Basic Science Concepts in Otitis Media Pathophysiology and Immunity: Role of Mucins and Inflammation

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Part I: The Innate Immunity in Otitis Media

The first line of defense against pathogens entering in the middle ear is innate immunity. It plays very diverse and important roles:

- Creating a physical and chemical barrier to pathogens: cellular barriers that are the epithelial surfaces and mucus layers on the top of epithelia
- Identifying pathogens with nonspecific receptors or sensing molecules
- Producing factors to activate inflammation, called pro-inflammatory mediators as cytokines and chemokines, to attract inflammatory cells
- Activate the process of adaptive immunity response by recruiting cells and presenting them antigens
- Kill pathogens and clean them from the tissue.

Several pathogens were identified in the middle ear of patients suffering from OM: diverse bacteria and viruses. Sometimes both at the same time have been found in middle ear effusions (MEEs) and seem to help each other [1]. Against these invaders, cellular and molecular barriers, recognition molecules and receptors, inflammatory mediators, and inducible effectors of the epithelium constitute the innate immune mechanisms that protect the middle ear.

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The First Line of Defense of the Innate Immunity: Cellular and Humoral Barriers

The very first lines of defenses of the innate immunity are physical and functional barriers. They are the epithelium of the middle ear and eventually the layer of mucoid gel on the top of it to protect the cells of the epithelium from the invasion by pathogens. The middle ear cavity of healthy patients does not contain liquid. Contrary to the airways, this line of defense has to be activated in the middle ear.

The Middle Ear Epithelium

The epithelium of the middle ear is mostly a single layer of cubical squamous cells. Some patches of the middle ear epithelium, and especially close to the Eustachian tube, gradually change in a pseudostratified columnar epithelium similar to the mucociliary epithelium of respiratory epithelia [2]. This epithelium is constituted of basal cells, goblet cells producing mucins, and other cells that can be ciliated or not. On top of these patches, mucus is present and protects these regions of the epithelium from the infection. The ciliated cells ensure the movement of the mucus in direction to the Eustachian tube orifice where

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it is evacuated from the middle ear to the oral cavity.

Mucus glands, a normal feature of the Eustachian tube, can also be present in the middle ear of patients with OM. They constitute invaginations of the epithelium in the lamina propria, regions very rich in goblet cells that are able to produce large quantities of mucins. In healthy subjects that did not have prior disease of the middle ear, very few mucus glands are usually observed, whereas in subjects that had a history of OM events, these glands are more numerous [3]. This suggests that in the middle ear, mucus glands probably appear after several episodes of OM and then remain even after the disease is resolved. In patients with chronic suppurative otitis media (CSOM), the density of mucus glands is very high [4] and appears as a sequelae of CSOM [5]. Studies of the structure of mucus glands in the middle ear showed that the epithelium first invaginates at the location of high-density goblet cells and then different ramifications develop to lead to different structure types and sizes. Nevertheless, it was noticed that mucus glands of the middle ear can degenerate and lose their ability to produce mucins [3] likely because when OM resolves, the ear contains less factors sustaining inflammation and mucin production.

As explained before, the healthy middle ear epithelium contains few goblet cells that are concentrated in some patches of mucociliary epithelium mainly close to the Eustachian tube. But in the case of OM, the simple layer epithelium remodels into a pseudostratified epithelium. Several studies have demonstrated by histology techniques (Hematoxilyn and eosin staining on cuts of paraffin-embedded tissues) that the middle ear epithelium exhibits more secretory cells as well as ciliated cells in numerous parts of the middle ear epithelium [6–8]. Secretory cells are positive to periodic acid Schiff (PAS) staining detecting the presence of glycoconjugates, which are mainly in mucin proteins. Smirnova et al. [9] demonstrated that these mucins are secreted in the MEEs as they were PAS positive using a slot blot.

The Mucus in the Middle Ear: An Important Role for Mucins

OM is characterized by the presence of fluid in the middle ear cavity, called effusions that can be serous or mucous. Serous effusions do not contain mucins and are not viscous. On the contrary, mucous effusions are highly viscous and contain a high content of mucins [10]. An in vitro test of transportability of a bead under magnetic attraction also indicated that mucous effusions are less transportable than serous ones, suggesting that mucous effusions are difficult to clear in the middle ear [11]. Serous effusions contain proteins similar to the blood, so it is suggested that serous fluids are the result of a passive transudate of blood components in the middle ear due to a negative pressure in the middle ear likely because of the Eustachian blockade during inflammatory OM [12, 13]. Even if there are some conflicting findings from different research groups, serous effusions are believed to show better outcomes of the disease, whereas mucoid effusions are suggested to predict chronic otitis media with effusions (COME) [11, 14]. The study of Matkovic et al. [15] also contributed to this hypothesis as among 108 effusions collected, only 6% were mucoid for patients having OM diagnosed for less than 3 months, whereas 95% were mucoid for patients having the disease for more than 3 months. The large differences in medical outcomes and effusion and middle ear mucosa (MEM) characteristics prove that various cellular and molecular pathways act in the evolution of the disease. The production of large amounts of mucins necessitates the differentiation of goblet cells in the epithelium and the development of mucin glands. Indeed, the reabsorption of water in serous fluids and the concentration of their proteins are believed to participate to turning serous effusions into mucous ones [16]. Contrary to serous effusions, mucous effusion production necessitates the active process of producing mucins (exudates). But as a large number of proteins from the blood are also present in the mucous effusions (as albumin the predominant one), a passive diffusion of proteins and liquid is also probably implicated in the accumulation of mucous effusions.

Ion transport and water channels are also believed to play an important role in bringing water in the middle ear cavity and participate to serous and mucous effusion production. The healthy middle ear has to be kept without fluid contrary to the inner ear for a good transmission of the sound vibrations. Herman et al. [17] suggested the importance of water channels and ion transports. Experiments conducted in Mongolian gerbil's middle ear cells showed that the absorption of fluid in the middle ear was dependent on an osmotic gradient created by sodium and potassium adenylpyrophosphatase (ATPase)-dependent channels. The impairment of this ion flux has been shown in the lungs of rabbits in response to hydrogen peroxide that is produced during oxidative processes as well as hypoxia, a condition likely to appear in OM [18]. The aquaporins (AQP)1, 4, and 5, channels regulating the water homeostasis in cells, were detected in the Eustachian tube and MEM of rats as well as the epithelial sodium channels (ENaCs) [19]. In experimental OM in rats induced by Eustachian tube obstruction, ENaC and AQP were deregulated from 1 to 8 weeks after Eustachian tube obstruction, suggesting their implication in the water imbalance leading to fluid presence in the middle ear [20].

Effusions are composed of mucins but contain other proteins (antibacterial proteins, cytokines, etc.), lipids, deoxyribonucleic acid (DNA), and bacterial components [10, 14, 21], some of these substances being remains of dead bacteria and epithelial cells. Mucins, the major macromolecular component of epithelial mucus, are very high-molecular-weight proteins constituted of a backbone where numerous sugar side chains are added as a posttranslational modification (glycosylation with glycotransferases enzymes). These glycoconjugates are linked to the mucins in the Golgi and are then stored in secretory granules, waiting to have a signal to merge with the membrane and be released in the extracellular compartment [22]. Mucins are widely studied as their regulation is often a key determinant of diseases as cancer, lung diseases, and gastrointestinal diseases.

Mucins are classified by their protein backbone that is encoded by different mucin genes called MUC. MUC transcripts are big (until 15 kilo bases), so is the protein backbone, accounting for 15–50 % of mucin mass, and can contain 400 to more than 11,000 amino acids [22].The major posttranslational modification of mucins is O-glycosylations, consisting in O-glycans attached to tandem repeats rich in serine and threonine all along the backbone. N-glycosylation is also observed but in a lesser extent. More than 20 human MUC genes have been identified—about the same number in mice. Considering the size of their gene transcript and protein backbone, but also their many glycoconjugates, the analysis of the MUC proteins is difficult. In the respiratory tract, 12 MUC genes have been identified and less in the ear, probably because mucins are more studied in the airways. Studies of mucins in the MEM and MEEs seem to show that MUC5B is the predominant mucin in the ear of patients having OM, whereas healthy subjects show very low levels of mucin [6–8, 23, 24]. But other mucins have been detected, apparently in lower amounts, which are MUC5AC, MUC2, and MUC4 [7, 13, 24, 25]. MUC5B has been detected by transcript analysis and protein assay: Preciado et al. [23] detected MUC5B protein by mass spectrometry in MEEs from COME patients and Lin et al. [7] by immunohistochemistry on the mucosa of patients with mucoid OM, whereas non-inflamed mucosa did not react with either of the antibodies anti-MUC5B and anti-MUC4. It has been noticed that the Eustachian tube of mucoid OM patients had MUC5B, MUC4, and also MUC5AC and MUC1 glycoproteins [8]. From the same study, electron microscopy of secretions from COME patients showed the presence of chain-like polymeric mucin. Some studies have detected the presence of messenger ribonucleic acid (mRNA) transcripts of MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC7, MUC8, MUC9, MUC11, MUC13, MUC15, MUC16, MUC18, MUC19, and MUC20 [24–26], but gene expression does not always reflect the protein production and secretion. Indeed, Thornton et al. [27] showed that mucin gene expression in the airways was not always correlated to the presence

of the protein. Mucins can be secreted or attached to the cell membrane. Among the mucins detected in the middle ear, we can notice that MUC5B and MUC5AC are secreted mucins, whereas MUC1 and MUC4 and are membrane-tethered mucins [22].

