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Panel 4: Recent Advances in Otitis Media in Molecular Biology, Biochemistry, Genetics, and Animal Models

Jian-Dong Li, MD, PhD¹, Ann Hermansson, MD, PhD², Allen F. Ryan, PhD³, Lauren O. Bakaletz, PhD⁴, Steve D. Brown, PhD⁵, Michael T. Cheeseman, DVM, PhD⁵, Steven K. Juhn, MD⁶, Timothy T. K. Jung, MD, PhD⁷, David J. Lim, MD⁸, Jae Hyang Lim, DVM, PhD¹, Jizhen Lin, MD⁶, Sung-Kyun Moon, MD, PhD⁸, and J. Christopher Post, MD, PhD⁹

¹Center for Inflammation, Immunity & Infection, and Department of Biology, Georgia State University, Atlanta, Georgia, USA

²Department of Otorhinolaryngology, Lund University Hospital, Lund, Sweden

³Surgery/Otolaryngology, University of California, San Diego, La Jolla, California, USA

⁴Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital and Department of Pediatrics, College of Medicine, The Center for Microbial Interface Biology, The Ohio State University, Columbus, Ohio, USA

⁵MRC Mammalian Genetics Unit, MRC Harwell, Harwell, UK

⁶Department of Otolaryngology, University of Minnesota Medical School, Minneapolis, Minnesota, USA

⁷Department of Otolaryngology–Head & Neck Surgery, Loma Linda University School of Medicine and Jerry L. Pettis Memorial Veterans Affairs Medical Center, Loma Linda, California, USA

⁸Division of Clinical and Translational Research, House Ear Institute, Los Angeles, California, USA

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Corresponding Author: Jian-Dong Li, MD, PhD, Center for Inflammation, Immunity & Infection, Georgia State University, 100 Piedmont Avenue, Atlanta, GA 30303, USA. jdli@gsu.edu.

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Author Contributions

Jian-Dong Li, literature search and review, preparation and discussion of the manuscript, correction and refinement; **Ann Hermansson**, literature search and review, preparation and discussion of the manuscript; **Allen F. Ryan**, literature search and review, preparation and discussion of the manuscript; **Lauren O. Bakaletz**, literature search and review, preparation and discussion of the manuscript; **Steve D. Brown**, literature search and review, preparation and discussion of the manuscript; **Michael T. Cheeseman**, literature search and review, preparation and discussion of the manuscript; **Steven K. Juhn**, literature search and review, preparation and discussion of the manuscript; **Timothy T. K. Jung**, literature search and review, preparation and discussion of the manuscript; **David J. Lim**, literature search and review, preparation and discussion of the manuscript; **Jae Hyang Lim**, literature search and review, preparation and discussion of the manuscript; **Jizhen Lin**, literature search and review, preparation and discussion of the manuscript; **Sung-Kyun Moon**, literature search and review, preparation and discussion of the manuscript; **J. Christopher Post**, literature search and review, preparation and discussion of the manuscript.

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⁹Pediatric Otolaryngology and Center for Genomic Sciences, Allegheny General Hospital, Pittsburgh, Pennsylvania, USA

Abstract

Background—Otitis media (OM) is the most common childhood bacterial infection and also the leading cause of conductive hearing loss in children. Currently, there is an urgent need for developing novel therapeutic agents for treating OM based on full understanding of molecular pathogenesis in the areas of molecular biology, biochemistry, genetics, and animal model studies in OM.

Objective—To provide a state-of-the-art review concerning recent advances in OM in the areas of molecular biology, biochemistry, genetics, and animal model studies and to discuss the future directions of OM studies in these areas.

Data Sources and Review Methods—A structured search of the current literature (since June 2007). The authors searched PubMed for published literature in the areas of molecular biology, biochemistry, genetics, and animal model studies in OM.

Results—Over the past 4 years, significant progress has been made in the areas of molecular biology, biochemistry, genetics, and animal model studies in OM. These studies brought new insights into our understanding of the molecular and biochemical mechanisms underlying the molecular pathogenesis of OM and helped identify novel therapeutic targets for OM.

Conclusions and Implications for Practice—Our understanding of the molecular pathogenesis of OM has been significantly advanced, particularly in the areas of inflammation, innate immunity, mucus overproduction, mucosal hyperplasia, middle ear and inner ear interaction, genetics, genome sequencing, and animal model studies. Although these studies are still in their experimental stages, they help identify new potential therapeutic targets. Future preclinical and clinical studies will help to translate these exciting experimental research findings into clinical applications.

Keywords

otitis media; molecular biology; biochemistry; inflammation; cytokine; chemokine; innate immunity; cell signaling; tissue remodeling

The host responses to viral and bacterial pathogens during otitis media (OM) are determined, in large part, by the expression of genes during host-pathogen interaction. The identification of genomic and expressed sequences and the recent advances in the experimental methods of molecular biology, genomics, proteomics, biochemistry, and genetics permit the assessment of gene activity in humans and animals experiencing OM. The increasing availability of genome information on pathogens also allows assessment of OM-related bacterial and viral gene expression and activity. Finally, molecular, genetic, and biochemical tools allow the manipulation of genes and their products in both hosts and pathogens. This provides powerful methods for evaluating the functional roles of genes in this disease. Significant progress has been made in the areas of molecular, genetic, and biochemical studies in OM over the past 4 years.

