Panel 5: Immunology

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract

Objective. To perform a state-of-the-art review of the literature from January 2012 through May 2015 on studies that advanced our knowledge of the innate and adaptive immunology related to otitis media. This review also proposes future directions for research in this area.

Data Sources. PubMed database of the National Library of Medicine.

Review Methods. Three subpanels comprising experts in the field focused on sections relevant to cytokines, innate immunity, and adaptive immunity. The review focused on animal, cell line, and human studies and was critical in relation to the recommendations from the previous publication and for determination of the proposed goals and priorities. The panel met at the 18th International Symposium on Recent Advances in Otitis Media in June 2015 to consolidate its prior search results and discuss, plan, and refine the review. The panel approved the final draft.

Conclusion. From 2012 to 2014, tremendous progresses in immunology of otitis media were established—especially in the areas of innate immunity associated with the pathogenesis of otitis media.

Implications for Practice. The advances of the past 4 years formed the basis for a series of short- and long-term research goals in an effort to guide the field. Accomplishing these goals will provide opportunities for the development of novel interventions, including new ways to better treat and prevent otitis media, especially for recurrent otitis media.

Keywords

otitis media, immunity, inflammation, cytokines

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(OME), and chronic suppurative OM. In 4 years (2011-2015), remarkable advances in understanding the innate immunology of the ear and its correlates with the pathogenesis of OM were undertaken.

**Human and Human Cell Line Studies.** **Viral-Bacterial Coinfection and TLRs.** The relationship between viral and bacterial coinfection remained a constant theme with advances in understanding a role for differences in TLR expression and function in OM-prone children. The key findings of the studies described here are summarized in **Table 1.** The studies suggest that lower innate and adaptive immune responses to respiratory syncytial virus (RSV), but not parainfluenza virus, in OM-prone children might slow the kinetics of viral clearance from the nasopharynx and allow for viral interference with antibacterial immune responses. These corresponded to changes in TLR-3/7 transcripts and decreased expression of HLA-DR (human leukocyte antigen D-related) on antigen-presenting cells, contributing to increased frequency of AOMs. Age was a differentiating factor, with lower expression levels of TLR-4, TLR-5, TLR-9, and nucleotide-binding oligomerization domain mRNAs in children with OME aged 2 to 7 years associated with colonization, nitric oxide synthase, and cytokines. In contrast, respiratory mucosa TLR-2, TLR-3, TLR-4, TLR-5, and TLR-7 were lower, and NOD2 higher, only in children with chronic suppurative OM. It was proposed that reduced TLR levels in the ME mucosa might cause weak host responses to the bacteria and be associated with susceptibility COM but also that single-nucleotide polymorphisms on particular receptors may play a role.

Bacteria have been reported to manipulate signaling where nontypeable *Haemophilus influenzae* (NTHi) upregulates β-defensin 2 via TLR-2 signaling, and in human ME epithelial cells, β-defensin 2 production can be inhibited by NOD2 silencing but augmented by NOD2 overexpression. Cytoplasmic release of internalized NTHi was involved in pathogenesis with NOD2-mediated β-defensin 2 regulation contributing to the protection against NTHi-induced OM.

Biofilm is known to play a role in the persistence of bacteria in the MEs of children with COM and/or rAOM. The relationship between the airway epithelia and the colonizing bacteria influenced whether biofilm formation occurred, with a report that contact with respiratory epithelial cells increased biofilm formation during coinfection with NTHi and *Streptococcus pneumoniae* (*Spn*). The key contributor to the increased biofilm formation was the upregulation of epithelial cell factors that activated the bacteria.

Differences in the clinical settings, study design, and stringencies for defining the OM populations and responses in vivo, in situ, or in vitro remained a challenge for comparing data for the innate immune responses of individuals associated with OM. Study designs range from patient referrals with limited prior history or standardization of diagnosis to rigorous stringencies for confirming of every AOM event. Nevertheless, the consistency of identified innate immunity problems within the continuum of OM from AOM to OME and COM clearly shows the importance of the innate immune response.

**Nasopharyngeal Mucosal Repair Response.** Studies of nasopharyngeal mucosal repair responses between age-matched stringently defined OM-prone and non–OM prone young children who progressed to ME infection reported that OM-prone children with *Spn* OM demonstrated significantly lower epidermal growth factor, epidermal growth factor receptor, and angiogenin cytokine concentrations in nasal washes. While higher expressions of TLR-2/4 transcripts were also observed in the nasal epithelium of OM-prone children and in the polymorphonuclear cells of their nasal secretions, these children had lower expressions of proinflammatory cytokines interleukin 6 (IL-6) and IL-8 in the nasopharynx. The lower chemotactic- and proinflammatory-associated cytokines suggest a lower capacity to signal the innate immune system. A lower propensity for repair responses during viral infection in the nasopharynx, combined with diminished innate inflammatory responses, could potentiate *Spn* pathogenesis in the OM-prone child.

One study reported that influenza virus HA facilitated disease by inducing a proinflammatory response in the ME cavity in a replication-dependent manner and that influenza infection mediated pneumococcal replication. Demonstrated that levels of lactate dehydrogenase in the nasopharynx were positively associated with AOM risk, suggesting that the severity of nasopharyngeal inflammatory injury during upper respiratory infections contributes to the development of AOM and that reduction of inflammatory injury may reduce the risk for AOM.

**Regulatory T Cells.** Regulation of immune responses associated with regulatory T cells (Tregs) diminishes immune responses to microbial infection and normal protective inflammatory responses and may contribute to the chronicity of infection. The presence and role of Tregs in the adenoids of children has been speculated as a contributing factor to COM. Studies have not been possible in children <2 years of age, when AOM and OME occur most commonly. However, FOXP3+ Tregs were studied from adenoidal tissues associated with pneumococcal carriage in older children in 2 studies. The expression of Treg cell–related markers—including FOXP3, CD25, CD39, CD127, and CTLA4—revealed higher numbers of CD25(high)FOXP3(high) Treg cells expressing higher CD39 and CTLA4 in adenoidal mononuclear cells than in peripheral blood mononuclear cells. Pneumococcal carriage correlated with higher proportions of Treg cells and higher CD39 and CTLA-4 expression than those that were culture negative. It was suggested that Treg cells with an effector/memory phenotype that possesses a potent inhibitory effect existed in the adenoidal tissue. Another study found that, in the adenoids of children who were positive or negative for pneumococcal carriage, FOXP3+ Treg cells were upregulated and Th17 cells downregulated in the pneumococcus-positive group. After stimulation, the increment in FOXP3+ Treg cells in the pneumococcus-positive group was significantly greater than that in the
pneumococcus-negative group, with Th17 cells having a lower increment. Additionally, IL-17A and IL-6 levels were higher in the pneumococcus-negative group, and the authors concluded that carriage in children might be closely associated with the expressions of Foxp3+ Treg and Th17 cells in the adenoid.