The overproduction of mucins leads to mucoid effusions that are hard to clear by the middle ear. Efforts have been made to try to prevent mucin overproduction, but a recent article pointed to the necessary presence of MUC5B in the innate immune response of airways and the ear. A study directed by Dr. Christopher Evans showed that the knockout of *Muc5b* in mice had a fast and dramatic effect on the mortality and morbidity due to infection of the airways leading to systemic infection [28]. Histology of the lungs showed an overproduction of *Muc5ac* probably to compensate the lack of *Muc5b,* but failed to protect the airways from infection. The ears were also infected by different bacteria and contained liquid as well as signs of inflammation. Thus, *Muc5b* glycoprotein is needed in the airways and the ear to protect mice against bacterial invasion and shows its central role in the innate immunity.

Antimicrobial Molecules in Effusions

MEEs contain other molecules that participate to the defense against pathogens. Antibacterial proteins efficiently kill bacteria and are very important in the innate immunity mechanisms. Defensins are broad-spectrum antimicrobial peptides, small (30–45 amino acids), rich in cationic amino acids, and stabilized by disulfide bounds that protect them from proteases [29]. Defensins have antimicrobial properties towards bacteria and viruses, are able to inhibit some bacteria toxins [30], and have pro-inflammatory activities stimulating cytokine and chemokine production [31]. Surprisingly, defensins have not been studied in patient samples, but in vivo and in vitro studies of experimental OM showed their induction in response to bacterial infection of the middle ear [32, 33]. Human β-defensin 2 (HBD2) was studied in vitro in order to determine the molecular pathways implicated in its induction. In human middle ear epithelial cells (HMEEsCs), HBD2 is under the control of the pro-inflammatory cytokine interleukin (IL)1-β that activates Raf-MEK1/2 (mitogen-activated protein kinase kinase), the mitogen-activated protein kinase (MAPK) pathway [34]. *Non-typable Haemophilus influenzae (NTHi)* is also able to induce the expression of HBD2 first activating the toll-like receptor (TLR) 2 and then inducing protein 38 (p38) MAPK pathway [32]. HBD1 and HBD2 have also shown their ability to reduce *Streptococcus pneumonia (SP), Haemophilus influenzae (Hi),* and *Moraxella catarrhalis (MC)* growth in a liquid broth assay [35]. In Chinchilla, the orthologue of human β-defensin 3, chinchilla β-defensin 1, CBD1, had potent antimicrobial activity against *SP*, *Hi,* and *MC* [36]. Furthermore, chinchillas pretreated with recombinant CBD1 resulted in lower colonization of *NTHi* in the nasapharynx [37]. But bacteria are able to resist to defensins when they are growing in biofilms: Jones et al. [38] demonstrated that HBD3 binds to extracellular DNA constituting the matrix of *NTHi* biofilms, leading to the sequestration of HBD3 and thus diminishing the biological activity of an important defense of innate immunity.

Other antibacterial molecules are also part of the innate immune defense. Among them, the lysozyme is a cathelicidin (a cationic peptide) that has various effects, primarily damaging the membrane of bacteria. Lysozyme is present in MEEs of pediatric patients having OM, especially in mucous effusions compared to serous ones [12, 13, 39]. Giebink et al. [40] showed that the concentrations of lysozyme are more important in the MEEs of patients with COME positive for bacteria culture and suggested that this antibacterial agent was not only produced by polymorphonuclear leukocytes but also by the middle ear epithelium that accounted for 50–80 % of the lysozyme in the middle ear. Experimental OM in animals also demonstrated higher lysozyme detection in the middle ear: in response to *MC* in the Guiney pig [41] and in response to *SP* in chinchilla, this study also showed that more lysozyme were observed even when heat-killed bacteria were injected in the middle ear, suggesting that the production of lysozyme might be activated in response to membrane components of bacteria. Furthermore, mouse depleted of lyso-

zyme also showed a higher susceptibility to OM development after *SP* infection [42], underlining the importance of lysozyme in the innate immune defense against bacteria.

Finally, some other antibacterial molecules poorly studied seem to play a role in the middle ear defense to pathogens: surfactant proteins as short palate, lung, and nasal epithelium clone (SPLUNC)-1, small cationic peptides, halocidin, and xylitol [29, 43].

Recognition of Pathogens

The System of the Complement

The complement system is a biochemical cascade composed of several peptides normally present as inactive forms. This system can be activated by different sequential cascades of enzymatic reactions in which proteins are sequentially cleaved and activated. The resulting effector molecules are C3a and C5a, also called anaphylatoxins. They are the most potent activation products of the complement that are able to induce a large diversity of effects as bacterial cytotoxicity, induction of pro-inflammatory cytokines production, and inflammatory cell activation [44]. The activation of the complement system depends on three pathways. The classical pathway consists in the recognition of immunoglobulin IgG and IgM complexes formed around pathogens that activate the C1 complex, activating C4 molecules to induce the activation of C3 and C5. The alternative pathway is triggered by carbohydrates, lipids, and proteins found on pathogens: C3 mediates the activation of the cascade. And the lectin pathway recognizes sugars at the microbial surface and leads to the activation of C4, C3, and finally C5.

Mediators of the complement activation have been found in MEEs and the MEM. Recently, He et al. [45] analyzed molecules of the component in effusions of children with recurrent OM by the enzyme-linked immunosorbent assay (ELISA). High amounts of C3a, C5a, and sC5-b9 were detected in the MEEs of patients having OM for more than 6 weeks. The concentration of C5a was also strongly correlated to the concentration of IL-6 and IL-8 pro-inflammatory cytokines, suggesting a link between complement activation and the inflammatory effect they induce. Complement transcript induction was also observed in HMEEsCs in vitro in response to *SP* and *influenza A virus (IAV)*. Another study was conducted on effusions of patients with COME to assay the complement activation (C3a and C3 cleavage fragments) by ELISA and western blot analysis [46]. High concentrations of complement molecules were found and C3 activation was evaluated at 40 % of the total amount of C3 protein. They also noticed that C3a concentration was higher when effusions stayed longer in the ear and when children had multiple tube insertions, pointing C3a levels as a marker of the chronicity of OM. The complement activation leads to lysis of pathogens; this has been verified by Niarko-Markela and Meri [47] with erythrocytes of Guiney pigs exposed to MEEs from patients with otitis media with effusion (OME). Thirteen of the 38 MEEs tested had direct endogenous hemolytic activity, and 27 enhanced serum-initiated lysis. They also detected high levels of terminal complement complexes demonstrating the strong activation of the complement.

Receptors of the Innate Immunity

Multiple cell types and especially epithelial cells that are in contact with the external environment express innate immune receptors as Toll Like Receptors (TLRs). In the mucosal environment, mast cells and dendritic cells also express TLRs. The TLRs are pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs). The activation of TLRs leads to the production of molecules also implicated in the innate immune response, as chemokines, cytokines, interferons (IFNs), and antimicrobial molecules described before. TLRs are type I transmembrane receptors with an extracellular N-terminal region with leucin-rich repeats and an intracellular toll-IL-1 receptor (TIR) domain. TLRs can form homodimers or heterodimers. The homodimers of TLR4, TLR5, TLR11, and the heterodimers of TLR2-TLR1 or TLR2-TLR6 bind to their respective ligands at the cell surface,

whereas TLR3, TLR7-TLR8, TLR9, and TLR13 localize to the endosomes, where they sense microbial and host-derived nucleic acids. TLR4 localizes at both the plasma membrane and the endosomes. They each recognize specific types of PAMPs, for example, TLR1-TLR2 and TLR1-TLR6 recognize acylated peptides, TLR4 recognizes lipopolysaccharide (LPS), TLR3 targets double-stranded ribonucleic acid (RNA), and TLR9 recognizes bacterial DNA. TLR signaling is induced by their dimerization, dependent on ligand binding. All TLRs except TLR3 have a signaling pathway dependent on myeloid differentiation factor 88 (MyD88), activating the transcription factor NF-κB and then the expression of pro-inflammatory cytokines [48].

Several TLRs have been identified in the MEM and MEEs. TLR2, TLR4, TLR5, and TLR9 were found at the level of RNA and proteins in the MEM of both OM and non-OM patients [49]. For TLR2, TLR4, and TLR5, no difference of expression was found between non-OM and OM MEM, but their concentration was lower in the mucosa of patients with CSOM. In consequence, it was suggested that the clinical recovery of OM depends on TLR expression in the middle ear. There are conflicting evidences considering TLRs in MEEs and the correlation with the presence of bacteria. Lee et al. [50] observed effusions of patients with OME having lower TLR2, TLR6, and TLR9 mRNA when they are prone to have persistent OM and the level of TLRs is higher in culture-positive MEEs. Another study demonstrated the inverse for TLR9: less TLR9 mRNA is detected in culture-positive MEEs [51], whereas Lee et al. [52] failed to see any difference in TLR2, TLR4, TLR5, and TLR9 mRNA. Studying TLRs in MEEs might be accurate for assaying their presence in immune cells but does not take into account the epithelial cells playing an important role in the immune defense through TLRs. This might explain the differences described before. Nevertheless, animal studies showed that defects in TLR2 and TLR4 lead to the persistence of inflammation and mucosal metaplasia during OM [53].