Methodology/Search Strategy

We searched PubMed for published literatures. The starting date for the search was June 1, 2007, and the ending date, May 30, 2011. Search terms included *otitis media*, *molecular biology*, *biochemistry*, *inflammation*, *cytokine*, *chemokine*, *innate immunity*, *Toll-like receptor*, *host response*, *cell signaling*, *mucus*, *mucin*, *mucosal hyperplasia*, *tissue remodeling*, *cholesteatoma*, *genetics*, *chinchilla genome*, and *animal model*. The search included all published literature with the abstract available in English. No further exclusion selection criteria were applied.

Discussion

Recent Advances in the Molecular Biology and Genetics of Otitis Media

Significant advancements have been made in developing molecular, genetic, and biochemical tools that allow the manipulation of genes and their products in both hosts and pathogens. These technological advancements led to substantial progress in improving our understanding of the molecular basis of host responses in OM in a number of areas.

Innate Immunity

The innate immune system, known as the nonspecific immune system and the first line of defense, is an evolutionarily conserved mechanism that provides an early and effective response against invading microbial pathogens. Extensive studies on the role of innate immune molecules were carried out using in vitro and in vivo approaches. Genome-wide array studies using cells and knockout (KO) mice, partial characterization of complementary DNA (cDNA) library in normal and nontypeable *Haemophilus influenzae* (NTHi)-treated chinchilla middle ear (ME), and identification of SPLUNC having a role in the host defense against NTHi in the chinchilla are some of the highlights.

A number of studies have added to our understanding of innate immune defects as a risk factor for OM in children. Wiertsema and Leach¹ noted that several such defects are associated with increased risk of OM and may help to explain the high incidence of disease in Australian Aboriginal children. Prommalikit et al² noted that children with defects in either innate or cognate immunity were at increased risk for pneumococcal OM. A similar conclusion regarding innate immunity was reached by Ilia et al.³ Kim et al⁴ detected several innate immune receptors in OM effusions, including Toll-like receptor 9 (TLR9), as well as NOD1 and NOD2. Lee et al⁵ found that induction of host response by NTHi in human ME epithelial cells requires TLR2 signaling mediated by the TLR adaptor MyD88. Lee et al⁶ identified mutations in TLR2 and TLR4 in patients with OM.

Studies in animals have added in vivo evidence regarding the importance of innate immunity in OM resistance. Song et al⁷ observed the expression of TLR2 and TLR4 in the rat ME during OM. Hirano et al⁸ and Leichtle et al⁹ found that mutations in TLR4 resulted in prolongation of OM in the mouse. MacArthur et al¹⁰ observed spontaneous OM in mice with a TLR4 deficit and identified *Klebsiella oxytoca* as a pathogen. Leichtle et al¹¹ noted that lack of TLR2 resulted in a more profound deficit in OM recovery than lack of TLR4 in mice. Moreover, they found that lack of TLR2 blocked upregulation of TLR4 by ME

inoculation with NTHi. Leichtle et al investigated mice deficient in TLR9, an intracellular receptor for foreign DNA, and found prolonged OM induced by NTHi. Most TLRs signal through the adaptor molecule MyD88, with only TLR3 and TLR4 signaling through the related adaptor TRIF. Hernandez et al¹² found that mice deficient in MyD88 exhibited greatly prolonged OM induced by NTHi, whereas Leichtle et al¹¹ found a more modest deficit in OM recovery due to lack of TRIF. Activation of TLRs induces the expression of inflammatory cytokines, one of the most important of which is tumor necrosis factor (TNF). Leichtle et al¹³ found that mice deficient in TNF showed greatly prolonged OM that was only partially rescued by exogenous TNF. Interestingly, exogenous chemokine CCL3, normally expressed at high levels during OM but not expressed in TNF-deficient mice, completely rescued normal OM recovery. Genetic defects in complement genes also reduced the ability of mice to resist and recover from OM.^{14,15} In a chinchilla model, McGillivray et al¹⁶ found that dysregulation of the innate immune effector β -defensin by respiratory syncytial virus enhanced colonization of the upper airway by NTHi. It is clear that animals with innate immune defects are excellent models of persistent/recurrent OM. Moreover, the fact that defects in so many innate immune genes prolong OM in animal models underscores the importance of this system in resistance to, and recovery from, OM.

Inflammation

Appropriate inflammation is critical for host defense. However, uncontrolled and excessive inflammation is clearly detrimental to the host. Inflammation is a hallmark of OM, and dysregulated inflammation in the middle ear contributes significantly to the development and progression of OM. Therefore, maintaining adequate levels of inflammation is critical for preventing middle ear damage. Otitis media is a multifactorial disease arising from the complex interactions among otopathogens, environmental risk factors, and host genetic factors. Thus, it is of particular importance to understand the mechanisms by which these factors regulate immune/inflammatory responses in the middle ear, either alone or in combination.

Microbial Factors—Among many factors for OM, infection with otopathogens was found to be the most frequent cause of OM. Otitis media is a polymicrobial disease caused by bacterial and viral pathogens. Although single-pathogen infection is typical, it is common to have coinfections with more than one type of bacterial pathogens (up to 55%) as well as bacterial and viral coinfections (up to 70%).¹⁷ Moreover, otopathogens can not only colonize together but also synergize with each other to induce host responses in multiple ways. For example, one of the most common bacterial otopathogens, *Streptococcus pneumoniae*, synergistically enhances NTHi-induced inflammation via upregulating TLR2 expression, one of the major host receptors for the NTHi pathogen.¹⁸ The incidence of OM by *S pneumoniae* is also significantly enhanced by other otopathogens, particularly by *Moraxella catarrhalis*.¹⁹ In addition, it has also been found that coinfection with NTHi facilitates pneumococcal biofilm formation and increases its persistence on middle ear mucosal surfaces. The enhanced biofilm persistence also correlates well with delayed emergence of opaque colony variants within the bacterial population and a decrease in the systemic infection.²⁰ Of particular interest are the clinical trials studying the use of spray bacteriotherapy. Nasal spray with probiotic bacteria (eg, *Streptococcus sanguinis* or

Lactobacillus rhamnosus) showed significant therapeutic effects, including complete or significant recovery from serous OM.²¹ Although the underlying mechanism for the observed effect remains to be investigated, the study suggests that the microbial physiology in the middle ear inflammation is far more complex than what we initially thought. Therefore, this complexity should be taken into consideration in designing treatments for controlling infection and inflammation in OM treatment.