### Table 1. TLRs and Innate Immune Moieties: Impact of OM.

<table>
<thead>
<tr>
<th>Clinical Group</th>
<th>Measurements</th>
<th>Key Findings</th>
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<tr>
<td>AOM in OM-prone (n = 42) and non–OM prone (n = 267) children</td>
<td>RSV and parainfluenza and AOM by Spn and NTHi</td>
<td>RSV but not parainfluenza OM-prone children had diminished T-cell responses to that correlated with lower TLR-3/7 transcript and decreased expression of human leukocyte antigen–antigen D related on antigen-presenting cells. RSV interfered with the Spn phagocytic capacity. TLR-2, -4, -6, and -9 mRNAs were significantly lower in the OM-prone than in the non–OM prone group (P &lt; .05).</td>
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<tr>
<td>OM with effusion in 4 age groups: 0-2, 2-4, 4-7, and &gt;7 y (n = 95)</td>
<td>Expression of mRNAs for TLRs, NOD-like receptors, and cytokines in middle ear effusion and correlated with age, sex, presence of bacteria, and accompanying disease</td>
<td>32.6% of patients had detectable bacteria. Receptor and cytokine mRNAs lower in children aged 2-7 years. TLR-2, TLR-9, NOD-1, NOD-2, IL-1, IL-6, and TNF-alpha mRNAs in middle ear effusion were lower for 2-4 and 4-7 y than 0-2 and &gt;7 y (P &lt; .05). TLR-4, TLR-5, TLR-9, and NOD-1 mRNAs lower in culture positive children (P &lt; .05).</td>
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<td>Non-OM (n = 68), COM (n = 71), CSOM (n = 72)</td>
<td>Collected during tympanotomy or mastoidectomy. RT-PCR for TLR-2, TLR-4, TLR-5, TLR-9, TNF-α, IL-1β, IFN-γ, IL-6</td>
<td>The cellular composition of the ME mucosa differed among the 3 groups. CSOM group presented suppurative inflammation in the rudimentary stroma, with infiltration of monocytes and macrophages. mRNA and protein levels of TLR-2, TLR-4, and TLR-5 between the non-OM and COM showed no differences but were lower in the CSOM. TNF-α, IL-1β, IFN-γ, IL-6 upregulated in the CSOM group.</td>
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<td>COM (n = 47) and controls (n = 46)</td>
<td>Middle-ear mucosa TLR-4, TLR-5, TLR-7, NOD-2, Nalp3, and TLR-3 mRNA based on RT-PCR</td>
<td>TLR-3, TR-4, TLR-5, and TLR-7 lower in COM children. NOD-2 was upregulated in COM</td>
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<tr>
<td>SNPs of genes in children with OM: OM (n = 70) and non-OM (n = 70); OM prone (n = 317) and non–OM prone (n = 336)</td>
<td>Genomic DNA was extracted from blood samples or buccal epithelial cells for RT-PCR genotyping or Sequenom MassARRAY.</td>
<td>TLR-2, TLR-4, TLR-9, and CD-14 gene SNPs were not more prevalent in OM-prone children. CX3CR1 (Thr280Met) SNP with a jointly interactive group of IL-10 (-1082) SNP, IL-1β (-511) wild-type genotype, and white race was associated with OM-proneness. IL-1β (-31) SNP was associated with increased risk for frequent upper respiratory infections, but IL-10 (-592), IL-1β (-511), IL-5 (-746), and IL-8 (-251) SNPs were associated with decreased risk of upper respiratory infections. IL-1β (-31), CX3CR1 (Thr280Met), IL-10 (-1082), and IL-1β (-511) SNPs were associated with increased risk for OM proneness.</td>
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<tr>
<td>Human middle ear epithelial cell line</td>
<td>Gene silencing</td>
<td>NTHi upregulates β-defensin 2 via TLR-2 signaling. Human middle ear epithelial cell line inhibited NTHi-induced β-defensin 2 production by NOD2 silencing but augmented it by NOD2 overexpression. β-defensin 2 upregulation was attenuated by cytochalasin D and α-hemolysin. Silencing NOD2 blocked α-hemolysin-mediated enhancement of NTHi-induced β-defensin 2 upregulation.</td>
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Abbreviations: AOM, acute otitis media; COM, chronic otitis media; CSOM, chronic suppurative otitis media; IL, interleukin; NTHi, nontypeable Haemophilus influenzae; OM, otitis media; RSV, respiratory syncytial virus; RT-PCR, reverse transcription polymerase chain reaction; SNP, single-nucleotide polymorphism; TLR, Toll-like receptor.
It is difficult to obtain control tissues for comparative studies of the adenoid, with removal of adenoids due to hypertrophy (AH) being 1 source. The correlation of adenoidal lymphocyte subsets and symptom severity was found to have a negative linear correlation between the Th17/Treg ratio and the patients’ clinical scores, which was independent of age and sex in older children. With the above findings, there appears to be a relationship between Treg and Th17 cells in the nasopharynx and disease status.

**Transcription Signatures.** Two studies that analyzed the transcriptome signatures elicited from peripheral blood mononuclear cells of children (10-15 months) before and at onset of AOM caused by *Spn* or NTHi found that 5% to 6% of total 29,187 genes analyzed were differentially regulated >2-fold at the onset of AOM when compared with the preinfection healthy state in the same children and were dominated by genes involved with the immune response. Signature differences were observed in children infected by NTHi or *Spn*, with a downregulation of genes associated with antibacterial activity for NTHi and an upregulation in responses observed with *Spn* infection.

