI. This coinfection results in poor antibiotic response, which is hypothesized to be due to reduced penetration of the middle ear in virus-infected children. An additional theory is that the inflammatory mediators released by viruses result in delayed resolution of OM. Animal models have shown that polymicrobial infection additionally results in hypersecretion and increased viscosity of mucus. Similar findings have been seen among humans with influenza A infections. However, in humans, the biochemical quality of the secretions is modified as opposed to changes that occur simply in the quantity or viscosity of mucus. Changes in secretions in human mucosa are thought to be due to viral neuraminidase. Such polymicrobial interactions increase the virulence of pathogens while evading the host responses.

### 5.6 Biofilm formation

Biofilms provide an additional immunologic evasion mechanism. Beyond increasing bacterial adherence and pathogenicity, biofilms have been found to be integral in the pathogenesis of OM. Biofilms have been demonstrated to contribute to the inability of the host immune system to clear bacteria during COM. Specifically, biofilms created by *S. pneumoniae* and *H. influenzae* appear to help these pathogens to avoid complement immunity and phagocytosis. *S. pneumoniae* has many different protein variants (namely, PspA, PspC, and Phts e PLY) that provide protection against complement-mediated microbial killing. Such bacterial proteins interweave in a complex interplay of reactions to inhibit complement from adequately protecting the host. For example, PspA is a factor that appears to be helpful in halting CRP and C3 convertase formation. PspC also appears to inhibit C3 convertase though by different target molecules, including, FH, C4BP, and Vitronecin. Finally, the Pfy factor targets C1q, IgG, and L-Ficolin, which keeps the host complement system away from the bacterial cells. Virulence peptide 1 (vp1) perpetuates biofilm development in the middle ear. At the current time, vp1 is hypothesized to detect local amino acid levels. However, the precise molecular mechanisms through which vp1 influences biofilms and virulence are still not known.

Besides bacterial proteins, extracellular DNA (eDNA) and associated DNAbil have been hypothesized to be involved in biofilm architecture and structural integrity. eDNA itself is a structural component of the extracellular polymeric substance. The eDNA is stabilized by eDNA strands, forming a meshwork of crisscrossing strands. In *H. Influenzae*, isogenic mutants (ΔcomE) have demonstrated a decreased presence of eDNA and type IV pilus (Tfp) in silico. The nonisogenic mutant, however, demonstrated elevated levels of fractal structures, which appears to have a role in enabling biofilm nutrient exchange, and feedback mechanisms. Extracellular RNA also plays an important role in the initial steps of biofilm synthesis, though its overall function is less important. Overall, the biofilm synthesized by *NTHi* has been found to release extracellular DNA and a β-glucan.

In addition to eDNA, the other genetic association related to biofilm formation in NTHi involves the activation of lipooligosaccharides (LOS), which are located on the surface of bacteria. Phosphorylcholine (PCho) is found on some LOS and it has been found that PCho+ aids in stabilizing NTHi biofilms in animal models. *NTHi* pathogenicity can be further enhanced by mutations in *luxS*. This influences the quorum signaling pathway and can result in the development as well as the establishment of biofilms. Biofilms can be particularly pathogenic in the case of OM, as the infections are polymicrobial and can hence create synergistic reactions, increased growth of organisms, antimicrobial tolerance, increased virulence and persistence, as well as exaggerated levels of exopolysaccharide (EPS). Biofilms have been found to be associated with adenoid hypertrophy and middle ear effusion. Syntrophy may also occur, which supports the idea that the growth of one product increases the growth of another, contributing to infections with elevated pathogenicity. A specific example of this is a coinfection with *N. meningitidis* and *S. pneumoniae*. NanA is required for adherence of influenza as well as for the interplay of pathogenicity between the two organisms.