Host Genetic Factors—There is also considerable evidence for a genetic predisposition for the development of OM. However, little is known about the genetic factors underlying susceptibility to OM. Recently, many studies have been focused on identifying the genetic factors underlying the regulation of immune/inflammatory responses in the middle ear, and they have identified a number of genetic factors regulating immune/inflammatory responses in the pathogenesis of OM. However, the mechanism underlying regulation of inflammation in vivo by these genetic factors is not well understood. For instance, TNF- α and TNF receptor pathways have been found to be critical for inflammation in the middle ear. Recent studies found that infliximab, the monoclonal TNF- α antibody, reduces inflammatory responses in experimental OM in rats.²² However, deficiency of TNF resulted in prolonged inflammation following infection of the middle ear with NTHi, whereas TNF-deficient mice failed to upregulate both TLRs and the downstream genes and proteins, such as CCL3, resulting in defects in both inflammatory cell recruitment and macrophage activation. Of particular interest in this study is that in vivo administration of rCCL3 to TNF-deficient mice restores their ability to control OM caused by NTHi.¹³ Moreover, genetic studies with TNF mutant mice also showed that TNF and TNF receptors are required for appropriate regulation of caspase genes and are critical for the maintenance of mucosal architecture in both normal and infected ME.²³ The role of innate immune regulators in inflammation is also demonstrated in the recent study by Leichtle et al.¹¹ This study showed that TRIF (Tir domain-containing adaptor inducing interferon β)-deficient mice showed reduced mucosal hyperplasia and had decreased leukocyte infiltration into the middle ear in response to NTHi infection as compared with wild-type (WT) mice. However, mucosal hyperplasia was found to be more persistent and bacterial clearance was delayed in these TRIF-deficient mice.¹¹ Nevertheless, due to the functional and anatomical similarities between the ears of mice and humans, the mouse OM model has been found to be an excellent model system for unraveling the complex genetic susceptibilities underlying OM.²⁴ Mouse genetic mutants with phenotypes comparable to OM in humans may be helpful for the identification of the precise genetic determinants underlying the increased heritability to OM. Many innate immune factors regulating inflammation have been tested in the mouse model of OM using gene-knockout approaches. Shimada et al²⁵ found that lysozyme M deficiency led to an increased susceptibility to middle ear infection with *S pneumoniae* and resulted in severe middle ear inflammation by studying *S pneumoniae*-induced OM in lysozyme M-deficient mice. In addition to TLR4 mutation, which was originally found to contribute to the development of OM, MyD88, TLR2, and TLR9 have also been found to be critical for regulating inflammatory responses in the middle ear.^{12,26,27} MyD88, a universal TLR adaptor protein (except for TLR3), has also been found to be critical for middle ear inflammation, as evidenced by the experiment showing that MyD88 KO mice displayed prolonged ME mucosal thickening and delayed recruitment of neutrophils and macrophages

in OM.¹² TLR2 KO mice were also found to produce relatively low levels of proinflammatory cytokines following pneumococcal challenge, thus hindering the clearance of bacteria from the middle ear and leading to sepsis and a high mortality rate.²⁷ Deficiency of TLR9 resulted in significantly prolonged inflammatory responses in the mouse model of NTHi-induced OM, indicating that DNA sensing by TLR9 may contribute to the pathogenesis and recovery of OM.²⁸ In addition, cylindromatosis (CYLD), a negative regulator of nuclear factor- κ B (NF- κ B), has been shown to play an important role in regulating NTHi-induced inflammation in the pathogenesis of cholesteatoma in human patients.^{29,30} In a later study by Byun et al,²⁹ CYLD expression was found to be significantly low in the cholesteatoma epithelium of human patients while enhanced NF- κ B activation was observed,²⁹ suggesting that the downregulation of CYLD may contribute not only to the inflammatory responses against otopathogens but also to the tissue remodeling process in middle ear disease. In addition, single-gene mouse mutants with OM have identified a number of genes—namely, Eya4, Tlr4, p73, MyD88, Fas, E2f4, Plg, Fbxo11, and Evl1—as potential and biologically relevant candidates for human disease. Human polymorphisms in FBXO11, TLR4, and PAI1 genes have been identified to be significantly associated with human disease.^{31,32} Rpl38 (encoding a ribosomal protein of the large subunit) mutation exhibited the OM phenotype in Tail-short (Ts) mice.³³

MicroRNAs in OM—Recent advances in molecular biology have revealed the potential role of microRNAs (miRNAs) as an important regulator of inflammation in many pathological processes. In a recent study, Song et al³⁴ found that a number of miRNAs, which regulate important biological processes, including developmental process, acute inflammatory responses, and innate immune responses, were differentially regulated (either upregulated or downregulated) during lipopolysaccharide-induced acute inflammation in the human middle ear epithelial cells (HMEECs). This study suggests that miRNAs may play important roles in the regulation of inflammation in OM as it does in the other inflammatory diseases.