**Animal Studies.** Many significant advances have been made through the use of animal models, in which highly controlled OM can be created and enable the role of specific genes to be evaluated through genome manipulation. Details of the key findings are found in Table 2.

**Gene and Molecular Contributions.** From the onset of infection through to its complete resolution, advances have been made via gene arrays. Genes involved in innate and adaptive immunity—including innate immune receptors, downstream signaling molecules and effectors, pro- and anti-inflammatory genes, and antimicrobial compounds (especially cathelicids)—change throughout AOM.

Many genes and proteins have now been identified as playing essential roles in the effective response to infection, regulation of inflammation, and resolution of infection. These include macrophage migration inhibitory factor, the multifunctional gene Ecrg4, interaction with TLR-4, type 1 plasminogen activator inhibitor, secreted protein SPLUNC1, and the complement C5a receptor. The complement system has regained some prominence with factor b in the alternative pathway and C3, with both having a pivotal role in innate immune defense against *Spn*-based early-stage AOM.

Defensins—broad-spectrum cationic antimicrobial peptides—have been implicated in the prevention of and early response to AOM; however, the mechanisms by which defensins and other antimicrobial molecules mediate this protection have not been completely elucidated. Extracellular DNA is part of the biofilm matrix and serves as a structural component. Human β-defensin 3 is a cationic antimicrobial host defense protein critical to the protection of the ME. Extracellular DNA in NTHi biofilms sequestered β-defensin 3, thus diminishing the biological activity of an important effector of innate immunity, and the intratympanic viral vector delivery of β-defensin 2 attenuated experimental NTHi OM in an animal model.

**Innate Immunity.** Innate immune receptors in the resolution of OM have an important role in activating intracellular signaling cascades that produce the molecules involved in the resolution of infection, including cytokines, chemokines, and antimicrobials. TLR genes and their signaling molecules were generally upregulated in OM in murine studies. Of interest was TLR-9 with NTHi and DNA sensing in OM pathogenesis and recovery and the deletion of c-Jun N-terminal kinase 1 in delaying recovery from OM while c-Jun N-terminal kinase 2 deficiency prevented recovery (Table 2).

Demonstration that deletion of virtually any innate immune receptor, signaling molecule, or major effector delays OM recovery suggests that an intact innate immune signaling system is critical to the resolution of bacterial OM. Animal studies in general illustrated the diversity of innate immune processes that are required for the timely resolution of AOM. The contribution of NOD-like receptors, the inflammasome, and IL-1β activation in OM showed that activation of the adaptor molecule ASC played a critical role, particularly for IL-1β maturation, leukocyte recruitment, and macrophage phagocytosis activity. Inflammatory defects were linked to an increase in the degree and duration of ME mucosal hyperplasia and delayed bacterial clearance. Regulation through Treg cells has been less studied in the nasopharynx and ME than in other mucosal sites and was a short-term goal in the previous report. CD4+ CD25+ FoxP3+ Treg cells accumulated in the ME, with the percentage of Treg in the ME mucosa increasing for up to 2 months after infection, while levels of IL-1β, TNF-α, IL-10, and TGFβ were increased over the same period. Treg depletion resulted in 99.9% clearance of the bacteria and reduced inflammatory cytokines, confirming their role.

**Signaling Molecules That Mediate and Regulate Immunity and Inflammation**

Many studies of signaling and cytokine production involved in the pathogenesis of AOM and experimental OM focused on mechanisms of activation, inhibition, and control. Figure 1 shows the main receptors and signaling pathways that lead to transcription of the mediators of inflammation and infection responses.

**Human and Cell Line Studies.** Several studies investigated the production of cytokines and chemokines. Study of IL-6, IL-10, TNF-α, IFN-γ, and TGF-β1 genes found that IL-10 and TGF-β1 genotypes appeared to be related to the age of AOM onset, multiple AOM episodes, and insertion of tympanostomy tubes. Serum IL-10 was elevated during AOM associated with *Spn* but not NTHi or *Moraxella catarrhalis* (Mcat), with ME effusions (MEEs) mirroring but 10-fold higher than in serum. Other effector cytokines—IL-4, IFN-γ, and TNF-α—did not show the same increases at onset of AOM. This contrasts with a study reporting no differences in the secretion of IL-8, IL-6, and IL-10 from adenoidal cells from older children with OME as compared with hypertrophic adenoids, though a significant increase in the production of IL-5 and TNF-α with OME. Adding to the complexity, bacterial detection was associated with significantly elevated concentrations of IL-1B, IL-
Table 2. Animal Studies Identifying Key Innate Immunity Contributors in OM.