Role of the Inflammation in OM

Inflammation is a central innate immune response activated by pro-inflammatory mediators (chemokines, cytokines) in order to attract and activate immune cells, stimulate the various innate immune defenses, and initiate the adaptive immune response to pathogens. This is a very efficient process involving different mediators and cells, but also deleterious if it does not resolve when the pathogens are no longer present. Inflammation is suspected to participate to the absence of resolution of OM especially in the case of COME and CSOM.

Pro-inflammatory Cytokines and Chemokines in OM

Pro-inflammatory cytokines and chemokines are characteristic of the inflammatory process: They are induced at early stages of the innate immune response until advanced stages to sustain the inflammation and to stimulate the adaptive immune response. Cytokines are usually associated to different types of immune response and can be produced by different cell types: epithelial cells, macrophages, neutrophils, dendritic cells, etc. Thus, cytokines that are known to play an important role in the innate immunity are tumor necrosis factor α (TNF-α), interleukins IL-1, IL-10, IL-12, IFNs, and chemokines like IL-8. The adaptive immunity is usually characterized by the cytokines IL-2, IL-4, IL-5, transforming growth factor β (TGF-β), IL-10, and IFN-γ production, TGF-β and IL-10 being able to repress inflammation. In addition, granulocyte-macrophage-colony-stimulating factor (GM-CSF) and granulocyte-colony-stimulating factor (G-CSF) are cytokines known to stimulate the differentiation of hematopoetic cells. Pro-inflammatory cytokines and chemokines have been detected many times in MEEs; the Table 7.1 summarizes some of the more recent studies assaying the content of cytokine protein in MEEs or MEM by ELISA or their transcripts by polymerase chain reaction (PCR) [15, 49, 50, 54–61]. Among the 11 studies listed, 12 different cytokines were detected in samples collected from children or adults with acute otitis media (AOM), OME, COME, and CSOM. Despite the fact that it is complicated to compare

Table 7.1 Pro-inflammatory mediator detection in middle ear effusions (MEEs) or middle ear mucosa (MEM) of patients with otitis media (OM)

Cytokine	Detected in	References
IL-8	36 MEEs, COME patients, 92%	[54]
–	108 MEEs, OM +/− 3 months, +	[15]
–	96 MEEs, OME, + (RNA)	[50]
–	46 MEEs, OME, +	[55]
IL-6	20 MEEs, AOM, +	[56]
–	96 MEEs, OME, + (RNA)	[50]
–	72 ears, MEM, COM/CSOM, + (RNA)	[49]
–	75 MEEs, OME persistent and/or recurrent, 83%	[57]
IL-12	96 MEEs, OME, + (RNA)	[50]
–	80 MEEs, OME adults, 100%	[58]
IL-1β	36 MEEs, COME patients, 67%	[54]
–	108 MEEs, OM +/− 3 months, +	[15]
–	30 MEEs children, 38 MEEs adults OM, +	[59]
–	72 ears, MEM, COM/CSOM, + (RNA)	[49]
–	75 MEEs, OME persistent and/or recurrent, 58%	[57]
IL-2	108 MEEs, OM +/− 3 months, +	[15]
–	80 MEEs, OME adults, 75%	[58]
IL-4	80 MEEs, OME adults, 41%	[58]
–	26 MEEs, OME, +	[60]
IL-5	80 MEEs, OME adults, 52%	[58]
–	26 MEEs, OME, +	[60]
TNF-α	36 MEEs, COME patients, 77%	[54]
–	108 MEEs, OM +/− 3 months, +	[15]
–	30 MEEs children, 38 MEEs adults OM, +	[59]
–	96 MEEs, OME, + (RNA)	[50]
–	72 ears, MEM, COM/CSOM, + (RNA)	[49]
–	75 MEEs, OME persistent and/or recurrent, 38%	[57]
IFN-δ	108 MEEs, OM +/− 3 months, +	[15]
–	96 MEEs, OME, + (RNA)	[50]
–	72 ears, MEM, COM/CSOM, + (RNA)	[49]
–	80 MEEs, OME adults, 83%	[58]
–	75 MEEs, OME persistent and/or recurrent, 51%	[57]
TGF-β	45 MEEs, adults, OME, +	[61]
IL-10	108 MEEs, OM +/− 3 months, +	[15]
–	96 MEEs, OME, + (RNA)	[50]
–	80 MEEs, OME adults, 18%	[58]
–	45 MEEs, adults, OME, +	[61]
TNF-β	36 MEEs, COME patients, 0%	[54]
–	108 MEEs, OM +/− 3 months, +	[15]

In the column "Detected in," the following information is given: number of MEEs or samples of MEM; type of OM detected; adult is specified—if nothing written, the samples come from children; % of samples positive for the analysis (+ means not specified in the study, assuming 100%)
OME otitis media with effusion, *AOM* acute otitis media, *COM* chronic otitis media, *COME* chronic otitis media with effusion, *CSOM* chronic suppurative otitis media, *RNA* ribonucleic acid

the quantity of cytokines in each study, it seems that IL-8, IL-6, IL-12, and IL-2 are detected in almost all the effusions. IL-1β, IL-4, IL-5, TNF-α, IFN-δ, TGF-β, and IL-10 were detected in less MEEs (40–80% for the studies detailing this parameter), and TNF-β showed conflicting evidences considering its presence. IL-8, IL-6, IL-12, IL-1β, TNF-α, IFN-δ, and IL-10 seem to be in

higher concentrations in culture-positive samples and CSOM that are usually characterized by the presence of a strong bacterial infection. IL-8 and IL-10 were detected in higher concentrations in mucoid effusions compared to serous ones.

MEEs contain a variety of cytokines acting both in promoting inflammation and regulating the adaptive immune response. Some of them are produced in very high content especially when the bacterial infection persists. The chronic stages of OM also show a diversity of cytokines in high concentration in the middle ear, suggesting a persistence of inflammation in absence of pathogens. The Eustachian tube obstruction due to inflammation and the low transportability of mucoid effusions might limit the efficiency of the clearance of killed pathogens, letting PAMPs in the middle ear that still stimulate the immune responses, so do the cytokines in mucoid fluids that might accumulate without the possibility of being cleared from the middle ear. Some defects in cytokine production, dependent on genetic and environmental influence, might also explain why children tend to be prone to recurrent and persistent OM. Cytokines exhibit strong effects that, if not balanced, can lead to a disproportionate immune response. These cytokines are produced by epithelial cells but also immune cells. They are granulocytes as neutrophils, basophils, and eosinophils and phagocytic cells as macrophages and dendritic cells, all detected in MEEs of patients with OME [60, 62–64].

Innate Immunity to Adaptive Immunity in OM: Activation of Lymphocytes

As described before, several immunoregulator cytokines are present in MEEs of patients, underlining the importance of the role of the adaptive system in OM. They can be divided in two groups: TH1 and TH2 (meaning lymphocyte T helper). They represent the ability of lymphocytes T to differentiate in TH1 type, inducing cell-mediated immunity and inflammation, or TH2 that mediates the humoral immunity through the production of antibodies by differentiated lymphocytes B. The different cytokines detected in the MEEs show the activation of both pathways. CD4+ T cells were detected in MEEs several times [9, 61], T cells that are naïve or differentiated. The lymphocyte subpopulation in MEEs was analyzed by flow cytometry in the study of Skotnicka et al. [65]. CD3+ T cells were dominating the population of lymphocytes, and the T helpers CD4+ were the majority. The ratio of CD4+/CD8+ cells was significantly higher in MEEs, but the proportion of CD8+ cells was lower in MEEs than in blood. These immune cells are suspected to come from adenoids in patients presenting this abnormality, as the population of lymphocytes of adenoids is important and similar to the middle ear [66]. Lymphocytes B have been identified in the middle ear as well. A study assayed the presence of lymphocytes in relation to the presence of antibodies against specific bacteria in 238 MEEs of patients with AOM [67]. The percentage of lymphocytes was higher in the ears with bacteria-specific antibodies than in the ears without, which correlated with a faster resolution of OM. The activation of the TH2-specific pathway inducing the differentiation of lymphocytes B to produce specific antibodies seem to also play an important role in the resolution of OM.

Part II: Molecular and Cellular Mechanisms Implicated in OM Pathogenesis

OM is a very common disease in children that sometimes evolves into chronic OM for reasons not yet understood. In order to prevent the evolution of the disease in a chronic stage that is difficult to treat, we need to understand the mechanisms implicated in OM development in response to bacteria, and how their interaction evolves into chronic OM. The innate immune system plays a central role in OM, so researchers investigated the different mechanisms implicated in its activation during the infection of the middle ear. In vivo and in vitro models were developed to better understand how the middle ear epithelium responds to bacteria, hoping to find new strategies to treat patients with OM.