Sequencing Chinchilla Genome

The chinchilla OM model is a robust, reproducible, and polymicrobial model of OM. Recently, significant progress has been made in obtaining genomic information from sequencing, which will be valuable for further promoting our understanding of OM pathogenesis.³⁵

Mucosal hyperplasia: It is now well accepted that middle ear infection triggers mucous cell metaplasia. Experimentally, it has been shown that bacterial infection or cytokine challenge of the middle ear mucosa results in mucous cell metaplasia/hyperplasia. Cytokines, especially the proinflammatory cytokines and T-helper 2 subset–derived cytokines, are well known to be involved in mucous cell metaplasia/hyperplasia. One such example is that interleukin (IL)–10 knockout mice developed no mucous cell metaplasia/hyperplasia.³⁶ These studies suggest that cytokines derived from T-helper 2 lymphocytes play a critical role in the mucous cell metaplasia. Mucous cell metaplasia involves increased mucin expression, production, and release. It is becoming clear that inflammation stimulates epidermal growth factor receptor activation and IL-13 to induce transition of both Clara and

ciliated cells into goblet cells through the coordinated actions of the fork-head box transcription factor A2 (FoxA2), thyroid transcription factor 1 (TTF-1), SPDEF, and GABAA α R in which FoxA2 is downregulated and SPDEF and GABAA α R are upregulated, resulting in an upregulation of mucin MUC5AC, mucin chaperone trefoils, and CLCA.³⁷ Moreover, Tsuchiya et al³⁶ found that IL-10 plays a significant role in mucoid metaplasia of the middle ear. Lee et al³⁸ found that transforming growth factor- β (TGF- β) signaling is activated during mucosal hyperplasia. Furukawa et al³⁹ found that inhibition of JNK reduced mucosal hyperplasia during OM, implicating the involvement of JNK signaling. Ebmeyer et al²³ reported that TNFA deletion altered apoptosis and caspase expression during OM, suggesting that this factor is involved in the recovery of the mucosa from hyperplasia. Yaguchi et al⁴⁰ successfully generated an artificial mucosa for the regeneration of the damaged or absent ME lining.

Molecular Biology of Cholesteatoma—Interesting progress has also been made in cholesteatoma studies. One interesting finding is the identification of the transcription factor ID1 (inhibitor of differentiation 1) as an important regulator that drives the proliferation and/or immortalization of the skin keratinocytes,⁴¹ thereby conferring more aggressive and ever-proliferating characteristics to keratinocytes in the middle ear cavity. The upregulation of ID1 in the middle ear epithelial cells is triggered by infectious agents such as *S pneumoniae*, and this upregulation persists in the middle ear cholesteatoma matrix.⁴¹ Recent studies indicate that ID1 is involved in aggressive behaviors of the cholesteatomal matrix through regulation of NF- κ B activity, a key regulator for keratinocyte proliferation and differentiation, and suppression of p16^{INK4a} expression, a checkpoint protein for the cell cycle progression of keratinocytes.⁴² These factors contribute significantly to the pathogenesis of cholesteatoma. In addition, ID1 has also been suggested to play a role in the pathogenesis of the cholesteatomal development⁴² via a TGF- β -dependent mechanism in OM.³⁸

Mucus and mucin: Although undoubtedly important in the mucosal defense against microbes, it is clear that excessive and exacerbated production of mucus in OM contributes significantly to the pathogenesis of OM by overloading the host mucociliary escalator function and resulting in conductive hearing loss. Thus, mucus production in the middle ear must be tightly regulated. Understanding the molecular mechanisms underlying mucin production and regulation is important for the development of adequate therapies to prevent mucus overproduction.

Mucous Production—In humans, mucins are encoded by 20 mucin genes (MUCs). Among these, 16 MUC genes are found to be expressed in the normal human middle ear epithelium in vivo and also in HMEECs in vitro,⁴³ as well as mouse middle ear epithelium (MMEE)⁴⁴ and chinchilla middle ear epithelium (CMEE).³⁵ Three MUC genes (MUC6, MUC12, MUC17) were not expressed and MUC21 was not evaluated in the middle ear.⁴³ Although most of the mucin genes are expressed in the middle ear mucosa, the major mucin genes in OM appear to be MUC2, MUC5AC, and MUC5B.^{45–47} In addition, recent studies also suggest the involvement of other MUC genes, such as MUC1, MUC3, MUC4, MUC6, MUC7, MUC8, MUC9, MUC11, MUC12, and MUC19, in clinical human OM effusion,

cultured middle ear epithelial cells, and animal models with middle ear inflammation.^{43–50} However, their roles and the underlying regulatory mechanism in OM have yet to be fully investigated.

Regulation of Mucin Expression—Upregulation of mucin expression has been found to be induced by both microbial and host-derived factors, including OM pathogens, cytokines, and growth factors. Otitis media pathogens not only activate host positive signaling pathways but also suppress inhibitory signaling pathways to upregulate mucin expression as exemplified by *S pneumoniae* pneumolysin-mediated inhibition of the TLR4-dependent JNK signaling pathway via MAPK phosphatase-1 (MKP-1) to enhance extra-cellular signal-regulated kinase (ERK)–mediated MUC5AC expression.⁵¹ In addition, OM pathogens also cooperate with other pathogens or pathogenic factors to synergistically upregulate the expression of mucin. For example, NTHi and *S pneumoniae* synergistically induce MUC5AC expression.⁵² Epidermal growth factor (EGF) synergistically upregulates NTHi-induced MUC5AC expression.⁵³ Moreover, *S pneumoniae* was found to activate 2 activator protein 1 (AP-1) sites in the promoter region of the MUC5AC gene via distinct signaling pathways, leading to differential regulation of MUC5AC gene expression.⁵⁴ All of these studies demonstrate the complexity of the tight regulation of mucin gene expression via various mechanisms.

