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Mouse models for human otitis media

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Abstract

Otitis media (OM) remains the most common childhood disease and its annual costs exceed \$5 billion. Its potential for permanent hearing impairment also emphasizes the need to better understand and manage this disease. The pathogenesis of OM is multifactorial and includes infectious pathogens, anatomy, immunologic status, genetic predisposition, and environment. Recent progress in mouse model development is helping to elucidate the respective roles of these factors and to significantly contribute toward efforts of OM prevention and control. Genetic predisposition is recognized as an important factor in OM and increasing numbers of mouse models are helping to uncover the potential genetic bases for human OM. Furthermore, the completion of the mouse genome sequence has offered a powerful set of tools for investigating gene function and is generating a rich resource of mouse mutants for studying the genetic factors underlying OM.

Keywords

Otitis media; Immunology; Mice; Genetic predisposition to disease; Etiology

1. Introduction

Otitis media (OM) is a multifactorial disease whose pathogenesis is affected by Eustachian tube structure and function, host immune status, innate mucosal defense, pathogen virulence and strain, and genetic susceptibility loci (Zhang, et al., 2006a). Infectious disease can be viewed as a battle between hosts and pathogens, in which commands (encoded in the genomes of both host and pathogen) are executed by protein products, such as bacterial structural proteins, components of the host immune response and drug-resistance mechanisms of the bacterial pathogen. Thus, the transition from subclinical resident bacteria in the nasopharynx to inflammatory disease in the middle ear will include multiple cellular and molecular responses by both host and pathogen. One can think of the pathogen–host relationship as a delicate balance whereby neither completely overpowers the other. Bacteria common to middle ear disease are normally resident in the nasopharynx, but in subclinical numbers. These bacteria proliferate and invade the middle ear via the Eustachian tube only if something tips the balance in their favor. This is generally an upper respiratory tract viral infection in the host that changes mucosal conditions in such a way that bacteria can colonize. A morphologic or genetic defect in the host could also lead to bacterial proliferation or access to the middle ear. The fact that middle ear disease is so common implies a frequent, but thankfully transient, bacterial dominance in this balance. A clearer understanding of these pathogen-induced responses is

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necessary to manage such a disease and prevent its escalation to the point of permanent alteration in the host.

2. Bacterial PAMPs and induction of otitis media

Otitis media with effusion (OME) is an inflammatory disease characterized by bacterial activation of transcription factors, production and release of pro-inflammatory cytokines, cellular infiltration into the middle ear, and secretion of serous or mucin-rich effusions (Bluestone and Klein, 2001; Kubba et al., 2000). The most common bacteria invading the middle ear are *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*, accounting for 85% of acute infections (Daly et al., 1999b). Inflammation is mediated by the innate immune system and initiated by host immune cell toll-like receptors (TLRs) that are activated following interaction and binding with pathogen-associated molecular patterns (PAMPs), conserved molecules that are found on bacteria but are absent in host tissues (Aderem and Ulevitch, 2000; Akira, 2001; Sabroe et al., 2003). For example, TLR4 binds cell wall lipopolysaccharide (LPS) of gram-negative *H. influenzae*, TLR2 binds lipoteichoic acid in the peptidoglycan (PG) of gram-positive *S. pneumoniae*, and TLR9 binds unmethylated bacterial DNA from both gram-negative and gram-positive organisms (Akira et al., 2001; Takeshita et al., 2004; Takeuchi et al., 1999). Although once thought that a unique TLR exists for each bacterial species, studies in mice now show that different components of a bacterium may bind with different TLRs, e.g., gram-negative *H. influenzae* and gram-positive *S. pneumoniae* have PAMPs that bind to both TLR2 and TLR4 (Chen et al., 2004; Ha et al., 2007; Moon et al., 2006; Moon et al., 2007; Shuto et al., 2001; Wang et al., 2002). Also, the LPS of *Porphyromonas gingivalis* preferentially binds to TLR2 instead of TLR4 (Hirschfeld et al., 2001), while its major and minor fimbriae bind both receptors (Davey et al., 2008). Thus, the one bacterium–one TLR concept is no longer valid as multiple intracellular inflammatory pathways can be activated with a single bacterium. This is a key issue in understanding the inflammatory mechanisms in otitis media.

The interaction of TLRs and PAMPs initiates TLR-dependent cellular activation (Medzhitov et al., 1998) of nuclear factor-kappa beta (NF- κ B), a transcription factor for the genes of numerous pro-inflammatory products (Chen et al., 1999; Ghosh et al., 1998; Tak and Firestein, 2001). Other key intracellular inflammatory factors are MyD88, AP-1, and the mitogen-activated protein kinases (MAPK) p38 and JNK, all of which may serve in NF- κ B independent upregulation of inflammatory cytokines. The impact of this cellular activation is the production of dozens of inflammatory cytokines (Ghaheri et al., 2007a; Ghaheri et al., 2007b). However, the predominant cytokines of greatest interest in middle ear studies are tumor necrosis factor- α (TNF- α), IL-1 β , and IL-8 (Juhn et al., 1997; Smirnova et al., 2002). These cytokines play a central role in OME and have been identified in 77–91%, 67–97% and 92–100%, respectively, of chronic middle ear effusions (Kubba et al., 2000). Although the major cell type involved in this process is the macrophage, other responsive cells within the middle ear that have TLRs are the neutrophil, vascular endothelial cell, and mucosal epithelium (Pugin et al., 1993; Shuto et al., 2001). In addition to the inflammatory cytokines, NF- κ B activation leads to production of platelet activating factor (PAF), vascular endothelial growth factor (VEGF), oxygen radicals, nitric oxide synthase (NOS), and intercellular adhesion molecule-1 (ICAM-1), which facilitates cellular transudation across vascular walls (Akira, 2001; Chen et al., 1999; Ghosh et al., 1998; Jewett et al., 1999; Ryan et al., 1990; Takahashi et al., 1992).