In Vivo and In Vitro Models to Study OM Pathogenesis

Animal Models

Animals are useful models to investigate the cellular and molecular mechanisms implicated in OM. They permit to control the type of infection, the different stages of a disease, as well as the genetic background of the biological material. Comparing to in vitro studies, in vivo models allow taking into account the entire immune system and the interaction between different cell types being important in the resolution of infections. But we have to keep in mind that animal models have their limitations as their responses to pathogens might not be the same as the human ones, the differences being dependent on the species chosen. For the study of OM, rodents are widely used: chinchilla, mouse, rat, Guiney pig, and gerbil. According to the literature, mice and chinchillas are the main animals used in laboratories to study OM. Mouse is the first animal models used now as they are small, with a very controllable genetic background, easy to use in laboratories because a high diversity of reagents are compatible with this species. Nevertheless, mice have a very small middle ear, which is less convenient to induce OM by surgery as well as collecting MEEs. Chinchilla offers the possibility to have a bigger middle ear: the review of Ryan et al. [68] compared the middle ear volume observed in different studies. The average middle ear volume of the chinchilla is about 1.5 ml^3, whereas the one of a mouse is about 0.05 ml^3, so the middle ear volume of the chinchilla is 30 times bigger than the one of the mouse. It is consequently easier to manipulate the middle ear and recover MEEs that are sufficient in quantity to do several biological assays. The anatomy of the chinchilla ear has also been shown to be very close to the human one [69]; they do not often develop spontaneous OM [70], and they show similar responses to virus and bacteria in the course of OM compared to humans even if the pathogens colonizing humans are not usually the same as those of chinchillas (see [71]). Rats are also used in several studies and have the advantage of having a bigger middle ear and more availability of reagents than chinchillas. Several interesting studies used rats to do a time course analysis of OM development.

OM is often induced by experimental obstruction of the Eustachian tube, leading to a negative pressure in the middle ear [72, 73]. Infection by bacteria can be coupled to this procedure to mimic better human OM. Bacterial injection can be made through the tympanic membrane but damaging this membrane lets other contaminants the possibility to enter in the middle ear. Injection via the ventral bulla is preferred as it does not damage the tympanic membrane and avoids contaminations. But it necessitates skills in microsurgery to avoid damaging the vessels and airways around the bulla. Infections post surgery can occur and may modify the immune response in the middle ear. Considering these limitations, Stol et al. [74] developed a noninvasive murine model adapted from a previous rat model. They used a pressure cabin at 40 kPa which induced *pneumococci* translocation from the nasopharyngeal cavity to the middle ear; the maximum bacteria load appearing 96 h post infection with the bacteria. Inflammation was confirmed with the secretion of IL-1β and TNF-α in the middle ear. This model has the advantage to avoid the limitations due to the surgery but probably does not permit to have homogenous OM development between animals. Another disadvantage should be considered: other parts of the body might be affected by the difference of pressure, especially for medium- or long-term experiments. Finally, pressure cabins might not be easy to use, expensive to buy, and might not permit to expose enough animals at the same time.

Human pathogens are studied in animal models as relevant clinical strains. But they are evolutionary adapted to humans and usually not animals, which can bring bias in these experimental studies. Nevertheless, the effects observed in experimental OM induced in animals and especially mice are very close to the observations made in humans: OM induced in mice having different genetic backgrounds with different strains of *SP, Hi,* and *MC* have shown similar inflammatory and mucosal effects even if the duration of the

disease, the intensity of the responses, and the ability of resolution of OM where variable [68].

OM was also evaluated in mutant mouse strains; a strategy often used to investigate the implication of a specific gene in the apparition or the course of a disease. Mutations are natural or induced in laboratories. For the study of OM, we have to be careful choosing the type of mutations. Mutations in genes acting in the development of the middle ear might create some morphologic defects, influencing the responses of the middle ear. Mutating central genes in the immunity might also compromise the response to pathogens. And the deletion of a gene sharing similar functions with other genes sometimes leads to compensation mechanisms that may compensate the loss of functionality. Otherwise, this type of biological material offers great possibilities in studying spontaneous OM or pathogen-induced OM.

In Vitro Models

Transformed middle ear epithelial cells are now widely used to investigate the mechanisms implicated in bacteria effects, especially focusing on inflammatory and mucoid effects. This type of biological material permits to assay the effect of live bacteria, bacteria lysates, purified bacteria proteins, inflammatory mediators, etc., in a homogenous cell type which is useful but lacks the interaction with other cell types, especially the immune cells that produce mediators regulating epithelial cells. Knowing the limitations of cells in vitro, it is a very useful tool that gives us opportunities we cannot have with animals: the analysis of mechanisms implicated in a biological effect is more easy.

The human middle ear epithelial cell line HMEEC-1 was created by Dr. David Lim in 2002 using a retrovirus containing E6/E7 genes of human papillomavirus type 16 to transform primary middle ear epithelial cells from adults [75]. This type of transformation is known to regulate the cell cycle acting on the retinoblastoma (RB) tumor suppressor gene limiting the repression of the cell cycle and mediating the degradation of p53 protein also implicated in cell cycle repression [76, 77].

The mouse middle ear epithelial cell (mMEEsC) line was made by Dr. Jizhen Lin laboratory in 2005 [78]. Middle ear epithelial cells were isolated from mice and transformed by the large T-antigen of the simian virus 40 (SV40) A-gene. These cells have the property to be temperature sensitive: At 33 °C, the SV40 antigen is active and stimulates the cell cycle. But at 37/39 °C, SV40 is inactivated and cells differentiate, expressing markers of epithelial cells such as keratins and collagens. In our laboratory, we have noticed that these cells can be cultured several weeks at air liquid interface and form a single layer epithelium.

Other cell types were used: the middle ear cell line from chinchillas immortalized by SV40 [79] and the primary chinchilla middle ear epithelial cells (CMEEsCs) [80] or primary middle ear epithelial cells from adults successfully differentiated at air liquid interface in a ciliary and secretory epithelium [81]. Our laboratory tried to culture middle ear epithelial cells from children middle ear but because of the low amount of cells available during these procedures, we were unable to successfully grow them.

Interactions Between Pathogens and Ear Epithelial Cells

After having passed the eventual innate immune barriers in the middle ear, bacteria reach the middle ear epithelium. There, they adhere to the cells using adherence molecules varying depending on the bacteria. This part is focused on *NTHi* adhesion and invasion in airway and middle ear cells as *NTHi* is the main pathogen implicated in OM and as its interactions with epithelial cells has been widely studied.

NTHi is a gram-negative nonencapsulated bacterium that adheres and invades the middle ear. Several factors are necessary to its ability to invade epithelial cells. *NTHi* is able to secrete IgA proteases that increase its ability to adhere and invade the bronchial epithelial cells NCI-H292 [82]. Several factors produced by *NTHi* bind to host proteins: The protein F, a homolog of

SP lamin-binding proteins, is an adhesion factor that binds to the lamin of host cells [83]. Protein E has also been implicated in epithelial cell adhesion and the interaction with extracellular matrix proteins [84, 85]. Protein D, an outer membrane lipoprotein highly conserved, is important for *NTHi* adherence and is now used in *pneumococcal* polysaccharide conjugate vaccines that include monoacetylated protein D carriers, vaccines that showed their efficiency preventing OM development [86]. Finally, the phosphocholine (PCho) groups associated to the lipooligosaccharide of *NTHi* showed several times its implication in *NTHi* adherence as well as its ability to form biofilms [86–88]. PCho also present in *SP* was found to interact with the platelet-activating factor receptor (PAF receptor) as PAF present also PCho motifs recognized by this receptor [88]. In addition, the study of Van Schilfgaarde et al. [89] demonstrated that different clinical strains of *NTHi* elicited different patterns of adhesion, implying that some factors produced by specific strains might play a critical role in *NTHi* adhesion. They suggested that high molecular weight proteins are implicated in the virulence of the different clinical strains of *NTHi*.

NTHi has been detected on cells (adherence) and in cells (invasion). The presence of *NTHi* at the surface of epithelial cells was demonstrated by bacteria culture after infection [90], fluorescent microscopy techniques [89], and scanning electron microscopy, bacteria being mainly located on the top of non-ciliated cells [91]. Different molecular pathways in epithelial cells were found to play an important role in *NTHi* adhesion and invasion. The cytoskeleton with microtubules and actin were rearranged and necessary for *NTHi* virulence [90–92]. Macropinocytosis was demonstrated to be an important internalization mechanism of *NTHi* [91], and other studies found the implication of lipid rafts [92]. *NTHi* is also able to produce outer membrane vesicles that contain factors that will help the bacteria to invade hosts. These vesicles have a diameter of 20–200 nm and contain DNA, adhesins, and other enzymes [93]. These vesicles are internalized by caveolin-dependent mechanisms and elicit the production of immune proteins as IL-8 and the antibacterial protein LL-37, surprisingly enhancing *NTHi* invasion in epithelial cells. Thus, different mechanisms are implicated in *NTHi* internalization in epithelial cells and might be dependent on cell culture conditions and the *NTHi* strain used.