In addition, cytokines are also found to upregulate mucin. Interleukin-10 induces mucin overproduction via mediating NTHi- and *S pneumoniae*–induced mucous cell metaplasia and hyperplasia in mice.³⁶ Interleukin-1 β (IL-1 β) regulates mucus secretion via activation of the Na-K-2Cl-cotransporter (NKCC).⁵⁵ Exposure to cigarette smoke was thought as one of the potential risk factors for OM. The molecular mechanism by which cigarette smoke regulates mucin expression in the middle ear was not well understood. Recent studies have shown that cigarette smoke induces MUC5AC expression via EGFR⁵⁶ and upregulates MUC5B expression via NF- κ B pathways.⁵⁷ In addition to MUC gene expression, cigarette smoke also induces goblet cell proliferation and excess mucus secretion.⁵⁸ Moreover, host factors, such as gastric acids, were also found to upregulate MUC5B in middle ear epithelial cells.⁵⁹ Thus, it is evident that multiple factors, including both host- and non-host-derived factors contribute to the pathogenesis of OM.

Recent Advances in Biochemistry of Otitis Media

Significant progress in OM research has also been made in the area of biochemistry. These advances include the identification of biochemical markers during various stages of OM pathogenesis through the characterization of the components of chronic otitis media (COM) effusion (eg, mucins, cytokines, chemokines, bactericidal molecules, and growth factors) and the elucidation of the biochemical basis of middle ear and inner ear interaction.

Biochemical Basis of Otitis Media

Otitis media is an inflammatory response to either acute or persistent stimuli characterized by the accumulation of both cellular and chemical mediators in the middle ear cavity. It is caused by multiple factors, including bacterial or viral infection, eustachian tube dysfunction, allergy, and barotraumas. There are many cellular and biochemical events as a

result of tissue injury induced by inflammation. Central factors to the formation of inflammation are the presence of inflammatory mediators, which include proteins (glycoproteins), peptides, cytokines, arachidonic acid metabolites, macrophage migration inhibitory factor, nitric oxide, and free radicals. These compounds are produced by epithelial cells, middle ear mucosa, and infiltrating inflammatory cells.

Inflammatory Cytokines in Middle Ear Effusion—Inflammatory mediators are known to diffuse into the inner ear across the round window membrane, possibly causing damage to the cells of the inner ear and leading to various types of auditory dysfunction.^{60–63} The genes of numerous inflammatory cytokines are either up- or downregulated in murine inner ear cells in response to acute or chronic inflammation of the middle ear.⁶⁴

It appears that IL-1 β and TNF- α are involved in the initial phase of the inflammatory processes. Interleukin-2 is involved in T-cell proliferation and induces other cytokines, including IL-4, IL-5, IL-13, and granulocyte macrophage colony-stimulating factor (GM-CSF). These cytokines participate in the regulation of molecular and cellular processes involved in different types of chronic inflammation. The presence of cytokines in the middle ear cavity may cause disruption in the normal balance of inflammatory cytokines within the lateral wall and may hinder the recycling of ions causing hearing impairment. It has been reported that eosinophilia in middle ear effusions (MEEs), as well as in middle ear mucosa (MEM), is closely related to atopy and asthma. Upregulation of thymus and activation-regulated chemokine (TARC) in the cultured middle ear-derived fibroblasts by the Th2 cytokines (IL-4 and IL-13) was reported.⁶⁵ The production of these inflammatory mediators, along with the production of VEGF, causes an increase in the vascular permeability that results in the formation of MEE. Mucoid otitis media (MOM) middle ear effusions found in COM are characterized by the presence of mucoid glycoproteins. Both MUC5AC and MUC5B were detected in mucous middle ear effusions.⁶⁶ However, factors participating in the regulation of molecular and cellular processes leading to the chronic inflammation remain to be further investigated. The availability of MEE for laboratory analyses provides a means for identifying biochemical markers that may help characterize the types and stages of inflammation in OM.

Aquaporins—Aquaporins (AQPs) facilitate water movement within specific organs, allowing water to move along the osmotic gradient. They appear to be involved in the water balance of the middle ear. The expression of various AQPs (AQPs 1, 3, 4, 5, 7, 8, and 9) has been reported in the tissues and cell linings of tubotympanum.^{67–69} The role of these AQPs in the MEE formation needs to be further studied.

Apolactoferrin—Management of OM is one of the important issues to be investigated. Studies have been performed on the inhibition of certain biochemical components involved in the pathological changes in the middle ear cavity. The effect of administration of apolactoferrin, the iron-free form of lactoferrin, on the middle and inner ears after experimentally induced pneumococcal OM was also studied.⁷⁰ Bacterial counts of MEE and the number of inflammatory cells in the round window membrane (RWM) were significantly lower in the apolactoferrin-treated group compared with the control group.