Research into OM falls along two major experimental lines, evaluation of normal inflammatory mechanisms and altered disease progression due to abnormalities in the host. This latter case could be genetically-based malformations or immune system defects. The following review will therefore focus on what has been learned about middle ear inflammation by inducing OM in normal mice and examining alterations in this inflammation in mouse models in which a

genetic defect has occurred that isolates some parameter of this process, usually with a relationship to a known human defect.

3. Mouse models for induced OM

The bacterial induction of inflammatory cytokines is the key to understanding the innate immune response and progression of middle ear disease. Therefore, the induction of OME and related disease processes have been extensively studied in normal mice by injecting bacteria or bacterial products into mouse middle ears (MacArthur and Trune, 2006; Ryan et al., 2006). Although mice do not routinely become infected with human otitis media bacteria, they are used to extensively investigate the middle ear inflammatory response to better understand this human disease. Whole bacteria, alive or dead, are often used to model the comprehensive pathways involved in the innate immune response, while specific bacterial components are employed to isolate one pathway of the intracellular inflammatory cascade at a time. However, mouse strains differ in response to the same bacterial strain (Melhus and Ryan, 2003), so one must be aware of species differences in experimental design, interpretation, and comparison to other studies. For example, Swiss–Webster mice are comparatively resistant to bacterial OM, BALB/c are quite susceptible, and C57BL/6 mice exhibit intermediate responses. Furthermore, dozens of cytokines are upregulated in the innate immune response (Ghaeheri et al., 2007a), and each cytokine has a specific role in the subsequent inflammation. Thus, inoculation of the middle ear space with a single cytokine is helpful in isolating inflammatory processes specific to that cytokine. Major cytokines that have been investigated thus in the mouse middle ear response are TNF- α , IL-1 β , IL-6, IL-8, etc. (Johnson et al., 1997; Ryan et al., 2006; Smirnova et al., 2002; Watanabe et al., 2001).

Although several animal models of OM have been reported, including the chinchilla (Bakaletz et al., 1998; Giebink, 1999; Gitiban et al., 2005) and the rat (Clark et al., 2000), mice have been used successfully to elucidate virulence factors, mechanisms of bacterial adherence and invasion, induction of mediators of inflammation, and the specificity of immune responses to pathogens such as nontypeable *H. influenzae* (NTHi) (Green et al., 1993; Kyd et al., 1995; Wallace et al., 1989), *H. influenzae* type b (Loeb, 1987), *Pseudomonas aeruginosa* (Cripps et al., 1994), *S. pneumoniae* (Yamamoto et al., 1997), *Moraxella catarrhalis* (Kyd et al., 1999), and viruses such as respiratory syncytial virus (Gitiban et al., 2005). Increasingly, mice have been used to study OM because of the commercial availability of immunologic probes (Gu et al., 1998; Gu et al., 1996; Johnson et al., 1997; Klingman and Murphy, 1994; Krekorian et al., 1990; Kyd et al., 1999; Murphy et al., 1999). Non-genetic mouse models of OM have been generated in approximately 100 studies, of which over 30% used the BALB/c inbred strain (Chen et al., 1996; Gu et al., 1995; Hotomi et al., 2002; Ichimiya et al., 1999; Kataoka et al., 1991; Klingman and Murphy, 1994; Krekorian et al., 1991; Krekorian et al., 1990; Kurono et al., 1992; Kyd et al., 1999; Murphy et al., 1999; Ryan et al., 2006; Sarwar et al., 1992; Ward et al., 1976; Watanabe et al., 2001; MacArthur et al., 2006a). Mice have also been inoculated with bacteria or bacterial cell-wall antigens to induce immunity for studies of pathogen–host interactions (Gu et al., 1998; Gu et al., 1996; Holmes et al., 2001; Hotomi et al., 1998; Klingman and Murphy, 1994; Kodama et al., 2000; Krekorian et al., 1991; Krekorian et al., 1990; Murphy et al., 1999; Ryan et al., 2006; Sarwar et al., 1992), or with attenuated bacterial strains to seek broad (mucosal and systemic) immune protection (Roche et al., 2007). In studies using BALB/c mice, a detoxified lipopolysaccharide (LPS)-protein conjugate was suggested as a candidate for immunization against *M. catarrhalis* infection (Gu et al., 1998; Gu et al., 1996). Two novel major proteins of approximately 19 kDa and 16 kDa (named OMP J1 and OMP J2, respectively), which exist in two variant forms associated with particular genetic lineages, were identified in *M. catarrhalis*. Experiments using two OMP J2 mutants (one complement-resistant and the other complement-sensitive) indicated that both were less easily cleared from the lungs of mice than were their isogenic wild-type counterparts (Hays et al., 2005). Studies

using Swiss–Webster, BALB/c, or CD-1 mice have shown that bacterial adherence factors, such as surface protein pneumococcal surface adhesin A (PsaA), ubiquitous surface protein (Usp)A1, and ubiquitous surface protein A (Usp)A2, may have some role in adherence (Briles et al., 2000; Chen et al., 1996; Holmes et al., 2001; McMichael, 2000). It is not clear if TLRs are the binding sites in these adherence mechanisms, or if these specialized pathogen proteins are involved more with nasopharynx adherence and colonization.