<table>
<thead>
<tr>
<th>Study</th>
<th>Key Findings</th>
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<tbody>
<tr>
<td>Mouse AOM with NTHi&lt;sup&gt;21&lt;/sup&gt;</td>
<td>Regulation of hundreds of genes in first 6 h Over course of AOM, &gt;3600 genes regulated— including innate immune receptors, downstream signaling molecules, pro- and anti-inflammatory genes, antimicrobial compounds (particularly cathelicidins).</td>
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<td>Mouse AOM and blocking of macrophage MIF and lipopolysaccharide-induced OM&lt;sup&gt;23&lt;/sup&gt;</td>
<td>Blocking the activity of MIF by ISO-1 reduced inflammation through involvement of TLR-4 and NF-kB. Reduced MIF activity alleviated mouse AOM.</td>
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<td>Rat AOM with NTHi&lt;sup&gt;24,25&lt;/sup&gt;</td>
<td>Multifunctional gene Ecrg4 reduced leukocyte infiltration of middle ear, possibly through TLR-4, suggesting that Ecrg4 plays a role in coordinating the inflammatory and proliferative response to infection of mucosal epithelium.</td>
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<td>Mouse Spn OM, followed by IAV infection&lt;sup&gt;28&lt;/sup&gt;</td>
<td>Antecedent IAV infection enhanced the severity of Spn AOM. Deficiency in complement C1qa (C1qa&lt;sup&gt;-/-&lt;/sup&gt;) or factor B (Bf&lt;sup&gt;-/-&lt;/sup&gt;) exhibited delayed viral and bacterial clearance and increased mucosal damage. Spn increased complement activation following IAV infection and corresponded with increased C3a and C5a in serum and middle ear. Deficiency in C5a receptor enhanced bacterial clearance.</td>
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<tr>
<td>Mouse Spn OM&lt;sup&gt;29&lt;/sup&gt;</td>
<td>Spn AOM increased gene expression of factor B of the alternative complement pathway and C3 in middle ear.</td>
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<td>Mouse (knockout) and NTHi AOM&lt;sup&gt;26&lt;/sup&gt;</td>
<td>Type I plasminogen activator inhibitor (PAI-1) knockout reduced inflammation, and mice failed to terminate inflammation. Specifically, it affected expression of IL-1β, TNFα, and MIP-2 early in infection but higher at the later stage of OM. Mice showed significant pathologic changes of tympanosclerosis.</td>
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<tr>
<td>Mouse (knockout)&lt;sup&gt;27&lt;/sup&gt;</td>
<td>Decrease in SPLUNC1 secretion increased OM susceptibility.</td>
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<tr>
<td>Mouse AOM with NTHi&lt;sup&gt;32&lt;/sup&gt;</td>
<td>Deletion of TLR-9 prolonged inflammation and delayed bacterial clearance, indicating the role for DNA sensing in infection response.</td>
</tr>
<tr>
<td>Mouse AOM with NTHi&lt;sup&gt;32&lt;/sup&gt;</td>
<td>TLR genes and key signaling genes were upregulated in genome array study. Deletion of key innate immune genes resulted in persistent OM and lack of NTHi clearance.</td>
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<tr>
<td>Mouse chronic otitis media with ET obstruction with NTHi&lt;sup&gt;35&lt;/sup&gt;</td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; CD25&lt;sup&gt;-&lt;/sup&gt; FoxP3&lt;sup&gt;-&lt;/sup&gt; Treg accumulated in the middle ear; the percentage in the middle ear mucosa increased for up to 2 mo after infection. IL-1β, TNFα, IL-10, and TGFβ increased over same period. Treg depletion induced a 99.9% reduction of bacterial counts in middle ear effusions.</td>
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<tr>
<td>Mouse AOM with NTHi in knockout and with gene microarrays&lt;sup&gt;34&lt;/sup&gt;</td>
<td>Nod-like receptors upregulated early in NTHi OM and activated adaptor molecule ASC, essential in the inflammasomes to cleave and activate certain cytokines. Proforms of IL-1β and IL-18 were upregulated. Lack of Asc gene resulted in lack of IL-1β maturation and reduced leukocytes and macrophage phagocytosis. Inflammation defects associated with increased mucosal hyperplasia and delayed NTHi clearance.</td>
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<tr>
<td>Mouse knockout OM&lt;sup&gt;33&lt;/sup&gt;</td>
<td>Lack of Jnk1 gene enhanced, and Jnk2 delayed, neutrophil recruitment. Jnk1 deficiency delayed recovery, and Jnk2 deficiency prevented it. Both isoforms were required for the normal resolution of OM.</td>
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Abbreviations: AOM, acute otitis media; IAV, influenza A virus; IL, interleukin; NTHi, nontypeable Haemophilus influenzae; MIF, migration inhibitory factor; OM, otitis media; TLR, Toll-like receptor.

6, IL-8, IL-10, and IL-17a in MEE in a bacterial load— and species-dependent manner in children with rAOM or COM. Neither the presence of viruses nor a synergistic effect between viral-bacterial co-infections was observed to influence these results. Culture-positive MEE in AOM children had higher levels of mRNA for all cytokines and chemokines (9- to 300-fold) as compared with culture-negative MEE. Polymerase chain reaction–positive/culture-negative MEE for otopathogens showed that cytokine and chemokine levels were similar, but differences were found in TLR-2, TLR-4, and TLR-9 expression (6- to 31-fold) with higher levels of TLRs, cytokines, and chemokines detected in polybacterial infections. A contrasting study found that the expression of TLR-9 decreased and that IL-12, TNF-α, IFN-β, and IL-6 expression tended to decrease with the detection of bacteria with OME. Higher TLR-2 and TLR-4 transcripts and lower expression of IL-6 and IL-8 were observed in the nasal epithelium and polymorphonuclear cells from OM-prone children. A study of obese and non-obese children with OME reported, for obese children, decreased TLR-2-mediated expression of IL-6 mRNA; TLR-4-mediated expression of IL-6 and IL-10 mRNA; TLR-5-mediated expression of IL-6, IL-10, and TNF-α mRNA; TLR-9-mediated expression of IL-6 mRNA; and
NOD2-mediated expression of IL-6, IL-12, and TNF-α mRNA. However, levels of IgG, IgA, and IgM in the MEEs did not differ significantly. Comparison of mucosal fluids and peripheral blood responses from AOM children did reveal differences in the effector cytokine responses, IFNγ and IL-2, for patterned antigen-recall responses of CD4 T cells. The ME cytokine levels did not mirror the blood but were similar to the nasopharynx. Thus, immune responses appeared to diverge in the mucosal and blood compartments at the onset of a bacterial ME infection.

**Animal Studies.** Manipulating inflammation affects OM outcomes. Neutralization of granulocyte colony-stimulating factor in a mouse OM model reduced the levels of IL-1β and TNF and inhibited inflammation. Knockout of IL-17A in mice showed its importance to neutrophil recruitment and induced apoptosis. IL-17A acting through the p38 MAPK signaling pathway was conducive to bacterial clearance of Spn. IL-17A, produced by activated T cells, is associated with many chronic inflammatory diseases. In mice, cytokine genes and their protein products in the middle and inner ears increased at 6 hours for MIP-2, IL-6, IL-1β, IL-10, TNFα, and IL-1α during AOM to H influenzae. Interestingly, the related cytokines were present in the inner ear at higher concentrations (2- to 122-fold) by 18 hours before declining. Damage associated with this inflammatory response within the inner ear could contribute to the permanent hearing loss observed for some cases of acute and COM.