Since antibiotic-resistant bacteria have become a problem, the significant reduction of bacteria in the middle and inner ear, as well as reduced damage to the RWM compared with the controls, is noteworthy. Further studies, using a topical application of exogenous apolactoferrin alone or in combination with other antimicrobial and/or anti-inflammatory agents for the treatment of acute OM (AOM), would be helpful in evaluating their therapeutic potential.

Middle Ear and Inner Ear Interaction

It has been well known that patients with COM develop sensorineural hearing loss, suggesting an interaction between the middle and inner ear.

Human Studies—Paparella⁷¹ suggested that certain sudden deafness associated with vestibular symptoms can be caused by middle and inner ear interactions. It was postulated that the 2 most common causes of idiopathic sudden deafness were viral endolymphatic labyrinthitis and middle ear/inner ear interaction, commonly caused by infections or barometric trauma to the middle ear, generated by activities such as nose blowing or scuba diving. These events can damage the inner ear via the RWM, resulting in impairment to inner ear elements, including sensory cells.

Yoshida et al⁷² reported sensorineural hearing loss with COM, specifically examining the role infection and aging played in older patients. Bone conduction (BC) hearing thresholds of 180 preoperative patients (207 ears) with COM and 226 normal individuals (289 ears) were measured by audiometry, and the percentage of ears with BC thresholds being higher than normal range was evaluated in the COM group. In the COM group, the size of the perforation on the eardrum (n=196) and the cross-sectional area of the mastoid air cells based on the axial computed tomography (CT) image (n=103) were also measured and correlated with the results of the BC threshold. When compared with the control group, the percentage of ears with higher than normal BC thresholds tended to increase with age, ranging from 4.5% in the 20s to 34.1% in the 60s. The increase in the BC thresholds did not correlate with the size of eardrum perforation but instead was closely associated with the size of the mastoid air cells. The authors suggested that all measures for an early cure, including surgery, should be considered as rapidly as possible for patients with COM.

Joglekar et al⁶¹ described cochlear pathology in human temporal bones with OM. They used 614 temporal bones with OM and selected 47 with chronic and 35 with purulent OM following strict exclusion of subjects with a history of acoustic trauma, head trauma, ototoxic drugs, and other diseases affecting the cochlear labyrinth. Temporal bones with labyrinthine inflammatory changes were further evaluated for loss of hair cells and other histopathologic changes compared with age-matched controls. In all, 19% of temporal bones with chronic and 9% with purulent OM showed labyrinthine inflammatory changes. In chronic OM, inflammatory changes were as follows: 56% localized purulent, 22% localized serous, 11% generalized seropurulent, and 11% generalized serous. Inflammatory changes in temporal bones with purulent OM included 67% localized purulent and 33% generalized seropurulent. Pathological findings included serofibrinous precipitates and inflammatory cells in scala tympani of basal turn and cochlear aqueduct, significant loss of outer and inner

hair cells, and significant decrease in the area of stria vascularis in the basal turn of the cochlea, as compared with controls. They concluded that middle and inner ear interactions in OM can lead to cochlear pathology. More severe pathological changes observed in the basal turn of the cochlea are consistent with prevalence of sensorineural hearing loss at higher frequencies in patients with OM.

Animal Studies and Molecular Biological Basis—The existence and characterization of the blood labyrinthine barrier (BLB) have been previously reported. The integrity of this barrier is essential for auditory function. The inflammatory cytokines (IL-1 β and TNF- α) present on the round window membrane caused an increase in the permeability of the BLB and resulted in auditory dysfunction. It is quite conceivable that an elevation of cytokines in the middle ear cavity due to OM can cause hearing impairment.

MacArthur et al⁷³ investigated the control of COM and sensorineural hearing loss in C3H/HeJ mice using glucocorticoids vs mineralocorticoids. They used 7 to 17 mice per treatment group. Auditory brain stem response (ABR) thresholds were performed at baseline, 2 weeks, and 4 weeks. Histopathologic test results were evaluated on all mice ears at the end of the study. Analysis of variance (ANOVA) of ABR threshold change showed significant treatment effects ($P < .05$) by both steroid types at all time intervals and ABR frequencies except 4 weeks/8 kHz. Histologic assessment showed prednisolone-treated mice (62%) had a higher rate of clearance of middle and inner ear inflammation than did control mice (4%). They concluded that steroid treatments can improve the physiology of chronic middle and inner ear disease seen with COM.

Cytokines in the middle ear cavity may cause disruption in the normal balance of inflammatory cytokines within the lateral wall and may hinder the recycling of ions, causing hearing impairment. Recurrent AOM leads to sensorineural hearing loss by unknown mechanisms. It is widely accepted that inflammatory cytokines diffuse across the round window membrane to exert cytotoxic effects. Ghaheri et al⁷⁴ investigated cochlear cytokine gene expression in murine AOM. BALB/c mice underwent transtympanic injection of heat-killed *H influenzae* to create an acute inflammatory response. These mice were compared with a control group, in addition to a group of uninjected mice, and found to have otomicroscopic changes consistent with persistent or COM. The cochleas of these mice were obtained, their RNA harvested, and cytokine gene expression analyzed using prefabricated cDNA arrays. Four groups of mice (control, 3-day postinjection, 7-day postinjection, and mice with COM) with 5 mice in each group were analyzed. Numerous classes of genes were found to be upregulated or downregulated by more than 2-fold. Some genes differed from control mice by more than 10-fold. These genes included numerous fibroblast growth factors, interleukins, tumor necrosis factors, and colony-stimulating factors. They concluded that the genes of numerous inflammatory cytokines are either up- or downregulated in murine inner ear cells in response to either acute or chronic inflammation of the middle ear. Their study provides a novel site of production of cytokines that may be responsible for the damage seen in sensorineural hearing loss.