Other examples of mice used in OM research include studies of signal transduction mechanisms involved in OM pathogenesis (Bakaletz et al., 2002). For example, receptor tyrosine kinases were demonstrated to be involved in normal and pathologic angiogenesis in mice (Sudhoff et al., 2000). It was found that regulatory genes involved in middle ear development may also influence susceptibility to OM. Mouse models of OM were also used to identify the expression of defensins, which are antimicrobial peptides that play a major role in innate immunity. As the expression levels of beta-defensins 2–4 (mBD2, mBD3, mBD4) in tubotympanum were up-regulated in experimental mice with OM, they may play a protective role in the pathogenesis with OM (Jin Shin et al., 2006). In other research, mice immunized with the *S. pneumoniae* D39 strain were employed to identify novel *S. pneumoniae* antigens by screening a whole-genome lambda-display library, and Spr0075 was identified as an expressed *S. pneumoniae* gene product, having an antigenic function during infection (Beghetto et al., 2006).

Mouse middle ear epithelial cell lines have also been established for OM studies. The primary culture of middle ear epithelial cells was established from the middle ear mucosa of an Immortomouse derived from an SV40-bearing egg. The cultured cells were transduced by a temperature-sensitive large T-antigen mutant and cultured for >50 passages. Temperature-sensitive middle ear epithelial cell lines are essential for pathophysiologic studies of OM. The cell line is very useful for studying the pathogen–host interaction, receptor identification, signal transduction, cytokine/mucin production and cellular responses, especially for cell proliferation and differentiation (Tsuchiya et al., 2005).

3.1. Mouse studies of inner ear sequelae in otitis media

Currently, little is known of the mechanisms by which middle ear disease impacts the inner ear, although it presumably involves entry of pathogens and cytokines through the round window (Goycoolea, 2001; Juhn et al., 1997). The prevalence of sensorineural hearing loss in children can be as high as 40–55% in children who had multiple infections in their younger years, whereas the incidence of permanent threshold shift increases with chronic otitis media (MacAndie and O'Reilly, 1999; Paparella et al., 1984; Papp et al., 2003; Ryding et al., 2002; Sorri et al., 1995). Inner ear pathology is reported in 20–67% of temporal bones, and includes basal turn loss of hair cells, reduction in the stria vascularis and spiral ligament, and inflammatory cells in the perilymphatic spaces (Cureoglu et al., 2004; Juhn et al., 1997; Meyerhoff et al., 1978; Paparella et al., 1972).

Several mouse studies have helped determine how the inner ear is affected by middle ear disease. Significant inner ear damage occurs following live streptococcus delivery to the middle ear (Ichimiya et al., 1999). Similar cochlear inflammation is seen in C3H/HeJ mice whose TLR4 defect prevents clearance of gram-negative bacteria (MacArthur et al., 2006b; MacArthur et al., 2008a). Studies of both acute and chronic OM mice also showed sensorineural hearing loss, although largely transient in the acute mice (MacArthur et al., 2006a; MacArthur et al., 2008a). Both models also showed significant upregulation of inner ear cytokine gene expression (Ghaehri et al., 2007a; Ghaehri et al., 2007b), suggesting that inner ear tissues are capable of responding to immune stimulation. Several studies in mice have shown that fibrocytes in the lateral wall will produce cytokines upon bacterial stimulation, implicating fibrocytes as one of the cell types directly involved in inner ear inflammatory processes (Ichimiya et al., 2000; Ichimiya et al., 2003; Moon et al., 2007; Yoshida et al., 1999), although

several inner ear structures besides the lateral wall have shown staining for NF- κ B and inflammatory cytokines in mouse models of otitis media (Ghaheri et al., 2007a; Ghaheri et al., 2007b). Recent mouse studies also show that sensorineural hearing loss in acute and chronic OM inner ear pathology can be reversed with various steroids (MacArthur et al., in press, 2008b), suggesting potential treatments to prevent permanent hearing loss in patients.

4. Host genetic factors underlying otitis media

Although genetics are not generally considered a factor in the development of an infectious disease such as OM, many lines of evidence indicate that the genetic background of the host plays an important role in OM. For example, patients with recurrent OM usually exhibit some of the following characteristics: sibling history of frequent ear infections, Down syndrome, cleft palate, immunodeficiency, and polymorphisms of inflammatory genes (Daly, 1991; Daly et al., 1999a; Daly et al., 1999b; Emonts et al., 2007a; Patel et al., 2006). Racial differences also suggest a genetic contribution to OM susceptibility. OM frequency is unusually high in American Indians and Australian Aborigines and comparatively low in African Americans (Coates et al., 2002; Harris et al., 1998). A study of OM in Apache Indians in Arizona also suggests familial predisposition (Todd, 1987). Some of the most compelling evidence comes from a twin and triplet study which concluded that genetic traits play a major role in OM development and that OM susceptibility is inherited (Casselbrant et al., 1999). Various congenital and inherited syndromes also demonstrate a genetic influence on OM susceptibility. For example, one study found that 89% of 193 children with achondroplasia had at least one episode of OM within the first 2 years of life and that 24 of the 99 children who had OM in the first year of life had multiple episodes (Hunter et al., 1998).