In the middle ear, bacteria are found planktonic or organized in biofilms [94, 95]. The growth of bacteria in biofilms gives them the ability to hide from the immune system of the host and resist to antibiotics due to the extracellular matrix the bacteria create around them [95]. Biofilms of main pathogens in OM were detected in the middle ear of patients: *SP, Hi,* and *MC* but also *Staphylococcus aureus* and *Staphylococcus epidermidis.* But even if these bacteria are known to resist to high antibiotic quantities, we do not know yet how they can invade human cells. It is possible that biofilms are a defense mechanism to protect bacteria from a hostile inflammatory environment, offering them a niche to wait that immune responses decrease in order to better infect the host.

Regulation of Mucin Production and Mucous Cell Metaplasia in OM: Role of Pro-inflammatory Mediators

Inflammation and effusion production are characteristic of OM. As explained before, clinical inflammation seems to appear at the early stages of OM development when the middle ear tries to fight the infection by bacteria and/or viruses until more chronic stages even if the bacteria count seems lower. But this is not the case for mucin production that is mainly observed at later stages of OM course. Serous effusions are suspected to mainly come from the transudation of liquid and proteins from the blood, whereas mucous effusions need the active process of mucin production. Mucins are produced by goblet cells of the middle ear epithelium and mucus glands, which are easily detected in the MEM of patients with OME and COME, but at very low levels in healthy middle ears. These observations suggest

that several factors play a role in the remodeling of the epithelium of the middle ear. This part of the chapter will try to review the different factors implicated in mucin production and mucous cell metaplasia, focusing on the inflammatory mediators that seem to play a crucial role in this process.

Infection of the Middle Ear by Bacteria Results in Pro-inflammatory Mediators Expression and Secretion In In Vivo And In Vitro Models

MEEs from patients with OM contain high concentrations of a panel of pro-inflammatory cytokines (see Sect. 3.3.1). In vivo studies were conducted to try to replicate the conditions of infection occurring in OM in order to analyze the expression and secretion of pro-inflammatory cytokines in the MEM and in MEEs mainly in mice, chinchillas, and rats (Table 7.1 and 7.2).

Several studies assayed the effect of live or killed *NTHi* or its purified endotoxins in the mouse middle ear (Table 7.2) [73, 96–98]. They used different protocols to induce OM (transtympanic injection of bacteria or bacterial components coupled to Eustachian tube obstruction in some cases), and all found similar pro-inflammatory cytokines to be induced in the middle ear at the level of RNAs or proteins (by PCR of the MEM or ELISA assay of MEEs). The cytokines TNF-α, IL-1α, IL-1β, IL-10, IL-6, MIP-2, KC, and IFN-δ were upregulated in response to *NTHi,* at various time points depending on the conditions of OM induction: from 6 h to 2 months post *NTHi* injection. Mac Arthur et al. [96] did a time course study of cytokine expression in the MEM. They found the mRNAs of *Mip-2, Il-6,* and *Kc* upregulated at all time points, but the fold inductions were higher at earlier times. Preciado et al. [97] confirmed this high early effect assaying *Mip-2* at day 1 and day 7 after *NTHi* lysates injection. *SP* was also used in other studies to induce OM. The different techniques used to induce *SP* infection (pressure cabin, intranasal exposure, or transtympanic injection) showed pro-inflammatory effects as well (IL-1α, IL-1β, TNF-α, MIP-2, IL-2, IL-6), effects occurring from 24 h to 15 days after *SP* exposure [74, 96, 99, 100]. Stol et al. [74] underlined the early effect on cytokine production in middle ear homogenates as IL-1β and TNF-α were induced at 48 and 96 h after nasopharyngeal infection by *SP* but not at 144 h. Endotoxins from *Salmonella Typhimurium* or LPS were used to induce OM in three studies by transtympanic inoculation [101–103]. All the cytokines cited before were also upregulated in these experiments, assaying the protein concentration in MEEs or middle ear washes. These inductions were observed at early stages (1 day post injection) until 3 days. This suggests that exposing the MEM to purified bacteria proteins induces AOM resolving with time as there are no live bacteria that sustain the innate immune response.

Fewer studies were conducted in chinchilla and rat species; some examples are listed in Table 7.3. These animals are bigger than mice and allow injecting bacteria directly in the bulla without drilling the tympanic membrane. In chinchilla, low or high quantity of live or killed *SP* was able to induce the secretion of IL-1β, IL-6, and TNF-α until 3 days after infection in MEEs in animals having previous Eustachian tube obstruction [72, 104]. In one of the studies, IL-1β was shown to be induced early (6 h post infection), whereas IL-6, IL-8, and TNF-α appeared later [104]. In rats, *SP* or nonviable *NTHi* were potent to induce all the panel of cytokines described before from 6 h to 7 days post infection [105, 106]. Different time-dependent expression profiles of pro-inflammatory cytokines are observed but seem to point TNF-α and IL-1β as early cytokines in the response to bacterial challenge, and they are sustained during the disease process. This is supported by human MEEs analysis [107], TNF-α being considered as a biomarker for OM with effusion persistence and chronicity [57, 108].

The importance of cytokines in the development of OM has been further investigated using mutant mice or wild-type mice treated with receptor antagonists or neutralization antibody. The use of an antagonist of IL-1 receptor during OM mediated by *Hi* in chinchilla showed better resolution of OM. In another study, mucous cell metaplasia induced in mouse in response to *NTHi* or *SP* was also shown to be less important

Table 7.2 Pro-inflammatory mediators detected in mouse models of otitis media (OM)

Reference	Bacterial species/ component	OM induction	Duration of infection	Cytokines deregulated (time point)	Technique
[98]	Heat killed *Hi*	TTI one ear, other ear not injected as control	6 h	TNF-α, IL-1α, IL-10, IL1β, IL-6, MIP-2, KC	Gene chip (mRNA) on MEM
[101]	LPS	TTI, saline as control	3, 6, 12, or 24 h	IL-1β, TNF-α, MIP-2, KC (24h, other ND); GM-CSF (6h)	ELISA of ME wash
[74]	*SP*	Pressure cabin, nasopharyngeal infection	48, 96, 144 h after nasopharyngeal infection	IL-1β, TNF-α (48hrs and 96hrs)	ELISA of MEH
[73]	*NTHi* purified endotoxins	ETO and then TTI, saline as control	3 days, 2 weeks, 2 months	TNF-α (3 time points), IL-1β (3 days), IFN-δ not consistent	ELISA of MEEs, in situ hybridization MEM
[99]	*SP* then *Influenza virus*	Intranasal exposure, saline as control	15 days after *SP* infection	IL-1α, pro-IL-1β, TNF-α, MIP-2	PCR (mRNA) of MEM
[97]	*NTHi* lysates	TTI, saline as control	1 day and 7 days	MIP-2 most induced gene	Microarray (mRNA)
[102]	endotoxins from *Salmonella typhimurium*	TTI, saline as control	6 h, 12 h, 1 day, 3 days, 7 days, and 14 days	IL-1α (up to 3 days), TNF-α (day 1 and 3)	ELISA of MEEs
[103]	endotoxins from *Salmonella typhimurium*	TTI, saline as control	24 h	MIP-2, IL-1β, IL-6	ELISA of ME wash
[100]	*SP*	TTI one ear, other ear not injected as control	24 h	TNF-α, IL-1β, IL2, IL-6	PCR (mRNA) of MEM
[96]	Heat killed *Hi*	TTI one ear, other ear not injected or saline	6, 24, 72 h, 1 week	MIP-2, IL-6, KC all time points but more at 6 h	PCR (mRNA) of MEM

Saline as control means the control group was injected with saline in the middle ear
TTI transtympamic injection, *ETO* Eustachian tube obstruction, *MEH* middle ear homogenate, *MEEs* middle ear effusions, *MEM* middle ear mucosa, *PCR* polymerase chain reaction, *ELISA* enzyme-linked immunosorbent assay, *LPS* lipopolysaccharide, *SP Streptococcus pneumonia*, *TNF* tumor necrosis factor, *IFN* interferon, *IL* interleukin, *GM-CSF* granulocyte-macrophage-colony-stimulating factor, *Hi Haemophilus influenzae, NTHi non-typable Haemophilus influenzae, KC* kinase C, *mRNA* messenger ribonucleic acid, *MIP* macrophage inflammatory protein, *ND* not determined

in IL-10 null mice [109]. These results implicate IL-1α, IL-1β, and IL-10 in the persistence of OM. IL-1β is produced as a pro-protein attached to the plasma membrane and requires cleavage to be secreted. The inflammasome, multiprotein complex implicated in the activation of inflammation is able to cleave the pro-IL-1β. A recent article evaluated the implication of the inflammasome, mutating its adaptor apoptosis-associated speck-like protein containing a CARD *(Asc)* in mice [110]. The inflammatory defects observed were linked to an increase in the degree and duration of mucosal epithelial hyperplasia in the middle ear of *Asc221*-/- mice as well as a delay in bacterial clearance. This shows that even if an overproduction of IL-1β tends to delay OM resolution, its absence or the absence of the inflammasome is deleterious.