### 5.7 Glycans

Glycans are carbohydrate structures that are found on the surfaces of pathogen and host structures, and they are often involved in highly specific interactions. Bacteria present glycosylated molecules to their host. These molecules include polysaccharides, glycoproteins, LOS, and LPS. Pathogens utilize the glycans that are found on the surface of many host cells as sources of carbon, as bacterial toxin targets, and as locations of attachment and subsequent invasion. One such example is *H. influenzae*, which has been shown to obtain sialic acid from host cells in the inner ear of animal models. The host-acquired sialic acid is then used for catabolism and sialylation of the pathogen LOS. Sialic acid catabolism and the genes associated with this process (nanEK, nanA, siaA, nagBA) have been demonstrated to be integral to the pathogenicity of *H. influenzae*.

Changes in the bacterial capsular structure often underlie bacterial evasion of the host immune response. In some cases, these structures are inhibitory against immune functions of the host. Other mechanisms include bacteria that create surface glycosylation structures that mimic host glycans. In many cases this allows for subversion of host immune system recognition. This mechanism helps microbes avoid immune defenses such as macrophages recognition that, in particular, utilize glycan surfaces to recognize both gram-positive and gram-negative bacteria.

Capsular polysaccharides have also been observed to mimic glycan structures on host surfaces. A common mechanism that is illustrative of this concept involves variations of the group A Streptococcus (GAS) capsule. Hyaluronan (HA) is expressed on the GAS capsule. It has been observed that HA interacts with CD44 on host cells and that high molecular weight HA leads to decreased phagocytosis by macrophages. By comparison, low molecular weight HA leads to increased macrophage uptake.
Some bacteria alter protein glycosylation patterns and avoid cleavage by host proteases. NTHi appears to use glycosylation to protect its surface-exposed high molecular weight adhesin 1 (HMW1A).123,124 This action serves to attach HMW1A to the bacterial surface along with protecting it from degradation by the host.

NTHi peptidoglycans and an associated outer membrane protein (OMP) P6, serve as a ligand for TLR2.125 Likewise, LOS is a ligand for TLR2 and TLR4. As one might expect, polymorphisms associated with OMP P6 or LOS have been found to be associated with recurrent cases of OM.126 An important clinical implication of these findings is that patient groups with chronic middle ear infections such as CSOM appear to have lower baseline levels of protein and mRNA related to TLR2, TLR4, and TLR6.127

Commensal organisms often make use of glycan interactions with host cells, which provides for more long-standing avoidance techniques.112 Neisseria meningitidis, for example is a pathogen that is commensal in the nasopharynx of an estimated 5–10% of all humans.128,129 The N. meningitidis serogroup C conjugate (MCC) vaccine achieves protection by generating bactericidal antibodies that target the serogroup C capsule.130 Even with the use of the MCC vaccine, at least three N. meningitidis escape strains have been noted. The N. meningitidis resistance against the vaccine appears to be related to changes in capsular production. Resistance has been associated with the insertion sequence (IS), IS1301. IS1301 lies within the intergenic region of the sia and ctr operons. The addition of the IS results in increased capsular polysaccharide synthesis. Aside from the vaccine resistance that is attained through increased capsular production, the increased polysaccharide also has been observed to interfere with complement activation. Because sialic acid-containing capsules can interfere with complement cascade amplification, the alternative pathway is inhibited on the bacterial surface and consequent decreases of C3 and membrane attack complex have also been observed. Although modification of the glycans located on the surface of pathogens assists in the aforementioned evasion mechanisms, modification of host proteins has also been noted.

Bacteria can modify host glycans using enzymes such as glycosyltransferases and glycosidases.122 Of these enzymes, the most commonly studied are the neuraminidases. S. pneumoniae has been observed to produce at least 10 glycosidases, 3 of which are neuraminidases that are integral in nutrient acquisition and pathogenesis, for example, N- acetylmuraminyl lyase (NanA), sialidase B (NanB), and neuramidase C (NanC).113 S. pneumoniae uses these enzymes for critical aspects of colonization such as utilizing carbon sources on host mucosal surfaces and in generating biofilm architecture. S. pneumoniae has been associated with induction of infection secondary to influenza A. The influenza A neuraminidase activity on sialylated structures appears to facilitate the S. pneumoniae secondary infection.110

The methods by which glycans can be modified to evade the host immune system are diverse. Such mechanisms include the use of host carbohydrate structures as sources of carbon, targeting host glycans with pathogen toxins, using polysaccharides as sites of attachment or as an opportunity for mimicry of host structures.122 As molecular mimicry via glycan modification can impact either innate or adaptive immune function, investigation of these interactions could pave the way for the development of future therapies.