The ME undergoes extensive modifications during OM, potentially involving changes in the expression of many genes. The expression profiles from the MEs of hybrid mice infected with live NTHi showed that approximately 8% of the sampled transcripts profiled defined the signature of acute NTHi-induced OM over infection time. The earliest wave of gene expression, peaking 3 to 6 hours after infection, included genes related to immune and defense responding to biotic stimulus, interleukin, TLR and TNFR signaling, and NFκB and neutrophil activation. A cluster peaking at 6 and 48 hours was enriched for STAT1/2 and IRF1/7/8 targets, with enriched pathways for interferon and JAK/STAT signaling, antiviral responses, the inflammasome, and leukocyte chemotaxis. At 24 hours, genes involved in immune and inflammatory response, chemotaxis, phagocytosis, neutrophil activation, immunologic synapse formation, alternative complement activation, cytotoxicity, and reactive oxygen species production were upregulated. Genes that were downregulated were related to microtubule-based movement, ciliary morphogenesis, and cell projection organization consistent with de-differentiation and possibly the loss of ciliated epithelial cells. The peak at 48 to 72 hours involved genes of the alternative complement pathway, CCL2 signaling, chemotaxis, and phagocytosis. By 5 to 7 days, the ME mucosa had remodeled close to baseline, and the inflammatory infiltrate resolved, although some genes remained upregulated. Understanding the genes involved in the activation and

**Figure 1.** Cytokine production by cells. Schematic showing key receptors, signaling pathways, and cytokines produced by cells in response to infection, immune stimulation, and inflammation.
negative regulation of immune responses, the changes in epithelial and stromal cell markers, and the recruitment/function of neutrophils and macrophages over the course of OM will help identify new therapeutic targets.

**Adaptive Immunity**

**Human Studies.** Differences found in various studies of children with rAOM may in part relate to the diagnosis and criteria for being OM prone. Most studies have used the commonly accepted definitions, as well as parental, general practitioner, and ear/nose/throat reports to assess their cohorts. Debate remains in relation to criteria against which to compare studies, with the Rochester, New York, OM research group having conducted several large in-depth longitudinal studies against a definition of “stringently defined OM prone.”

**Antibody Responses.** Current pneumococcal conjugate vaccines reduce the incidence of AOM through elimination of pneumococcal serotypes in the nasopharynx and transudation of serum IgG to the nasopharynx and ME (the ME is devoid of any immunocompetent cells in normal mucosa). Table 3 presents the major studies and details their key findings. Differences in antibody ratios between the nasopharynx and ME fluids suggest that IgA in MEE could be predominantly derived from sera and possibly reflux via the eustachian tube.

The main otopathogens colonizing the nasopharynx and causing AOM following introduction of the pneumococcal conjugate vaccines are *Spn*, NTHi, and *Mcat*.

Low antibody titers to these are thought to enable bacteria to colonize the nasopharynx that may then disseminate through the eustachian tube into the ME resulting in AOM, though data are conflicting. The association of AOM and antibody levels has been inferred per the peak incidence of AOM that occurs around 1 to 2 years of age, coinciding with low immunoglobulin levels and reduced IgG2 in children with rAOM.

Immunization with the pneumococcal conjugate vaccines reduced OM due to *Spn* serotypes in the vaccines and, in some studies, NTHi following vaccination with the protein D (PD) containing PCV10, confirming the relevance of antibody.

Serum IgG titers to *Spn* proteins revealed that, at the AOM visit, anti-PhtD, anti-LytB, anti-PhtE, and anti-Ply IgG titers in otitis-prone children were lower when compared with non–OM prone and AOM–treatment failure (AOM-TF) children. Convalescent sera showed no change in titers (except for PhtE in children with AOM-TF), with about one-third of children in each cohort having a 2-fold rise in antigen-specific antibody. This indicated that OM-prone and AOM-TF children mounted less of an IgG serum antibody response to *Spn* proteins after AOM and nasopharyngeal colonization. In a similar study for NTHi, OM-prone children mounted less of an IgG serum antibody response toward PD, P6, and OMP26 after AOM, and OM-prone children had significantly lower IgG levels to P6 and OMP26 versus AOM-TF children.

Mucosal IgG and IgA levels to PhtD, PcpA, and Ply appeared to decrease slightly in children from 6 to 9 months of age and then gradually increase through 24 months of age, with children with *Spn* AOM having lower levels to the proteins than children asymptomatically colonized with *Spn*.

In contrast, in a different geography, *Spn* PspsA1, PspsA 2, CbpA, and Ply and NTHi P4, P6, and PD in children with a history of rAOM had significantly higher serum IgG levels against NTHi proteins P4, P6, and PD as compared with healthy controls, whereas there was no difference in antibody levels against *Spn* proteins. Antibody levels increased with age and colonization with *Spn* or NTHi. Another study found that the levels of antigen-specific IgG, IgA, and IgM to 10 *Mcat* proteins and 17 *Spn* proteins had extensive interindividual variation with no significant differences in anti-*Mcat* and anti-*Spn* serum or MEE IgG levels between the rAOM and COME groups. IgG, IgG1, and IgG2 levels measured in serum against 11 pneumococcal polysaccharides (7 serotypes in PCV7; 4 nonvaccine serotypes) in children had extensive interindividual variation with no significant differences in anti-*Mcat* and anti-*Spn* serum or MEE IgG levels between the rAOM and COME groups. IgG, IgG1, and IgG2 levels measured in serum against 11 pneumococcal polysaccharides (7 serotypes in PCV7; 4 nonvaccine serotypes) in children had extensive interindividual variation with no significant differences in anti-*Mcat* and anti-*Spn* serum or MEE IgG levels between the rAOM and COME groups. IgG, IgG1, and IgG2 levels measured in serum against 11 pneumococcal polysaccharides (7 serotypes in PCV7; 4 nonvaccine serotypes) in children had extensive interindividual variation with no significant differences in anti-*Mcat* and anti-*Spn* serum or MEE IgG levels between the rAOM and COME groups. IgG, IgG1, and IgG2 levels measured in serum against 11 pneumococcal polysaccharides (7 serotypes in PCV7; 4 nonvaccine serotypes) in children had extensive interindividual variation with no significant differences in anti-*Mcat* and anti-*Spn* serum or MEE IgG levels between the rAOM and COME groups. IgG, IgG1, and IgG2 levels measured in serum against 11 pneumococcal polysaccharides (7 serotypes in PCV7; 4 nonvaccine serotypes) in children had extensive interindividual variation with no significant differences in anti-*Mcat* and anti-*Spn* serum or MEE IgG levels between the rAOM and COME groups. IgG, IgG1, and IgG2 levels measured in serum against 11 pneumococcal polysaccharides (7 serotypes in PCV7; 4 nonvaccine serotypes) in children had extensive interindividual variation with no significant differences in anti-*Mcat* and anti-*Spn* serum or MEE IgG levels between the rAOM and COME groups. IgG, IgG1, and IgG2 levels measured in serum against 11 pneumococcal polysaccharides (7 serotypes in PCV7; 4 nonvaccine serotypes) in children had extensive interindividual variation with no significant differences in anti-*Mcat* and anti-*Spn* serum or MEE IgG levels between the rAOM and COME groups. IgG, IgG1, and IgG2 levels measured in serum against 11 pneumococcal polysaccharides (7 serotypes in PCV7; 4 nonvaccine serotypes) in children had extensive interindividual variation with no significant differences in anti-*Mcat* and anti-*Spn* serum or MEE IgG levels between the rAOM and COME groups. IgG, IgG1, and IgG2 levels measured in serum against 11 pneumococcal polysaccharides (7 serotypes in PCV7; 4 nonvaccine serotypes) in children had extensive interindividual variation with no significant differences.
cells were lower in OM-prone children and that ME cytokine levels did not mirror blood but were more similar to the nasopharynx. IL-2 production was higher in OM-prone children. The immune responses appear to diverge in the mucosal and blood compartments at the onset of a bacterial ME infection.