HMEEsCs treated with bacterial components also exhibited an overexpression of cytokine genes as well as protein secretion [34, 111, 112], showing that the epithelial cells of the MEEs

Table 7.3 Pro-inflammatory mediators detected in chinchilla and rat models of OM

Reference	Animal species	Bacterial species/ component	OM induction	Duration of infection	Cytokines deregulated (time point)	Technique
[72]	Chinchilla	Heat killed *SP*	ETO and then injection superior bulla, saline as control	3 days after *SP* injection	IL-1β and TNF-α	ELISA of MEEs
[104]	Chinchilla	Low quantity *SP*	ETO and then injection superior bulla, saline as control	1–72 h	IL-1β, (6 h); IL-6, IL-8, TNF-α (72 h)	ELISA of MEEs
[105]	Rat	*SP*	ETO and 42 days after injection though bulla, saline as control	2 days and 7 days after *SP*	IL-1β, TNF-α, IL-6, IL-10, IL-8 in MEEs up to day 3. In MEM IL-1β same; IFN-δ, TNF-α W3/5 to W16; IL-6, IL-10, IL-8, TGF-β, MCP-1 biphasic	ELISA of MEEs and PCR on MEM (mRNA)
[106]	Rat	nonviable *NTHi*	Transbullar inoculation, saline as control	3, 6, 24, 48, 72 h after *NTHi*	In ME wash more IL-1β, IL-6, TNF-α (24 h, other time points ND), in MEM mRNA TNF-α (up to 6 h); IL-1α, IL-8 (up to 24 h); IL-1β, IL-6 (up to 48 h); IL-10 (up to 72 h)	ELISA of ME wash and PCR of MEM

Saline as control means the control group was injected with saline in the middle ear
ETO Eustachian tube obstruction, *ME wash* middle ear wash, *MEEs* middle ear effusions, *MEM* middle ear mucosa, *PCR* polymerase chain reaction, *ELISA* enzyme-linked immunosorbent assay, *SP Streptococcus pneumonia*, *TNF* tumor necrosis factor, *IL* interleukin, *NTHi non-typable Haemophilus influenzae, mRNA* messenger ribonucleic acid, *W* week, *MCP-1* Monocyte chemoattractant protein 1

alone are able to produce pro-inflammatory cytokines in response to bacteria exposure, the TLRs playing an important role in this effect [113, 114].

Infection of the Middle Ear by Bacteria Induces Mucin Production and Mucous Cell Metaplasia

Several laboratories have demonstrated that the infection of the middle ear of animals by *NTHi, SP* live or killed, or LPS results in middle ear mucous metaplasia similar to patient samples described in Sect. 2.1. In mice, the transtympanic injection of *NTHi* lysates or its purified endotoxins resulted in middle ear thickening and mucous cell metaplasia starting at day 3 after infection until 2 months [73, 97]. This was confirmed using rat models infected by *NTHi, SP,* or *MC* as rats inoculated with these bacteria developed high middle ear secretory capacity showing the presence of mucins by the detection of carbohydrates and a high goblet cell density with thickness of the MEEs [115–119]. After *NTHi, SP,* or *MC* infection, the middle ear goblet cell density reached a peak at 2 months after infection and remained until 6 months. Thus, the middle ear is subject to a gradual remodeling that can persist a long time after a single injection of bacteria. Hunter et al. [119] showed that 7 days after a single injection of LPS, rats with Eustachian tube obstruction developed middle ear goblet cell metaplasia and hyperplasia, but not the control group. These results underline the high responsiveness of the middle ear epithelium to a single injection of bacterial component when the Eustachian tube is obstructed.

In vitro experiments have been conducted to analyze the mucoid effect of live or lysed bacteria on HMEEsCs-1. The exposure to *NTHi, SP,* or *MC* revealed a potency of the HMEEsC-1 to activate the promoter of MUC5AC and the transcription of its mRNA [113, 120–125]. Coculture of HMEEC-1 with live *SP* also demonstrated an induction of MUC5AC mRNA and promoter [126]. Kerschner et al. [120] extended the study to other mucins in this cell type: they found mRNA induction of MUC5AC, MUC5B, and MUC2, this being more relevant with the clinical observations of mucins in the middle ear of patients with OM. None of these studies analyzed the mucin proteins. Importantly, mucins have more of a biological effect if they are secreted. It has been shown in airway cells that mucins can be stored in vesicles before being secreted [22], and as mentioned before, the activation of mucin genes does not always reflect the production of the protein [27]. The predominant mucin in the MEEs MUC5B is also very poorly studied and needs more attention in terms of its genetic regulation, as from it appears that MUC5AC plays less of an important role in OM. But MUC5B glycoprotein production in the middle ear is probably mainly dependent on mucus glands that are hard to model in vitro. A way to address this question would be to use a glandular model in three-dimensional (3D) gel as already described for human primary bronchial epithelial cells grown in a basement membrane matrix [127]. After 22 days of growth, the bronchial cells differentiated into glandular acini with a lumen and were able to secrete MUC5B in the lumen.

Inflammatory Mediators Regulate Mucin Production and Mucous Cell Metaplasia

Inflammation is activated very quickly after infection of the middle ear, whereas mucin production, dependent on mucous cell metaplasia, occurs later. The hypothesis suggested by several researchers is that inflammation drives mucous cell metaplasia in the middle ear during OM and in consequence increases the production of mucins in effusions. A simple way to address this question is to use animals exposed to a pro-inflammatory cytokine in the middle ear instead of bacterial components. A first experiment was done by Catanzaro et al. [128] in Guiney pigs. The animals were exposed to human recombinant IL-1, IL-2, or TNF-α injected through the tympanic membrane, and the ears were observed until 72 h post injection. IL-2 and TNF-α induced effusions in the ear that resolved at 48 h for TNF-α and 72 h for IL-2. IL-1 did not have any effect. The experiment was repeated in mice by Watanabe et al. [129] that injected IL-1β and compared it to *NTHi* LPS effect. Similar pathological changes were observed in the two groups compared to controls and showed an inhibition of effusion production in presence of IL-1 receptor antagonist. TNF-α effects were studied several times in rats: injection of TNF-α in the middle ear induced effusion production, subepithelial edema, neutrophil infiltration, and MUC2 mRNA in the MEM [130, 131]. Coupled with Eustachian tube obstruction, TNF-α injection stimulated mucous cell metaplasia and hyperplasia with abundant production of mucin glycoproteins [132]. IL-8 pro-inflammatory cytokine was also shown to induce the thickening of the middle ear epithelium and inflammatory cell infiltration in mice, effects comparable but stronger compared to heat-killed *SP* [133].

Similar experiments were conducted in vitro to analyze the effect of pro-inflammatory cytokine exposure on mucin expression in the middle ear epithelium. HMEEsC-1, normal MEEsC, or chinchilla MEEsC exposed to IL-1β or TNF-α showed an increase of mRNA production of MUC2, MUC5AC, MUC8, and/or MUC19 [80, 134, 135]. In addition, MUC19 mRNA was induced in HMEEsC-1 and chinchilla MEEsC in response to IL-6 and IL-8 [80]. The total mucin glycoprotein content, more relevant with the biological effect of mucins, was increased by the incubation of chinchilla MEEsC or HMEEsC-1 with IL-1β or TNF-α detected by PAS staining and scintillography technique [134, 136]. Nakamura et al. [137] analyzed the ability of middle ear epithelial cells to differentiate in goblet cells in vitro in response to TNF-α. He showed that the co-exposure of mMEEsC to TNF-α and retinoic acid differentiated the epithelial cells in mucus-like cells, whereas retinoic acid alone did not,

demonstrating that TNF-α participates in mucous cell differentiation. Smirnova et al. [62] used a goblet cell type from the human colon, HT29-MTX cells, in order to assay the effect of IL-8 on cells already differentiated in mucin-producing cells. They demonstrated an increase of MUC5B and MUC5AC secretion in response to IL-8 in a dose- and time-dependent manner, which was sustained until 5 days.

These studies show that several cytokines including IL-1β and TNF-α induce middle ear metaplasia and hyperplasia as well as effusion production containing mucins in vivo. Further analyses in in vitro models of middle ear epithelium demonstrated that these cytokines induce mRNA expression of mucins, stimulate the secretion of mucins by goblet cells, and are able to participate to mucous cell differentiation. All together, these studies point pro-inflammatory cytokines as a key determinant in middle ear mucous cell metaplasia and mucin production in OM. The signaling pathways implicated in these effects will be developed in the next parts.

Role of the Innate Immune Receptors TLRs

As mentioned, TLR2, TLR4, TLR5, and TLR9 were found at the level of RNA and proteins in the MEM of both OM and non-OM patients [49]. The TLRs are receptors that recognize similar patterns in pathogens, they are the first sensors of infection in the middle ear. In animals, TLRs were demonstrated to play a critical role in OM. Mutant mice were used to investigate the impact of deficiencies in TLRs in OM. Mice mutated for TLR4, TLR2, or TLR9 showed a more profound and persistent inflammation with impaired bacterial clearance when infected by *NTHi* or *SP* [138–141]. The early TNF-α induction observed in wild-type mice was not occurring in TLR2-/- and TLR4-/- mice. Leichtle et al. [139] showed that TLR2-/- mice had a delayed IL-10 expression and a prolonged failure to clear bacteria, whereas TLR4-/- mice had only an early bacteria clearance impairment. TLR4-/- mice were also characterized by an absence of TLR2 induction, suggesting an involvement of TLR4 in TLR2 activation.