Studies of several human syndromes have also contributed to identifying genes that might be involved in predisposition to OM. Kartagener's syndrome is an autosomal recessive heritable disorder with impaired function of the mucociliary system of the Eustachian tube. In a study of Kartagener's syndrome, all 27 affected children developed chronic sinusitis and OM (Mygind and Pedersen, 1983). More recently, mutations in the dynein heavy-chain gene (*DNAH5*) were identified in Kartagener's syndrome families, aided by genetic mapping information of the homologous gene in the mouse, *Mdnah5* (Olbrich et al., 2002; Vaughan et al., 1996). Indeed, a null mutation of the *Mdnah5* mouse gene exhibited the OM phenotype. Gene expression studies have demonstrated that surfactant protein A, which plays a role in innate host defense in the lung, is also expressed in the Eustachian tube (Ramet et al., 2001). The frequency of specific surfactant protein A haplotypes and genotypes has been shown to differ between children with recurrent OM and those in a control population in Finland (Alho et al., 1991). Van der Woude syndrome (VWS) and popliteal pterygium syndrome (PPS) are autosomal dominant clefting disorders caused by mutations in the IRF6 (Interferon Regulatory Factor 6) gene. The IRF gene family consists of nine members encoding transcription factors that share a highly conserved helix–turn–helix DNA-binding domain and a less conserved protein-binding domain. Most IRFs regulate the expression of interferon- α and - β after viral infection. By detection of the expression of IRF6 in different tissues at different stages, researchers found that the zebrafish IRF6 expression was consistent with the observation of lip pits in human VWS patients, as well as the reports of alae nasi, otitis media and sensorineural hearing loss in some patients (Ben et al., 2005).

Although the above studies suggest that genetic factors contribute to OM, human genetics approaches are limited in the ability to undertake systematic investigations of the genetic pathways and pathological mechanisms involved in middle ear disease. For example, genome-wide association studies in human populations with the aim of identifying genetic loci underlying OM are fraught with significant logistical and practical difficulties. Moreover, genetic investigations in the human population are compounded by uncontrollable

environmental factors. While none of these difficulties are completely insurmountable, there are significant advantages to the parallel development of mouse models of OM. The mouse can play a key role in unraveling the genetic etiology of OM, information that can be translated to studies of the genetics of OM in the human population — for example by assessing candidate genes identified in the mouse in association studies in human families. Moreover, a diverse panel of mouse genetic models will provide an important platform for drug discovery and the development of alternative therapeutic strategies for human OM.

With these and other limitations in mind, natural or induced mutations offer the opportunity to study the role of an ever-increasing number of genes (Ryan et al., 2006, Zheng et al. 2006a,b). None of the studies with OM models mentioned above addressed the underlying genetic basis for host susceptibility. However, the most significant advantage of using mouse OM models over other model organisms is that there is an extensive genetic toolkit available for manipulating the mouse genome and studying the relationship between genes and disease susceptibility (Cox and Brown, 2003; Parkinson and Brown, 2002; Whitfield et al., 2005). For example, a significant difference in middle ear inflammation and effusion formation in response to gram-positive bacterial cell wall product (peptidoglycan-polysaccharide) was reported between two genetically different strains of rats (Clark et al., 2000). If this work had been carried out in the mouse, the gene (s) underlying the observed differences could have been identified using the well-developed high-resolution mapping, positional cloning and genetic manipulation tools available.

Evidence shows that mice with certain deficiencies are susceptible to OM. Ebmeyer et al. (2005) found that mast cell-deficient mice exhibited a significantly reduced middle ear (ME) response to bacterial infection, when compared to wild-type controls. Moreover, this deficit could be corrected by transplanting mast cells derived from the bone marrow of wild-type animals, which populated the ME mucosa. This indicates that mast cells are an important component of the innate immune response of the ME cavity during the initial stages of OM. Rivkin et al. (2005) found that mucosal hyperplasia during OM was enhanced in *lpr/lpr* mice when compared to wild-type controls. In addition, the recovery of the mucosa was significantly delayed in Fas-deficient mice. The results suggest that Fas-mediated apoptosis plays a role in remodeling of the ME mucosa during OM-induced mucosal growth and that recovery of the mucosa during OM resolution involves apoptosis mediated by death ligands and receptors. In another study, the role of the plasminogen (plg)/plasmin system in spontaneous development of chronic otitis media was investigated by analysis of plg-deficient mice. Essentially all of the wild-type control mice kept a healthy middle ear status; whereas all the plg-deficient mice gradually developed chronic otitis media with varying degrees of inflammatory changes during an 18-week observation period. Five bacterial strains were identified in middle ear cavity exudates of six plg-deficient mice, suggesting that plg plays an essential role in protecting against spontaneous development of chronic otitis media (Eriksson et al., 2006). Moreover, lysosomal neuraminidase deficiency may result in the alteration of ear morphology. External auditory canal obstruction, otitis media and ossicle changes may cause conductive hearing loss, and defects in lysosomal storage of neurons, stria vascularis, spiral limbus, Reissner's membrane and basilar membrane cells may contribute to sensorineural deafness (Guo et al., 2005).