Previous gene expression array studies have shown that cytokine genes might be upregulated in the cochleas of mice with acute and chronic OM. This finding implies that

the inner ear could manifest a direct inflammatory response to OM that may cause sensorineural damage. Therefore, to better understand inner ear cytokine gene expression during OM, quantitative real-time polymerase chain reaction and immunohistochemistry were used in mouse models to evaluate middle and inner ear inflammatory and remodeling cytokines. MacArthur et al⁶⁴ reported altered expression of middle and inner ear cytokines in mouse OM. They induced AOM in BALB/c mice by a transtympanic injection of *S pneumoniae* in one ear while the other ear was used as a control. C3H/HeJ mice were screened for unilateral COM, with the noninfected ear serving as a control. Both acute and chronic OM caused both the middle ear and inner tissues in these 2 mouse models to overexpress numerous cytokine genes related to tissue remodeling (TNF- α , bone morphogenetic proteins, fibroblast growth factors) and angiogenesis (VEGF), as well as inflammatory cell proliferation (IL-1 β , IL-2, IL-6). Immunohistochemistry confirmed that both the middle ear and inner ear tissues expressed these cytokines. They concluded that cochlear tissues do express cytokine mRNA that contributes to the inflammation and remodeling that occur in association with middle ear disease, thereby providing a potential molecular basis for the transient and permanent sensorineural hearing loss often reported with acute and chronic OM.

Recent Advances in Animal Models of Otitis Media

Animal models of OM are important research tools, since they allow access to the entire course of the disease and are subject to experimental manipulation. Because of their importance, there has been continuous and significant work done to develop additional and improved animal models for this condition. Those advances include (1) animal model of spontaneous OM using MyD88 and TLR2 KO mice; (2) animal model of induced OM with increased severity and duration using TLR2, 4, 9, Trif, dynactin subunit 4 (DYA4) KO mice and ribosomal protein L38 (RPL38) mutant mice; (3) animal model of polymicrobial infection, including virus and bacteria; and (4) animal model of viral infection.

Mouse Genetics and Animal Models

Genetic Resources—Recently, novel OM genes in chemical (ENU) mutagenesis programs linked to phenotypic screens for deafness and vestibular signs were discovered. The gene targeting and the international efforts, including the European Mouse Disease Clinic (EUMODIC), Knockout Mouse Project (KOMP), and North American Conditional *Mouse* Mutagenesis (NORCOM) programs, to produce KO of all genes in the mouse genome are gaining momentum. In addition, embryonic stem cells are a publicly available resource for the scientific community. The International Mouse Phenotyping Consortium (IMPC) is funded by the National Institutes of Health (NIH), MRC Wellcome Trust, and Genome Canada. The 3 consortia—UC Davis–Toronto, Regeneron–Jax, and Baylor–Sanger Wellcome Trust–MRC Harwell—are funded to share the work. Phase 1 (2011–2016) aims to turn 5000 targeted embryonic stem cells into mutant mice and also perform the baseline phenotyping (includes ABRs). Studies at Sanger Wellcome Trust indicate that this effort will lead to the identification of novel sensorineural and OM mutants. Phase 2 (2016–2021) of the program will aim to deliver an additional 15,000 mutants.

Translation of Genetic Insights—The findings from mouse studies can be applied in a myriad of ways to humans through genetic studies. Candidate genes for human disease association studies (eg, polymorphisms in F-box protein 11 are associated with recurrent or chronic OM replicated in 2 separate populations in the United States and Australia) have been identified. Identification of genetic pathways and mechanisms; the interest in identifying the gene underlying *Jeff*, a dominant mouse mutant displaying chronic OM; and a TGF- β signaling mutant highlighted the possible role of TGF- β -induced factor, another TGF- β signaling pathway member (TGF- β -induced factor KO used for the study had an OM phenotype). Possibility of testing the candidate genes identified in *genome-wide association studies* (GWAS) by making mouse models (eg, fat mass obesity gene) is a great opportunity. New regulatory mechanisms were also identified (eg, identification of Evi1 as a negative regulator of NF- κ B).⁷⁵

Enhanced Animal Models—In addition to KO alleles, EUCOMM alleles will produce a β -galactosidase (LacZ) reporter mouse by crossing to a ubiquitous Cre recombinase line and a conditional floxed allele (the sandwiching of a DNA sequence between 2 lox P sites) by breeding with an Flp recombinase transgene mouse. Availability of various Cre recombinase driver lines will provide opportunities for site-specific or temporal gene deletion. Hypomorphic and hypermorphic alleles can be generated by screening of the mutagen ENU archive and regenerating mice by in vitro fertilization from sperm. Acute OM challenge models and enhanced models for challenge studies with human pathogens (eg, C3H/HeJ TLR4-deficient background enhances susceptibility to gram-negative bacteria) will provide a platform for studying pathogen-specific patterns. Transgenic mice with humanized receptors (eg, rhinovirus) and chronic OM models (*Junbo* and *Jeff*, 2 novel deaf mutant mice) that developed chronic lifelong disease are new additions to the growing list of animal models.

Translational Opportunities

When designing and developing new treatments, testing the efficacy of drugs (eg, moderation of acute inflammatory response using EGFR kinase inhibitor in the NTHi-challenged model and moderation of hearing loss in the chronic model using VEGFR kinase inhibitor in the *Junbo* mutant mouse) is critical. New models for vaccine research (eg, proposed plan to establish a chronic NTHi infection in *Junbo* mouse model) and testing novel drug delivery systems will be crucial in shaping the translational approaches.