AOM and OME cases are frequently preceded by a viral upper respiratory infections. Described early, RSV and parainfluenza diminished total RSV-specific IgG, with higher viral nasal burdens and lower IgG-neutralizing capacity in OM-prone children; there was also diminished

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### Table 3. Antibody Studies in Children with Otitis Media.

<table>
<thead>
<tr>
<th>Clinical Group</th>
<th>Measurements</th>
<th>Key Findings</th>
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<tbody>
<tr>
<td>AOM (n = 137)</td>
<td>IgG, IgA, and sIgA to <em>S. pneumoniae</em> serotypes from nasopharynx, middle ear, and serum</td>
<td>IgA levels were higher and IgG levels lower in nasal wash. IgA antibodies in the MEE and nasal wash were virtually identical; thus, IgA in MEE was derived predominantly from serum and the nasopharynx by reflux via the eustachian tube.</td>
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<tr>
<td>Randomized AOM (n = 34), OM prone (n = 35), and AOM-TF (n = 25) in population (n = 268)</td>
<td>Serum IgG to 5 <em>S. pneumoniae</em> proteins: PhtD, LytB, PcpA, PhhE, and Ply</td>
<td>Anti-PhtD, -LytB, -PhhE, and -Ply IgG titers in OM-prone &lt; non-OM prone (P &lt; .05) and AOM-TF (P &lt; .05). Non-OM prone children with increased anti-PhtD, PcpA, PhhE, and Ply IgG (P &lt; .001) between 6 and 24 mo were associated with nasopharyngeal colonization and AOM. OM-prone and AOM-TF children mounted lower IgG responses.</td>
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<td>AOM: 176 children between 6 and 24 mo with 76% (n = 133) for both health and AOM visits and 24% (n = 43) for AOM</td>
<td>Nasopharynx (n = 424) and MEE (n = 152) IgG and IgA to <em>S. pneumoniae</em> proteins: PhtD, PcpA, and Ply. Samples from 234 health and 208 AOM visits.</td>
<td><em>S. pneumoniae</em> nasopharynx colonization was associated with higher mucosal antibody levels to all 3 proteins. Children with <em>S. pneumoniae</em> AOM had &lt;5- to 8-fold IgG and &lt;3- to 6-fold IgA to the 3 proteins vs non-OM and Spn colonization. MEE and nasopharynx levels correlated. Spn AOM had &lt; IgG titers than AOM from other pathogens.</td>
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<td>rAOM, &lt;3 y of age (n = 166); ≥3 episodes of AOM and ventilation tube insertion, MEE (n = 144); healthy controls (n = 61)</td>
<td>Serum and MEE IgG, IgG1, and IgG2 to 11 <em>S. pneumoniae</em> polysaccharides by multiplex bead-based assay.</td>
<td>Specific IgG, IgG1, and IgG2 levels were similar in children with or without rAOM, except IgG and IgG1 to serotype 5 (higher with rAOM: P = .02 and P = .05, respectively). MEE specific IgG correlated with serum titers.</td>
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<tr>
<td>rAOM &lt;3 y of age (median, 7 episodes and ventilation tube insertion; n = 172) and healthy age-matched controls (n = 63)</td>
<td>Serum IgG to <em>S. pneumoniae</em> (PspA1, PspA2, CbpA, and Ply), <em>N. meningitidis</em> (P4, P6, and PD), multiplex bead assay</td>
<td>rAOM had higher IgG levels to NTHi P4 (P &lt; .001), P6 (P = 0.01), and PD (P = 0.01) vs healthy controls. No difference for PspA1, PspA 2, CbpA, and Ply. All children’s IgG increased with age and was higher (1.8-3.4 times) in children colonized with <em>S. pneumoniae</em> or NTHi, including rAOM PD IgG lower in AOM in OM prone vs AOM-TF (P &lt; .01) and non–OM prone (P &lt; .03). P6 and OMP26 IgG lower in OM prone vs AOM-TF (P &lt; .02 and P &lt; .04, respectively). Non–OM prone children had increased PD IgG post-AOM but not OM prone and AOM-TF. Longitudinal PD, P6, and OMP26 IgG had &lt;2-fold increases over time in OM prone and &gt;4-fold increases in non–OM prone (P &lt; .001).</td>
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<tr>
<td>AOM-TF (n = 26), rAOM (n = 32), non-Randomized AOM-TF (n = 27); longitudinal from age 6 to 30 mo (n = 10, OM prone; n = 150, non–OM prone)</td>
<td>Serum antibody response to outer membrane proteins PD, P6, and OMP26 of NTHi</td>
<td>Specific IgG, IgG1, and IgG2 levels were similar in children with or without rAOM, except IgG and IgG1 to serotype 5 (higher with rAOM: P = .02 and P = .05, respectively). MEE specific IgG correlated with serum titers.</td>
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<tr>
<td>AOM (n = 42) and COME (n = 114), &lt;5 y, ventilation tube insertion indicated</td>
<td>MEE, nasopharynx, and serum to proteins from <em>M. catarrhalis</em>: UspA1 557-704, UspA2 165-318, MID 764-913, MID 962-1200, MhbB, MhcA, Mcp51-333, and Hac 385-863 through Luminex xMAP technology; <em>S. pneumoniae</em>: PspC/CbpA, α-enolase, hyaluronidase, IgA1 protease, NanA, Ply, PdbD, PpmA, PsaA, PspA, Pht proteins SPI-003 (PhtD) and BVH-3 (PhtE), SlaA, SP0189, SP0376, SP1651, and Pilus A</td>
<td>Antigen-specific IgG, IgA, and IgM showed extensive interindividual variation. No significant differences in anti-Mcat and anti-<em>S. pneumoniae</em> serum and MEE were observed between the rAOM or COME groups for all antigens tested nor for colonization and serum IgG. A strong correlation occurred between antigen-specific serum and MEE IgG levels, indicating serum transudation for MEE.</td>
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**Abbreviations:** AOM, acute otitis media; COME, chronic otitis media with effusion; chronic; MEE, middle ear effusion; NTHi, nontypeable *Haemophilus influenzae*; OM, otitis media; PD, protein D; rAOM, acute otitis media recurrent; TF, treatment failure.
T-cell responses to RSV. It was concluded that lower adaptive and innate immune responses to RSV in OM-prone children might slow the kinetics of clearance and allow for viral interference with antibacterial immune responses. In children at high risk of developing rAOM, 48 of 60 developed rAOM, and of these, 31 developed severe rAOM (6 AOM episodes in a year). Low levels of IgG2, Clq, and mannan-binding lectin were found up to 8 times as often as expected. A study investigating adults with a history of rAOM during childhood and with low levels of IgG2 (ie, adults had no difference with regard to IgG2 concentrations or specific antibody levels) concluded that immunoglobulin patterns normalized as adults.