Several groups have used genetic mouse models to study OM. Outbred Mcr:(ICR) breeder mice (Harkness and Wagner, 1975), inbred strains C3H/HeJ (Mitchell et al., 1997), and B6;129 mice (Haines et al., 2001) have been reported to have relatively high incidence rates of OM. Several CBA/J colonies had to be destroyed because the incidence of purulent OM was as high as 62% (McGinn et al., 1992), and CBA/CaJ was recommended as a replacement to provide appropriate controls in hearing studies. There have been more than a dozen middle ear studies using LP/J mice (Brodie et al., 1993; Henry and Chole, 1987; Steel et al., 1987), but none of

these studies included genetic characterization of the OM susceptibility traits. Similarly, some studies have generated pathogen-challenged OM models in BALB/c mice, but none of these were designed to identify the gene(s) affecting host susceptibility to OM (Chen et al., 1996; Kyd et al., 1999; Sabirov et al., 2001).

Several molecules were identified in middle ear effusions as inflammatory mediators, including the cytokines IL-1 β , IL-2, IL-6, IL-8, TNF α , interferon- γ , and TGF β (Ball et al., 1997; Maxwell et al., 1997; Nassif et al., 1997; Storgaard et al., 1997). The results of these studies indicate that these mediators play an important role in the pathogenesis of OM. It is possible that genetic differences between mouse strains could affect the production of these mediators as part of the immune response to pathogens, which, in turn, could affect susceptibility to OM. Gene knockout mouse models (<http://www.jax.org/imr/index.html>) have been produced in most genes of the molecules mentioned above, such as IL-1 β , IL-2, IL-6, IL-8, PsaA, (Usp)A1, and (Usp)A2, but few of the knockout mouse models have been used for OM studies.

4.1. Toll-like receptors and inflammation

Recent studies have demonstrated the relevance of TLRs to human innate immune response and how their dysfunction leads to chronic inflammation and sepsis (Schroder and Schumann, 2005). Several investigations have shown that 10% of humans have a TLR4 polymorphism that causes hyporesponsiveness to the endotoxin from gram-negative bacteria (Arbour et al., 2000; Cook et al., 2003; Lorenz et al., 2002a; Schwartz, 2002). This could explain the known genetic relationship of otitis media between siblings and parents (Bluestone and Klein, 2001), where as much as 60–70% of otitis media liability is due to genetic background (Casselbrant et al., 1999). Recent studies suggest that polymorphisms in TLR4, as well as other inflammatory cytokine genes, may predispose individuals to otitis media (Daly et al., 2004; Emonts et al., 2007a; Patel et al., 2006). Chronic infectious disease is also seen in people with TLR2 and TLR9 defects (Schroder and Schumann, 2005), but little is known of any predilection to otitis media. Polymorphisms also have been identified for intracellular inflammatory mediators and cytokines, such as IRAK-4, PADI, SP-A, TNF- α , IL-1 α , IL-1 β , IL-6, IL-10, many of which have been correlated with otitis media in children (Daly et al., 2004; Emonts et al., 2007a; Emonts et al., 2007b; Joki-Erkila et al., 2002; Ku et al., 2007; Lindberg et al., 1994; Patel et al., 2006; Pettigrew et al., 2006).

Early studies of the TLRs suggested once they were bound by their specific bacterial ligand, they induced identical inflammatory responses through NF- κ B upregulation. This no longer appears to be the case in that other multiple pathways may be activated in otitis media (Ha et al., 2007; Koga et al., 2008; Kweon et al., 2006; Shuto et al., 2001; Wang et al., 2002), particularly via MAP kinase P38 induction of cytokine gene expression (Chen et al., 2004; Moon et al., 2006). Also, some TLRs function in combination with other TLRs or cytokines in a synergistic mechanism to amplify inflammation (Kweon et al., 2006; Moon et al., 2006).

Differentiating inflammatory pathways in otitis media has been made easier with null mice for the various TLRs. There are now mouse strains available that have non-functional TLR2, TLR4, TLR9, and MyD88. Defects in these receptors and adapter proteins are critical for bacterial clearance (Hernandez et al., 2008; Leichtle et al., in press). Recent mouse studies have shown that cytokine and inflammatory cell profiles vary depending on the TLR involved, particularly with cytokine-producing cell kinetics (Hirschfeld et al., 2001; Melhus and Ryan, 2000; Melhus and Ryan, 2001). These models are in addition to natural mutations in TLR4 (C3H/HeJ). In fact, the C3H/HeJ mouse is often the experimental animal model for studies of the human TLR4 defect (Lorenz et al., 2002b; Schwartz, 2002). The C3H/HeJ mouse, which has a single amino acid substitution in TLR4, does have chronic otitis media (Ghaheri et al., 2007b; Hirano et al., 2007; MacArthur et al., 2006b; MacArthur et al., 2008a).

Also, TLR2 and TLR9 appear to work in cooperation so that their differential inflammatory pathways lead to greater immune defects when the two nulls are combined (Bafica et al., 2006; Bafica et al., 2005; Leichtle et al., in press). TNF α and IL-6 are affected to a greater degree by the TLR2 null than when only TLR9 is nonfunctional. Thus their cytokine and immune cell profiles are quite different. Furthermore, TLR4 responses to LPS in the various mouse strains are different, leading to the conclusion that other genetic factors work in combination with this receptor for specific inflammatory responses (Lorenz et al., 2001).