MyD88 and TRIF proteins, adaptors of TLRs that mediate parallel signaling pathways, were also mutated. MyD88 mediates IL-1β induction, whereas TRIF mediates IFN responses. TRIF-/- and MyD88-/- mice both showed a reduced but more persistent mucosal metaplasia and impairment to clear bacteria [142, 143]. If we compare the mucosal effects of these mutated mice to wild-type ones, we see that TLR2-/- mice as well as MyD88-/- mice have a sustained and higher thickening of the middle ear epithelium after *NTHi* inoculation in the ear compared to wild-type mice, whereas milder effects were observed for TLR4 or TRIF deficient mice [53].

The signaling pathways leading to pro-inflammatory mediators and mucin gene induction were studied in vitro, focusing on MUC5AC. Figure 7.1 shows a summary of *NTHi* effect on HMEEsC-1 and other cell types as airway cells, adapted from the results of five studies directed by Dr. Jian Dong Li [112, 114, 121, 122, 144]. *NTHi* seem to activate the TLR2 but not the TLR4 and require the mediators MyD88, interleukin-1 receptor-associated kinase 1 (IRAK), and TNF receptor-associated factor protein 6 (TRAF6) to activate p38 and nuclear factor κB (NF-κB) pathways, resulting in pro-inflammatory mediators and MUC5AC promoter activation and in consequence the initiation of their transcription. Other studies also implicated the central transcription factor in inflammation NF-κB in response to bacterial components or cigarette smoke in middle ear cells [145–147]. The protein kinase C (PKC) pathway activates CARD-containing MAGUK protein 1 (CARMA-1) that seems to be implicated in inflammasome assembly [148], and extracellular signal-regulated kinase (ERK) was also implicated in *NTHi* effects as well as the Tβ receptors (TβR) that dimerizes in response to *NTHi* and activates Smad3/Smad4 to induce MUC2. The outer membrane protein (OMP)-6 of *NTHi* demonstrated its ability to induce several biomarkers of these pathways, suggesting the high importance of this protein in *NTHi* biological effects in middle ear epithelial cells.

Interestingly, *SP* infection revealed different responses. Figure 7.2 summarizes the results of four studies on the mechanisms of MUC5AC in-

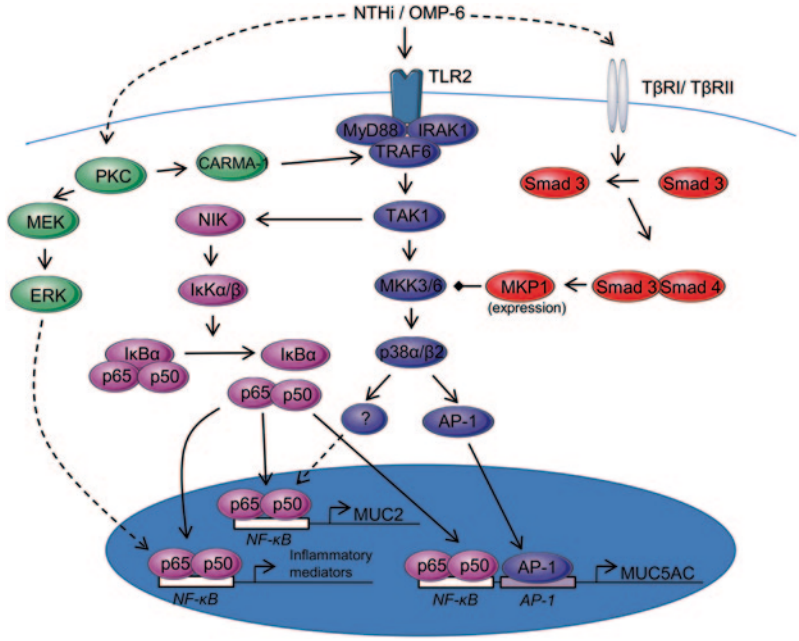

Fig. 7.1 Molecular pathways implicated in *non-typable Haemophilus influenzae (NTHi)* inflammatory and mucoid effects in human ear and airway epithelial cells. Four main molecular pathways have been identified in response to *NTHi* or its outer membrane protein *(OMP)-6:*1. Toll-like receptor 2 *(TLR2)* activates myeloid differentiation factor 88 *(MyD88),* interleukin-1 receptor-associated kinase 1 *(IRAK1),* TNF receptor-associated factor protein 6 *(TRAF6),* TGF-β-activated kinase *(TAK1),* MAP kinase kinase 3/6 *(MKK3/6),* p38, activator protein 1 *(AP-1)* leading to MUC5AC and MUC2 transcription activation.2. *TAK1* induces a parallel signaling pathway activating NF-κB-inducing kinase *(NIK),* IκKα/β (inhibitory IκB kinase), IκB phosphorylation detaching from p65/p50 that translocates to the nucleus to activate the transcription of MUC5AC and MUC2.3. TβRI/TβRII dimerization is induced by *NTHi*. It activates Smad3 that binds to Smad4 and induces the expression of MAP kinase phosphatase 1 *(MKP1)* inhibiting *MKK3/6* activation.4. *NTHi* activates protein kinase C *(PKC)* that activates CARD-containing MAGUK protein 1 *(CARMA-1),* inducing TRAF6 signaling pathway. *PKC* also activates MAPK/ERK kinase *(MEK)* and then extracellular signal-regulated kinase *(ERK)* leading to the increase of p65/p50 binding to inflammatory mediator promoters.(Based on [112, 114, 121, 122, 144])

duction in response to *SP* lysates [113, 125, 126, 149]. Contrary to *NTHi, SP* activated TLR4 and not the TLR2 pathway. Nevertheless, the mediators MyD88, IRAK1, and TRAF6 were also implicated in TLR4 effects. Inhibitory IκB kinase (IκKα/β) was shown to be phosphorylated, leading to ERK activation by IκKα and repression by IκKβ. ERK activation resulted in MUC5AC transcription probably via the activator protein 1 (AP-1) factors. Jun kinase (JNK) was also activated in response to *SP* via TRAF6/p21-activated kinase 4 (PAK4), repressing the activation of MUC5AC. The protein MAP kinase phosphatase 1 (MKP1) was also demonstrated to repress ERK and JNK activation, conferring to the epithelial cells the ability to limit MUC5AC induction in response to *SP* via different signaling pathways.

In consequence, from our knowledge TLRs especially TLR2 and TLR4 play an important role in the innate immune response to *NTHi* and *SP*. These receptors activate a quick inflammatory response to attract immune cells and clear pathogens. They regulate the production of proinflammatory mediators as well as mucin genes (MUC2 and MUC5AC). MUC5B is the predominant mucin in the MEEs of patients with OM; thus, it is important to further investigate the mechanisms of its regulation as well. TLR mutations in mice demonstrated a persistence of the middle ear metaplasia and hyperplasia, underlining the importance of its activation in the in-

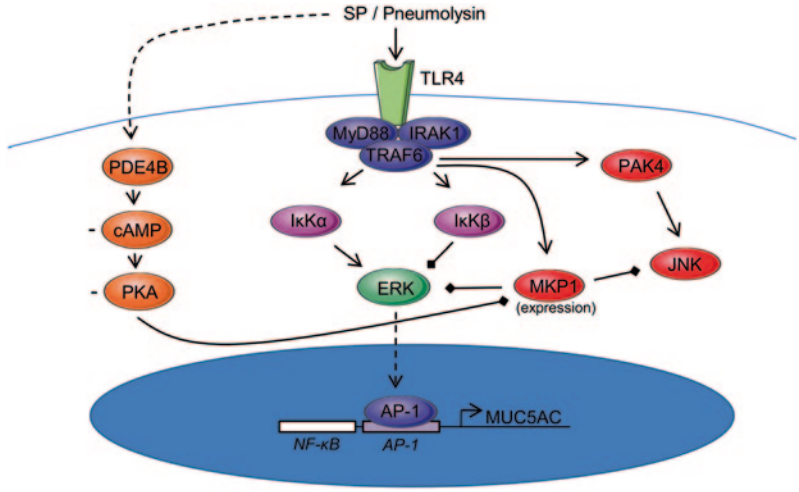

Fig. 7.2 Molecular pathways implicated in *Streptococcus pneumoniae (SP)* mucoid effects in human ear and airway cells. *SP* or its membrane protein pneumolysin activates three main signaling pathways:1. Toll-like receptor 4 *(TLR4)* activates myeloid differentiation factor 88 *(MyD88),* interleukin-1 receptor-associated kinase 1 *(IRAK1),* TNF receptor-associated factor protein 6 *(TRAF6),* inhibitory IκB kinase *(IκKα/β)* that activates or represses extracellular signal-regulated kinase *(ERK)* leading to activator protein 1 *(AP-1)* translocation to the nucleus to bind to its responsive element in MUC5AC promoter and activate its transcription.2. *TRAF6* activates p21-activated kinase 4 *(PAK4)* and Jun kinase *(JNK)*. *TRAF6* also induces the expression of MAP kinase phosphatase 1 *(MKP1)* that represses *JNK* and *ERK*.3. *SP* also activates phosphodiesterase 4B *(PDE4B)* that reduces cyclic adenosine monophosphate *(cAMP)* content leading to less activation of protein kinase A *(PKA)* and the repression of *MKP1* expression.(Based on [113, 125, 126, 149])

flammatory responses during bacterial infection. Nevertheless, after the immune system resolved the bacterial infection, the inflammation has to stop. If it persists, it is likely that the middle ear epithelium remodels and exhibits a high number of goblet cells producing an excess of mucins.