Responses to Pediatric Vaccines. Data from studies described earlier that measured IgG to pediatric vaccine antigens in OM-prone and non–OM prone children found that IgG-protective titers from several vaccines were decreased in OM-prone versus non–OM prone children. A higher percentage of OM-prone children had nonprotective antibody values that persisted until 24 months of age despite routine boosters. A study investigating adults with a history of rAOM and with low levels of IgG2 (ie, adults had no difference with regard to IgG2 concentrations or specific antibody levels) concluded that immunoglobulin patterns normalized as adults.

OME and Adenoidal Hypertrophy. IL-7 plays a pivotal role for activation and proliferation of T and B cells, with IL-7R (CD127 and CD132) expression an important marker of immunologic function. Evaluation of CD4+ and CD8+ T cells in AH of older children suffering with OME for 3 months or without OME found that the percentages of CD4+ and CD8+CD127 receptor and CD4+CD132 expression but not CD8+CD132 in AH with OME were higher than in the AH alone. The increased percentage of T lymphocytes with IL-7R expression (CD127 and CD132) in AH seems to influence the quantity of lymphocytes and disturb the immunologic function of tonsils, which can influence the course of OME.

CD69 is an early inducible cell surface glycoprotein during lymphoid activation with changes to its expression indicating reduced T-cell activation. It has been found that dendritic cells and lymphocytes from adenoid and peripheral blood in older children with AH and OME were lower for CD3+CD69+, CD4+CD69+, CD8+CD69+, and CD19+CD69+ cells in adenoids from OME, suggesting reduced T-cell activation. Differences were also observed between cells from the adenoids and peripheral blood in patients with OME for BDCA-2+/CD123+ cells and CD3+ and CD19+ activation markers. The reduced activation of adenoidal T cells from patients with OME may result in a suppression of the Th2-type response.

Animal Studies. Neutrophils play an important protective role in OM; however, recent studies suggest that neutrophil extracellular traps (NETs) may contribute to bacterial persistence in the ME. A mouse OM model with Influenza A virus facilitated OM by inducing a proinflammatory response in the ME that mediated Spn replication. With B cell–deficient infant mice, antibodies were found to play a crucial role in facilitating Spn replication due to antibody-dependent NET formation in the ME, which, instead of clearing the infection, allows the bacteria to replicate. It remains unclear whether the OM-inducing antibodies were natural low-affinity antibodies or high-affinity antibodies produced in response to non-Spn antigens or autoantibodies. These NETs are potential therapeutic targets with transsympanic administration of a DNase effectively reducing the bacterial load in the ME.

Chitosan, as a mucosal delivery system to form chitosan-PsaA nanoparticles, was used to immunize BALB/c mice intranasally against Spn. Compared with mice immunized with naked PsaA, levels of IFN-γ, IL-17A, and IL-4 in spleen lymphocytes, serum IgG, and mucosal IgA Psa-specific antibodies were enhanced significantly in the chitosan–PsaA mice. They had increased protection against AOM with Spn challenge, improved survival following intraperitoneal challenge, and induced mucosal and systemic immune responses.

Conclusion

Research advances that improve our understanding of the innate and acquired immunologic conditions associated with OM, susceptibility to disease, and progress to chronic or severe disease states were made. Significant findings in understanding the mechanisms associated with the innate immunity and pathogenesis of OM were particularly advanced through genome array studies, enabling detailed understanding of the sequencing of regulated events. Antigen-specific antibody and cellular immune studies provided new information on the importance of the acquired immune system and potential areas where immune suppression rather than immunologic deficiencies may be contributors to disease progression.

Recommendations and Implications for Practice

An update on the implications for practice and recommendations from the 2011 symposium can be found in the Supplementary Materials at the end of this article. Research recommendations and goals for improving knowledge and clinical outcomes as determined at the 2015 symposium are as follows.