Although C3H/HeJ mice were reported years ago (Mitchell et al., 1997) to have a high incidence of spontaneous otitis media, it was not until the discovery of TLRs that the immune basis for this strain was understood. The C3H/HeJ mouse has a single amino acid substitution in its TLR4, making it insensitive to LPS (Haziot et al., 2001; Poltorak et al., 1998). As a result, these mice cannot efficiently clear gram-negative bacteria. The middle ear shows extensive inflammatory cell infiltration, fibrosis, mucosal hypertrophy and degeneration, fluid, breakdown of the round window membrane, and inner ear inflammation (MacArthur et al., 2006b). All C3H/HeJ tympanocentesis and blood specimens grew gram-negative *Klebsiella oxytoca*, which was confirmed by PCR (MacArthur et al., 2008a). Electron microscopy of the middle ears revealed abundant rod-shaped *Klebsiella* bacteria, both free and being engulfed by neutrophils. *K. oxytoca* is a common gram-negative bacterium of mice. This confirmed that spontaneous chronic otitis media in the C3H/HeJ mouse is a gram-negative bacterial process, suggesting that its TLR4 defect causes susceptibility to gram-negative bacterial infection. Bacteria also were seen in the inner ear, demonstrating the connection between chronic otitis media and sensorineural damage.

5. Genetic approaches to studying mouse models of otitis media

The completion of the mouse genome sequence has enabled a more or less complete annotation of mouse genes (Waterston et al., 2002). With the increased availability of knockout and transgenic mice, and the large amount of data to indicate that human disease is accurately modeled in the mouse, the mouse model is increasingly becoming a model of choice (MacArthur and Trune, 2006). This development coupled with the tools to introduce defined, targeted alterations into the mouse genome means that we are able for the first time to contemplate undertaking a systematic assessment of gene function using large-scale mouse mutagenesis, phenotype assessment of mutant strains and the identification of disease models from the mutants created.

There are two distinct approaches to determining gene function in the mouse — gene-driven and phenotype-driven (Barbaric et al., 2007; Bradley et al., 1984; Gu et al., 1993; Hrabe de Angelis et al., 2000; Nolan et al., 2000; Thomas and Capecchi, 1987; Wiles et al., 2000). In the gene-driven approach, a specific lesion introduced into the mouse genome is the start-point for an analysis of the resulting phenotype. Gene-driven approaches include gene-traps and knock-out and knock-in mutations (Hernandez et al., 2008; Leichtle et al., in press; Bradley et al., 1984; Gu et al., 1993; Thomas and Capecchi, 1987; Wiles et al., 2000). It is feasible for such targeted approaches to be scaled in order to generate mutations for every gene in the mouse genome. Moreover, targeting constructs can be manipulated in order to introduce conditional mutations so that mutational effect can be explored in both a time-dependent and tissue-specific manner (Gu et al., 1993). There has been much recent discussion on the development of international programs to generate mutant lines for all mouse genes (Austin et al., 2004; Auwerx et al., 2004). In Europe, the EUCOMM (European Conditional Mouse Mutagenesis program) is undertaking the generation of conditional mutations for 20,000 genes and began in 2006. In Canada, a similar program (NORCOMM) will also get underway shortly, while in the US the KOMP (Knock-out Mouse program) initiative is considering proposals.

The mutant lines produced from all of these programs will be a major resource for studying gene function and generating diverse disease models.

In contrast, the phenotype-driven approach undertakes screens of large collections of randomly mutagenized mouse genomes, commonly produced by chemical ENU mutagenesis, for disease phenotypes of interest (Hrabe de Angelis et al., 2000; Nolan et al., 2000). Thus, the phenotype of interest is the start-point of the study irrespective of the underlying lesion responsible. Upon discovery of an interesting mutant phenotype, the underlying gene is identified and investigated further. Importantly, phenotype-driven approaches do not make any a priori assumptions about the relationship between gene and phenotype and are therefore a relatively powerful route for discovering novel gene function and genetic pathways. So in the case of OM, where the genetic etiology is very poorly understood, this approach would be expected to yield benefits. An additional advantage of ENU is that it introduces point mutations and has the capacity to reveal many of the gene-phenotype relationships at an individual locus by the introduction of a range of null, hypomorphic, gain-of-function and dominant negative mutations. By analysis of the published data of randomly acting mutagens, a study showed that ENU-induced mutations identified in phenotype-driven screens were in genes that had higher coding sequence length and higher exon number than the average for the mouse genome. Data also showed that ENU-induced mutations were more likely to be found in genes that had a higher G+C content and neighboring base analysis revealed that the identified ENU mutations were more often directly flanked by G or C nucleotides. ENU mutations from phenotype-driven and gene-driven screens were predominantly A:T to T:A transversions or A:T to G:C transitions. Knowledge of the spectrum of mutations that ENU elicits will assist in positional cloning of ENU-induced mutations by allowing prioritization of candidate genes based on some of their inherent features (Barbaric et al., 2007).