5.8 | Neutrophils and eosinophils

OM pathogens are also able to escape host defenses by manipulating the host’s innate immune system. Neutrophils are typically the first responders and form neutrophil extracellular traps (NETs) to kill pathogens. In AOM, animal models demonstrated that NETs and fibrin formation are initial host defenses in middle ear infections.131–133 However, a persistence of NETs and fibrin contribute to biofilm formation, and hence chronic disease and effusion.131 This can be attributed to the fact that NETs lead to a thicker effusion, which creates a more fertile ground for biofilm formation.132 The end result is an association between NETs, COM and middle ear effusions.133 Respiratory pathogens, such as S. pneumoniae and S. aureus appear to prevent the release of NETs, at least in part due to nuclease impeding antibacterial proteins and breaking down the NETs.134,135 Although NETs have been more extensively studied in pulmonary diseases as targets for therapy,136,137 their role in OM, especially in CSOM, is still not clear and warrants further studies.

In addition to NETs, eosinophil extracellular traps (ETs) have been implicated in the pathogenesis of OM. ETs are associated with eosinophilic OM (EOM) and appear to facilitate the release of eosinophilic granules as well as DNA traps to destroy pathogens.138–141 Eosinophils and mast cells, which both contribute to Th2 host cell immunity, are often found in COM infections.141 Eosinophils in the middle ear are activated by cytokines such as IL-5 and eotaxin. Eventually, eosinophils undergo extracellular trap cell death, also known as EToxis.140 EToxis involves cell death that results in the creation of extracellular traps (ETs), which play an important role in eosinophilic OM.140 The mechanism by which these eosinophilic traps promote evasion of immunity by otopathogens is through an increased amount of eosinophilic secretions, resulting in increased viscosity.128 This increased viscosity will hinder the clearance of otopathogens by the mucociliary system and will also prevent the penetration of antimicrobial compounds. Furthermore, the end product of EToxis is the expulsion of proteins, cytokines, chromatin, and lipid mediators, all of which are components of inflammation, perpetuating damage of surrounding tissue.129 These findings suggest that fibrin, NETs, and ETs could serve as a potential avenue for therapy against OM.131

6 | IMMUNE EVASION BY COM PATHOGENS SPECIFICALLY OTOPATHGENIC P. AERUGINOSA

The precise mechanisms underlying the pathogenesis of COM are still far from clear. There is a need to initiate research studies in this area. A few studies have started to decipher the molecular mechanisms that can lead to COM/CSOM. Otopathogenic P. aeruginosa has been demonstrated to activate the PKC pathway by phosphorylation of PKC-alpha leading to the invasion of human middle ear epithelial cells. The ability
of otopathogenic *P. aeruginosa* to activate PKC pathway is dependent on OprF expression. The results of this study demonstrate the crucial role that the PKC pathway and OprF expression plays in the pathogenesis of COM/CSOM. The PKC pathway is responsible for the activation of many subsequent signaling cascades that can further activate the expression of proinflammatory cytokines. Specifically, cytokines such as TNF-α and IL-1β can contribute to chronic inflammation. It has also been demonstrated that otopathogenic *P. aeruginosa* enters and multiplies inside human and mouse primary macrophages that is dependent on both microtubule and actin dependent processes. The colonization of macrophages by otopathogenic *P. aeruginosa* will lead to evasion of potent immune responses and may contribute to persistence of infection observed during CSOM. COM/CSOM is also associated with increased biofilm formation, which further explains why COM infections are so difficult to treat. However, it remains to be seen how COM pathogens such as *P. aeruginosa* and *S. aureus* manipulate host immunity to promote biofilm formation in the middle ear leading to subversion of immune responses and induced infection.