Research Goals

High Priority

- Study how viral and bacterial pathogens alter pathways of innate and acquired immune systems, including inflammation, and the role of these in the pathogenesis of AOM, OME, and chronic suppurative OM
- Perform research to understand the induction of excessive inflammation and the identification of
lack of resolution, with the objective of developing intervention strategies

- Characterize the pathways of innate immunity associated with OM and determine the impact on acquired immune responses and the clinical relevance
- Elucidate the roles of different lymphocyte types in host responses, protection, and memory responses for all OM conditions
- Study trafficking of immune cells to the ME, its relationship with trafficking to the nasopharynx, and the partitioning of the roles of the systemic and mucosal immune systems
- Clarify the role of the eustachian tube as an immunologic gateway between the ME and nasopharynx

Recommendations.

- Focus efforts on global approaches to understanding the fundamental immunology of OM
- Perform research to investigate the potential of novel adjuvants to elicit protective responses to prevent OM
- Study the role of allergy in OM in induction of inflammatory response and relatedness to inflammation caused by a viral infection
- Perform research to understand the specific immunologic benefits of breastfeeding that might be mechanisms beyond antibody for developing novel therapeutic supplements
- Study the impact of cigarette smoke and other environmental pollutants on immune systems and their role in the development of OM
- Determine if children who get OM become older adults that are more susceptible to pulmonary diseases/infections

Supplementary Material

Summary of Progress on Goals from 2011 Symposium

1. *The role of various inflammatory mediators and their mechanisms of action in the pathogenesis of acute otitis media (AOM) following viral upper respiratory infections need to be further studied.* There has been limited progress that includes viral-bacterial studies but not a substantive body of work on the relationships between the relevant viruses and the host. More studies are still needed to understand what is happening in the nasopharynx, the middle ear (ME), and the role of the systemic immune system. Many observational studies have been undertaken and it is recommended that research is still needed to understand the mechanisms that are triggered that explain the observations. This should cover both otitis media with effusion (OME) and chronic suppurative otitis media (CSOM) conditions and consider the relationship of viral and bacterial loads to innate and acquired immune responses.

2. *Study how viral and bacterial pathogens alter pathways of innate immunity and the role of these alterations in pathogenesis.* Progress has been made through further studies that extend our knowledge of acquired immunity, including more reports that demonstrate differences in polymicrobial mixes by population group and geographic location. Considered as a high priority is research involving complex infections and the contribution of the presence of multiple microbes during infection. In particular to understand the induction and lack of resolution of inflammation as a main contributor to morbidity in otitis media (OM) and the mechanisms that might be modulated. The translation of knowledge of the mechanisms involved into intervention protocols and therapies is still not close.

3. *Characterize pathways of innate immunity as they relate to OM.* Good progress has been made in understanding Toll-like receptor (TLR) pathways and regulation as better tools become available. More work is still required on how the regulation works mechanistically and to determine the pathways and their impact on the acquired immune responses. Studies are still needed that are able to make the relevant connections between the bench studies and the clinical findings.

4. *Create an overall integrated map of the cytokines, chemokines, mediators, and signaling pathways relevant to the host response in OM.* Much work has been done for both OM and other respiratory sites in relation to cytokines, chemokines, signaling pathways, and mediators generally in relation to responses associated with specific viruses and/or bacteria. It is timely to bring this body of information together to confirm links and determine gaps.

5. *Elucidate the role of Tregs and the T17 axis in the host response and in protection from OM.* A lot of good work is now being reported and is still at relatively early stages. The role of adenoids in not clear, in cellular immunity, studies are needed to broaden to investigate more types of T cells (now approximately 34 populations) to consider their roles and contributions to OM and non-OM prone. Gaps in our knowledge also remain for B cells and their differences in receptors and internal pathways differences that reduce memory responses. It is recommended that more is done with mutant animal models, not just those that have been directly connected to OM.

6. *Study trafficking of immune cells to the nasopharynx and the ME; and Characterize how the ME interacts in the common mucosal immune system.* Some descriptive studies on what is happening in the adenoids and tonsils have been undertaken. A lot of work is being done using mouse models for innate cell trafficking and phagocytic killing.
More studies are needed on the importance of the systemic immune system to infection in the ME and on understanding the relationship between the systemic and mucosal systems in OM as physiologically, the ME mucosa is very different from the mucosa and proximity to lymphoid tissues of other mucosal sites.

7. **Focus efforts on global approaches to understanding the fundamental immunology of OM.** Many studies are being undertaken but are not necessarily systematized for ease of comparative analysis. Differences in outcomes remain according to factors of geography, ethnicity, and culture. Approaches to enable comparative studies globally, and particularly in those susceptible first peoples (eg, Indigenous Australians, Eskimos, etc) would be an advantage to elucidating the key factors that contribute to susceptibility to OM.

8. **Study the role of allergy in OM.** Some recent work on IgE and the role of eosinophils in OM as well as connections between sinusitis and OM. Further investigations of the relationship between inflammation associated with allergy, which potentially mimics a viral infection, and possible contribution to bacterial OM conditions are required. It was determined that there would be merit to investigating potential immune signatures of nasopharyngeal secretions at time of OM and to compare viral and allergy inflammatory signatures and relation to OM.

**Author Contributions**

**Jennelle M. Kyd**, substantial contributions to leading the coordination of the postsymposium meeting, substantial contributions to analysis of information reviewed, writing and editing, final approval and accountability for all aspects of the work; **Muneki Hotomi**, substantial contributions to leading the coordination of the postsymposium meeting, contributions to analysis of information reviewed, writing and editing, final approval and accountability for all aspects of the work; **Masamitsu Kono**, substantial contributions to analysis of information reviewed, writing and editing, final approval, and accountability for all aspects of the work; **Arwa Kurabi**, substantial contributions to analysis of information reviewed, writing and editing, final approval, and accountability for all aspects of the work; **Michael Pichichero**, substantial coordination and analysis of the information reviewed, particularly innate and adaptive immunity in humans sections, writing and editing, final approval, and accountability for all aspects of the work; **Allen Ryan**, substantial coordination and analysis of the information reviewed, particularly the animal studies, writing and editing, final approval, and accountability for all aspects of the work; **W. Edward Swords**, substantial contributions to analysis of information reviewed, writing and editing, final approval, and accountability for all aspects of the work; **Ruth Thornton**, substantial contributions to analysis of information reviewed, writing and editing, final approval, and accountability for all aspects of the work.

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**Funding source:** International Society for Otitis Media.

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