Taken together, gene-driven and phenotype-driven approaches offer a powerful collection of tools for investigating gene function and can be expected to generate a rich resource of mouse mutants for the elucidation of genetic mechanisms underlying disease. Indeed, a phenotype-driven approach has recently been proven instrumental in the identification of pathogen susceptibility and resistance genes and their alleles. In each case, the work began by identifying mouse strains showing differing susceptibility to infection. In the first example, a 2'-5'-oligoadenylate synthetase 1B gene (*Oas1b*), was identified as *Flv* (flavivirus resistance gene) via analysis of the association between genotype and phenotype in nine mouse strains differing in susceptibility to flavivirus infection (Perelygin et al., 2002). This study showed that susceptible strains produce a protein lacking 30% of the C-terminal sequence due to a premature stop codon. In a second study, mouse strains susceptible and resistant to West Nile virus were identified and used to map the susceptibility locus. A C-terminal transition that results in a stop codon in exon 4 of a gene encoding the L1 isoform of *Oasl* (*Oasl1*) was identified as the cause of susceptibility in laboratory mice (Mashimo et al., 2002). These studies give confidence that the development of genetic mouse models of OM would contribute significantly to efforts to identify the genetic and biologic factors affecting OM. Moreover, having identified and characterized mouse models, we can investigate the epistatic effects of mutations by performing crosses between different mutant strains and thus further explore the multifactorial basis of disease (Schachern et al., 2007; Zheng and Johnson, 2001).

However, to date, no systematic screening has been performed for OM, as has been done for other disease susceptibilities (Alper et al., 2002; Chang et al., 2002; Mashimo et al., 2002; Nolan et al., 2000; Perelygin et al., 2002; Zheng et al., 1999). In particular, no effective rapid screening methodology has been established for the detection of OM in the mouse. Tympanometry and otoscopy have been used to screen mouse middle ear diseases (Zheng et al., 2007). Nevertheless, screening of large numbers of mutant mice from ENU mutagenesis programs for hearing impairment using relatively simple but high-throughput procedures, such

as the click-box test, might be expected to identify mutants showing a conductive hearing loss along with mice with sensorineural hearing defects (Brown and Hardisty, 2003). Indeed, the discovery of the deaf mouse mutant *Jeff* (*Jf*), a single locus model for OM, in an ENU program in the United Kingdom underlines the potential of large-scale mutagenesis projects for the identification of OM mouse models (Hardisty et al., 2003). The *Jf* mutant shows a significant conductive hearing loss by postnatal day 35 with fluid and pus in the middle ear cavity. *Jf* mice develop a chronic suppurative OM with severe inflammation of the mucoperiosteum. The *Jeff* locus maps to mouse chromosome 17. Further study demonstrates that *Jeff* carries a mutation in an F-box gene, *Fbxo11*, which is expressed in epithelial cells of the middle ears from late embryonic stages through day 13 of postnatal life. In contrast to *Jeff* heterozygotes, *Jeff* homozygotes show cleft palate, facial clefting and perinatal lethality. *FBXO11* is one of the first molecules to be identified, contributing to the genetic aetiology of OM (Hardisty-Hughes et al., 2006). Another group observed evidence consistent with an association between polymorphisms in *FBXO11* and chronic otitis media with effusion/recurrent otitis media (COME/ROM) by genotyping 13 SNPs across the 98.7 kb of genomic DNA encompassing *FBXO11* (Segade et al., 2006). Another mutant, *Junbo* (*Jbo*), with a very similar phenotype has been identified from the same ENU mutagenesis program. *Junbo* maps to chromosome 3, and recently a mutation in the gene encoding the transcription factor *Evi1* has been shown to underlie the OM phenotype (Hardisty-Hughes et al., 2006). Later, a group observed N-ethyl-N-nitrosourea-induced dominant mouse mutant *Junbo* with hearing loss resulting from chronic suppurative OM and otorrhea. They have identified the causal mutation, a missense change in the C-terminal zinc finger region of the transcription factor *Evi1*. This protein is expressed in middle ear basal epithelial cells, fibroblasts, and neutrophil leukocytes at postnatal days 13 and 21 when inflammatory changes are underway. The identification and characterization of the *Junbo* mutant elaborates a novel role for *Evi1* in mammalian disease and implicates a new pathway in genetic predisposition to OM (Parkinson et al., 2006).

This is a very provocative finding, since the *Evi1* transcription factor is a repressor of the Tgf β pathway which is already implicated in OM pathogenesis and from in vitro studies has been shown to be involved in signaling pathways controlling mucin production (see above and (Hardisty et al., 2003). Both these studies emphasize the potential benefits of continuing to screen mutants from large-scale ENU programs to identify additional models of OM.