Implications for Clinical Practice

Otitis media is the most common childhood bacterial infection and also the leading cause of conductive hearing loss in children. Despite an obvious need for prophylactic measures, development of highly effective vaccines for OM still remains a great challenge. Moreover, inappropriate antibiotic treatment of OM has increased antibiotic resistance substantially. Currently, there are no effective therapeutic agents available for treating OM due to the poor understanding of the molecular, cellular, and biochemical basis of the pathogenesis of OM. Therefore, development of novel therapeutic strategies is urgently needed for treating OM based on fully investigating the molecular mechanism and identifying the key molecular

therapeutic targets. To this end, the following short- and long-term goals in the areas of molecular biology, genetics, biochemistry, and animal model studies need to be pursued. Successfully achieving these goals would ultimately lead to the development of novel therapeutics for treating OM.

Molecular Biology and Genetics of Otitis Media

Short-Term Goals

1. Further study of host gene expression in OM, including differences in the responses to various pathogens
2. Further study of bioinformatics analysis of gene networks activated in the ME during OM, using genomics and proteomics approaches
3. Further study of pathogen gene expression during OM, including viruses
4. Further study of the interaction of host and pathogen gene expression using both mouse and pathogen mutants as well as genomics such as gene arrays and deep sequencing
5. Identify the gene targets and functional consequences of various cell signaling networks in ME cells
6. Develop transfection, transduction, small interfering RNA (siRNA), and other technologies for in vivo up- and downregulation of genes as well as gene therapies in the ME
7. Use transgenic and mutant bacterial models to understand pathogenesis, virulence, and biofilm formation
8. Study regulatory sequences that target gene expression to the ME and epigenetic modification and its influence on OM pathogenesis
9. Further identify additional mutations that cause OM in human and animals
10. Further identify polymorphisms contributing to susceptibility to OM

Long-Term Goals

1. Understand how the complex cell signaling pathways and gene regulatory networks are induced to produce various outcomes in OM
2. Translate molecular findings on cell signaling and gene regulation during OM into improvements in patient care by targeting key regulators of pathogenesis and recovery

Biochemistry of Otitis Media

Short-Term Goals

1. Explore the link between pathogens and pathogen combinations and the production of key molecules such as cytokines, chemokines, growth factors, and bactericidal molecules

2. More precisely define mucus components and their modifications in various types of ME effusions and nasopharyngeal secretions
3. Explore combinatorial strategies for modifying the various phenotypes of ME cells
4. Explore new methods for high-throughput screening in ME cells to identify compounds that have therapeutic potentials

Long-Term Goals

1. Perform more detailed proteomic analysis of ME, eustachian tube, and nasopharynx during OM to explore posttranslational processing of gene products
2. Define the biochemistry of ME mucin degradation to aid in the development of treatments for mucoid OM
3. Identify diagnostic and prognostic markers of different stages and varieties of OM using various more advanced biochemical methods, including proteomic and glycobiology methods
4. Develop new biochemistry-based therapeutic strategies

Animal Models of Otitis Media

Short-Term Goals

1. Further standardize phenotype determination in mouse models of OM
2. Develop better animal models of chronic OM, including sequelae such as cholesteatoma
3. Develop better models of mucoid and serous OM (OME)
4. Develop models of conditional gene expression in the ME, including both site specificity and inducibility
5. Study polymicrobial effects in ME, ET, and nasopharynx in vivo
6. Identify the susceptibility of inbred mouse strains to induced OM
7. Generate mouse models of candidate OM disease genes

Long-Term Goals

1. Improve all of our existing animal models of OM and continue to develop new models
2. Use diversity of animal models to study OM and to ensure that differences between any one species and humans do not bias our data

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Appendix

Abbreviations

ABR	Auditory brain stem response
AOM	Acute otitis media
AP-1	Activator protein 1
AQPs	Aquaporins
BLB	Blood labyrinthine barrier
COM	Chronic otitis media
CYLD	Cylindromatosis
DYA4	Dynactin subunit 4
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinases

Evi1	Ecotropic viral integration site 1
EUMODIC	European Mouse Disease Clinic
FoxA2	Forkhead box transcription factor A2
GM-CSF	Granulocyte macrophage colony-stimulating factor
GWAS	Genome-wide association studies
HMEEC	Human middle ear epithelial cell
ID1	Inhibitor of differentiation 1
IL	Interleukin
JNK	c-Jun N-terminal kinases/stress-activated protein kinase
KOMP	Knockout mouse project
MAPK	Mitogen-activated protein kinase
MEE	Middle ear effusion
MEM	Middle ear mucosa
miRNA	MicroRNA
MKP-1	MAPK phosphatase-1
MOM	Mucoid otitis media
MyD88	Myeloid differentiation primary response gene 88
NF- κ B	Nuclear factor- κ B
NORCOM	North American Conditional Mouse Mutagenesis
NTHi	Nontypeable <i>Haemophilus influenzae</i>
RWM	Round window membrane

RPL38	Ribosomal protein L38
SPLUNC	Short palate lung and nasal epithelial clone
<i>S pneumoniae</i>	<i>Streptococcus pneumoniae</i>
TARC	Thymus and activation-regulated chemokine
TGF- β	Transforming growth factor- β
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor- α
TRIF	Tir domain-containing adaptor inducing interferon β
TTF-1	Thyroid transcription factor 1
VEGF	Vascular endothelial growth factor