7 | HOST GENETIC VARIABILITY IN SUSCEPTIBILITY TO OM

APC tumor suppressor gene deletions have also been associated with COM. For example, COM can result in an abnormal skin growth composed of stratified squamous keratinized epithelium known clinically as cholesteatoma. Children with cholesteatomas have genetic associations with a deletion in the APC tumor suppressor gene, as well as variations in the connexin gap-junction genes, *GJB2* and *GJB6*. These studies highlight the need for understanding the role of genetics in predisposition to COM/CSOM and in the evasion of host immune responses.

8 | CONCLUSION AND FUTURE DIRECTIONS

OM, one of the most common childhood infections, is characterized by mucus overproduction and elevated levels of inflammation within the middle ear. Common pathogens such as *S. pneumoniae*, *H. influenzae* and *Moraxella*, are capable of inducing OM due to a complex interplay between pathogens and host immunity, ultimately leading to evasion of potent immune responses. A wide variety of mechanisms such as biofilm formation, phase variation, and glycans have been implicated in subversion of host immune responses by AOM pathogens. Biofilms are composed of extracellular substance that forms a protective layer, which shields the pathogens from the host immune system as well as the interventional therapies. Identification and characterization of proteins such as DNABII and Type IV pili (Tfp) has led to the development of antibodies that target such structures, which subsequently has demonstrated the destruction of biofilms that are protective to *H. influenzae*. Although targeting either of these proteins independently has subtle yet different effects, such strategies supports the idea that eradication of biofilm formation from an “outside-in” approach may assist in clearing both adherent and planktonic generations of NTHi.

Although destruction of the biofilm structure may facilitate pathogen vulnerability, pharmaceutical interventions targeting intracellular pathways have also demonstrated potential as a therapeutic strategy for OM. Understanding the role of host pathways such as p38 MAPK and MAPK phosphatase MKP-1 as well as mucin signaling cascades and how natural compounds such as curcumin is able to target these pathways may help in developing novel treatment modalities for OM. Further investigation of such therapies is warranted, particularly those focused on increasing local drug concentration and improving targeted delivery within the middle ear.

In addition to biofilm formation and host pathways, phase variation allows bacterial pathogens to regulate their genetic makeup to escape destruction from the host immune system. Research has shown that ModA phase variation specifically gives NTHi pathogens the ability to evade host immunity, increasing pathogenesis and virulence. Further research focused on ModA phase variation of NTHi may allow for genetic manipulation to prevent and treat such infections. NTHi also has adhesins (type IV fimbriae) and Hia that help protect it from the host immune system. Further investigation of these structures and how NTHi infects host middle ear could be useful to create a vaccine. This type of vaccine could be used to prevent future OM infections by synthesizing multiple adhesin types.

Glycans are an additional extracellular component of bacterial pathogens that are an important structural component in the cell wall. As such, glycans have unique membrane proteins that can even be acquired from the host cell. Due to the varied nature of bacterial glycans and the role they play in evasion of the host immune system, it is possible that future research may allow for the modification of glycans as defense mechanism against OM pathogens.

Although a number of studies have highlighted the molecular mechanisms involved in subversion of immune response during AOM, our knowledge regarding COM is still very limited. Despite the high prevalence of COM, especially CSOM in both developed and developing countries, it is still an underexplored research area. There is an urgent need to perform research studies determining the molecular mechanisms employed by COM/CSOM pathogens such as *P. aeruginosa* to cause middle ear infection. Understanding the role of host immune cells and NETs in the pathophysiology of COM/CSOM will provide novel insights into the pathogenesis of the disease. As with other aspects of immunologic host evasion, focusing research on integral mechanisms of host evasion and resistance is likely to accelerate the overall knowledge, prevention, and treatment of COM/CSOM. The availability of novel effective treatment modalities beyond antibiotic therapy will lead to improved quality of life of many OM patients and their families.

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