Studies employing gene targeting or other transgenic modifications also support the feasibility of developing additional valuable genetic mouse models of OM. Chronic OM is commonly found in VCFS/DGS patients with 22q11 deletions (Funke et al., 2001). Mice overexpressing genes from this region exhibited OM. Interestingly, bacterial artificial chromosome (BAC) transgenic mice overexpressing the human *TBX1* transcription factor (a strong candidate for the equivalent of human VCFS/DGS in mice) and three other transgenes, had malformations similar to VCFS/DGS patients, thus providing a molecular basis for the pathogenesis and variable expressivity of the syndrome (Liao et al., 2004). Mice harboring a homologous deletion of the *p73* locus had a 100% incidence of OM on a C57BL/6 background. A feature of *p73*^{-/-} mice (Yang et al., 2000) was purulent OM at the earliest ages (postnatal day 2 pups), which persisted through adulthood. Microbiologic analysis of affected sites from *p73*^{-/-} weanlings (P21) revealed the presence of *Escherichia coli*, *Pasteurella aerogenes*, and micrococcal species. Despite these indications of inflammation and infection, no obvious deficiencies in lymphoid or granulocyte populations were detected in *p73*^{-/-} mice, indicating that there might be defects in other components of the natural immune system. Mice deficient in lymphocyte function-associated antigen 1, *LFA-1*^{-/-} (CD11a/CD18), have increased incidence of OM but also have significantly increased mortality (Prince et al., 2001). In addition to mice with spontaneous and ENU-induced mutations, The Jackson Laboratory maintains more than 1,000 existing transgenic and knockout mouse strains, which are freely available for screening for genetic models of OM.

Lysozyme knockout mice ($M^{-/-}$) showed an increased susceptibility to middle ear infection with *S. pneumoniae* 6B and resulted in severe middle ear inflammation, compared to wild-type mice. (Shimada et al., 2008). *Eya4* knockout ($-/-$) mice develop otitis media with effusion due to abnormal middle ear cavity and Eustachian tube dysmorphology (Depreux et al., 2008).

6. Future research directions based on mouse models of OM

6.1. Establish more genetic mouse models of OM

Establish the mouse OM models that may be susceptible to certain pathogens or a group of pathogens and can be reproduced spontaneously from generation to generation as in the case of *Jeff* (*Jf*), or *Junbo* (*Jbo*) (Hardisty et al., 2003, Hardisty-Hughes et al., 2006). More genetic OM models need to be established in order to better understand genome-related host-pathogen interactions and facilitate new therapeutic discoveries for better and alternative treatment of human OM conditions.

6.2. Identify the genes underlying OM susceptibility

In fact, the genes or proteins differentially expressed in an animal strain can be identified by methods such as DNA microarray (Ghaheri et al., 2007a; Zhang, 2006) and 2D-DIGE (Viswanathan et al., 2006). Because mRNA expression frequently does not correlate with the amount of protein expressed and does not allow identification of post-translational modification, proteomics technologies will play valuable roles in elucidation of physiologic or pathologic processes underlying otitis media in the mouse model (Thalmann, 2006). The novel approach called SSUMM—Subtractive Strategy Using Mouse Mutants proposed recently (Zheng et al., 2006a,b) will take advantage of the differences between control and affected or mutant samples. We predict that SSUMM would be a useful method in proteomics, especially in those cases in which the investigator must work with small numbers of diverse cell types from a tiny organ.

6.3. Mouse models for vaccine development

The mouse will undoubtedly play a major role in future work on OM vaccines. Considerable efforts have been made to develop effective vaccines against otitis media (Giebink et al., 2005; Murphy et al., 2005). The mechanism by which otitis media occurs is the colonization of bacteria within the nasopharynx and their subsequent entry into the middle ear via the Eustachian tube. Often the nasopharynx bacterial colonization follows a viral infection, which appears to establish the proper conditions for bacterial proliferation (Chonmaitree et al., 2008; Heikkinen, 2000). Thus, an understanding of nasopharyngeal immunology is critical, particularly since vaccines are often delivered intranasally. IgA is a key immunoglobulin produced by plasma cells and secreted into nasal and upper respiratory tract mucosa for vaccine-induced immune memory (Brandtzaeg, 2007). It serves a number of functions, including preventing pathogen attachment and facilitating pathogen neutralization and clearance. Mouse models null for various genes involved in IgA production and secretion are helping to define the mechanisms by which it protects against both viral and bacterial infections (Sun et al., 2004; Asahi et al., 2002).

Mouse models have been used extensively for studies of nasopharynx bacterial delivery, colonization parameters, viral preconditioning, and vaccine efficacy (Hirano et al., 1999; Meek et al., 1999; Sabirov and Metzger, 2008a; Sabirov and Metzger, 2008b). Vaccination of the mouse nasopharynx with several different components of human otitis media bacteria have been shown to be effective in reducing middle ear disease. These include *H. influenzae* P6 (Kodama et al., 2007; Kodama et al., 2006; Sabirov et al., 2001) and *S. pneumoniae* liposomes (Kurono et al., 1993) and polysaccharide (Sabirov and Metzger, 2006). Thus, TLRs play a role in the innate immune response to the vaccine components and development of the adaptive

immune response necessary for long term protection. Other nasopharynx insults, such as cigarette smoke, are also known to induce otitis media. Mouse studies have shown that TLR4 is at least one of the receptors that is involved in this inflammatory response (Doz et al., 2008; Preciado et al., 2008).

7. Conclusions

There can be much optimism that the genetic analysis of mouse models of OM will lead to the identification of novel loci in mouse and humans and a more profound understanding of the genetic aetiology of this complex, multi-factorial disease. The identification of these loci and the study of the specific genes involved might offer the possibility of screens to predict OM susceptibility, which will assist physicians in identifying those patients who are at risk for severe OM and, therefore, may benefit from prophylactic or targeted treatment. Moreover, the development of a new set of mouse mutants and a better understanding of the genetic basis for OM will deliver opportunities to devise novel therapies as well as provide relevant models for pre-clinical testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.brainres.2009.02.047.