Role of Hypoxia Mechanisms

Hypoxia is defined as an insufficient level of O_2 in the blood or a tissue. It induces responses of stress from cells trying to reduce their metabolism to save O_2 and try at the same time to bring more of it. The biomarkers of hypoxia are usually the transcription factor hypoxia inducible factor 1α (HIF-1α) and the secreted protein vascular endothelial growth factor (VEGF). During OM, the orifice of the Eustachian tube is often blocked, likely leading to a mild hypoxia in the middle ear. To investigate this direction, Sekiyama et al. [55] assayed the presence of VEGF in MEEs from patients with OME. VEGF was detected in high concentrations in MEEs and was associated to IL-8 secretion as well as endotoxins presence.

In vivo, the hypothesis that hypoxia regulates the responses observed during OM is supported by the fact that Eustachian tube obstruction induces MEEs and mucosal metaplasia [150]. This is also supported by the clinical observations of the Eustachian tube blockade during OM, due to inflammation, which stimulates the mucosal metaplasia of the middle ear [151]. A lower oxygenation of the middle ear can be a cause of hypoxia but the presence of a large amount of inflammatory cells during OM might participate to the consumption of O_2 as well. Kitaoka et al. [152] have also shown that *SP* consumes oxygen too, altogether the different cells (human cells and bacteria) additional to the Eustachian tube dysfunction are the cause of hypoxia.

Hypoxia induces stress responses dependent on oxygen radicals called reactive oxygen species as they react with the components of the cells (protein, lipids, DNA…) and damage them.

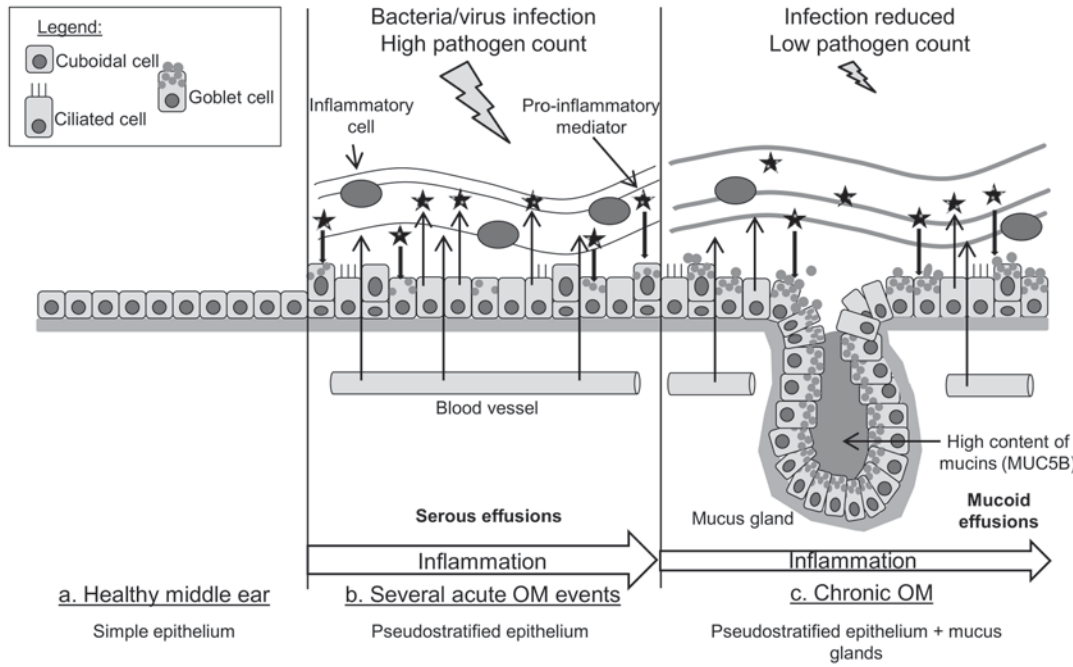

Fig. 7.3 Model suggested for the development of otitis media *(OM)* and the evolution into COME. **a** The healthy middle ear is represented in the part **a** as a single epithelium. The infection by bacteria induces inflammatory cytokine secretion in the middle ear which attracts inflammatory cells. **b** During middle ear inflammation, the Eustachian tube likely blocks and generates a negative pressure in the middle ear, leading to liquid and protein transudation from the blood, bringing liquid in the middle ear cavity. The pro-inflammatory mediators stimulate the middle ear epithelium to differentiate goblet cells. **c** In chronic stages of the disease, fewer bacteria are present in the middle ear. But the sustained inflammation remodeled the epithelium that has mucus glands producing large amounts of mucins, making the MEEs very viscous

High levels of lipid peroxidation, induced by an overproduction of reactive oxygen species, have been detected in MEEs of patients with OME [153, 154]. Balikci et al. [155] also showed evidence of protein oxidation in MEEs of patients with COME.

In vivo, rats with Eustachian tube blockade showed OM development and elevated expression of HIF-1α, VEGF, IL-1β, and TNF-α [156]. Two mutated mice were also characterized by the overexpression of the same hypoxia and inflammatory mediators: Jeff and Junbo mice [157]. Both mice having different mutations developed spontaneously OM and showed low O_2 levels in the middle ear and fluids as well as apoptotic polymorphonuclear inflammatory cells. The Junbo mice treated with an inhibitor of VEGF were seen to develop less hearing problems and mucosal metaplasia. The hypoxia was resolved by myringotomy of Junbo mice and associated with a reduction of the inflammation and the thickness of the MEM [158], showing the beneficial effect of middle ear oxygenation. The mechanisms under these effects were investigated with in vitro models grown in hypoxic conditions. Primary airway cells grown at 1% O_2 at air-liquid interface showed a dramatic differentiation in goblet cells positive for MUC5AC as well as a pseudostratified appearance and reduction of ciliated cells [159]. In another study, a bronchial cell line was demonstrated to secrete MUC5AC if cultured in hypoxic conditions, induction dependent on HIF-1α and Smad activation [160].

Hypoxia seems to participate in OM development, likely occurring during the inflammatory response of the middle ear epithelium. In addi-

tion to the innate immune response activated by pathogens, the increase in oxygen consumption in the middle ear coupled with the Eustachian tube obstruction might create a hypoxic environment. This can lead to the production of reactive oxygen species, creating some damages in the MEM, leading to the sustainment of inflammation.

In summary, from what we can summarize from the literature, we can suggest a model of OM development from very early responses to the chronic stage of the disease (Fig. 7.3). The healthy middle ear is a simple-layer epithelium (Fig. 7.3a) that keeps the middle ear without fluid which is assured by ion pumps and AQP water channels. When AOM events occur (Fig. 7.3b), the epithelial cells recognize pathogens with receptors which leads to the secretion of pro-inflammatory mediators. These mediators attract inflammatory cells (also producing pro-inflammatory mediators) and stimulate the remodeling of the epithelium, showing more cells producing mucins but also some ciliated cells. One of the consequences of the inflammation is to block the Eustachian tube thus impairing its function of clearance. A negative pressure in the middle ear likely occurs and induces the transudation of proteins and liquid from the blood. In addition, the impairment of ion pumps and water channels fail to reabsorb the water, letting serous fluids in the middle ear cavity. The infection can be managed by the immune system and the inflammation resolve, stopping the OM. If the inflammation persists, sometimes even in absence of pathogens or a low amount of bacteria as it has been described for COME, a more drastic remodeling of the middle ear epithelium occurs, leading to the production of more goblet cells as well as mucus glands that produce very large amounts of mucins (Fig. 7.3c). Proteins and water still diffuse from the blood vessels and participate in the production of a very viscous fluid due to the high content in mucins, mainly MUC5B.

Knowledge of mechanisms implicated in OM has increased dramatically over the past 20 years with new laboratory techniques for MEEs analysis, the use of mutant animals and in vitro models of middle ear epithelium. Nowadays, antibiotics are widely used but are not necessarily needed in certain cases like COME, where the bacteria infection has already been identified and treated by the innate immune system. Thus, efforts are needed to better understand what happens in the different stages of the disease to better guide patient treatment. This includes the study of MUC5B, the predominant mucin in MEEs, as there is a total lack of understanding of its regulation in OM. The recent study showing that the knoockout in MUC5B gene induces middle ear infection underlines this need [28]. The development of mucus glands probably produces a large amount of MUC5B present in MEEs. Their differentiation and regulation should also be addressed in OM with innovative cell culture strategies.

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