Noninvasive immunization strategies to target dendritic cells and protect against experimental otitis media due to nontypeable *Haemophilus influenzae*

**THESIS**

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Abstract

Nontypeable *Haemophilus influenzae* (NTHI) causes multiple diseases of the respiratory tract, including the pediatric disease otitis media (OM). As adherence is a critical step in pathogenesis, the development of vaccines to target adhesins expressed by this bacterium is of interest. We have designed candidates derived from the NTHI adhesins OMP P5 and Type IV pilus and investigated the potential for each to prevent or treat experimental NTHI-induced OM after noninvasive immunization regimens in a chinchilla model.

Characteristics of an immunogen and interactions with dendritic cells (DCs) can influence vaccine efficacy. The OmpA family of bacterial proteins is recently classified as a pathogen-associated molecular pattern. As NTHI OMP P5 is an OmpA homologue, we examined whether the efficacy afforded by immunization with OMP P5 candidates could be attributed to this characteristic. Chinchilla DCs had not been described; therefore we adapted well-established protocols to induce the differentiation of bone marrow precursor cells to DCs, which resulted in cells that were morphologically and phenotypically similar to DCs of other species. Both *in vitro* and *in vivo* we demonstrated DC maturation, migration and interaction with T-cells after stimulation with an OMP P5-derived immunogen. These data suggested an important role for chinchilla DCs in the development of protective immunity following immunization.

We next examined the protection afforded by intranasal immunization of chinchillas with the OMP P5-derived immunogen and observed significantly earlier
clearance of NTHI from the nasopharynges and middle ears of chinchillas, compared to controls. The mechanism for the observed protection was DC priming of T-cells within the nasal-associated lymphoid tissue. IN immunization with OMP P5-derived immunogen effectively induced systemic and mucosal immune responses that resulted in the observed resolution of experimental NTHI-induced OM.

As an additional noninvasive immunization strategy, we examined the efficacy afforded by transcutaneous immunization (TCI) with NTHI OMP P5- and Type IV pilus-derived immunogens delivered with a double mutant of *E. coli* heat labile enterotoxin as the adjuvant. Both systemic and mucosal immune responses were detected after application of formulations to the pinnae of chinchillas. This response was protective, as significantly fewer NTHI were detected within the nasopharynx and middle ears, compared to animals administered adjuvant alone. The mechanism for the observed immune response was the migration of DC-SIGN\(^+\) dermal DCs from the pinnae to local lymphoid tissues. Thus, TCI was effective to prevent experimental NTHI-induced OM.

The chronic nature of OM is due to biofilms formed within the middle ear; thus we evaluated the therapeutic potential of TCI. NTHI biofilms were established in the middle ears of chinchillas prior to TCI. Animals that received immunogen alone or immunogen plus adjuvant demonstrated markedly reduced or completely eliminated biofilms, compared to animals that received only adjuvant. These data advocate TCI as a therapeutic strategy to target ongoing OM. Collectively, these data show that noninvasive immunization strategies with NTHI adhesin-targeted immunogens could expand the use of vaccines to protect against as well as treat OM, and have potential for efficacy against other diseases of the respiratory tract due to NTHI.
Dedication

I wish to dedicate this body of work to the people who are most important in my life, those who have encouraged me, inspired me, pushed me and most of all, who love me.

I would not be the person I am today or have accomplished what I have without your support.

To Mom, Dad and Casey.

I also dedicate this to the memory of my sister, Jayme Lynn.

You are missed and loved.
I would like to thank Dr. Robert Munson, Jr. for agreeing to take on the challenge to be my advisor. This has not been an easy process for either of us and I truly appreciate all of the extra effort it required of you to direct me to this point. It was a battle hard fought and finally won.

Thank you to Dr. Larry Schlesinger, for your support throughout my long educational journey. I appreciate your advocacy.

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Remember to keep on swimming.

To Jennifer Neelans, who always makes sure we end up where we should be, thank you for your patience and sense of humor.

Most of all, I wish to express my heartfelt gratitude to Dr. Lauren Bakaletz. I am the scientist and leader I am today because of your guidance and never-ending support. I appreciate the many ‘opportunities’ you have offered to me, whether I felt I was ready for them or even wanted to take part, you clearly knew better than I. Please know these words cannot begin to convey my appreciation for all that you do.
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regions of the PilA protein of nontypeable *Haemophilus influenzae* (NTHI) to facilitate the design of two novel chimeric vaccine candidates. Vaccine. 28:279-289.


**Field of Study**

Major Field: Molecular Virology, Immunology and Medical Genetics
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<tbody>
<tr>
<td>AOM</td>
<td>acute otitis media</td>
</tr>
<tr>
<td>APC</td>
<td>alophecoerythin</td>
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<tr>
<td>AV</td>
<td>adenovirus</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CEACAM1</td>
<td>carcinoembryonic antigen-related cell adhesion molecule -1</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluoroscein succinimidyl ester</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
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<tr>
<td>chimV4</td>
<td>chimeric protein version 4</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
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<tr>
<td>CSOM</td>
<td>chronic suppurative otitis media</td>
</tr>
<tr>
<td>daPa</td>
<td>deca-Pascals</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<tr>
<td>DCs</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
</tr>
<tr>
<td>dmLT</td>
<td>double mutant of <em>Escherichia coli</em> heat labile enterotoxin</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPS</td>
<td>extracellular polymeric substance</td>
</tr>
<tr>
<td>ET</td>
<td>Eustachian tube</td>
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<tr>
<td>EU</td>
<td>endotoxin units</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>FSC</td>
<td>forward scatter</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage-colony stimulating factor</td>
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</table>
GMT: geometric mean titer
HRP: horse radish peroxidase
IACUC: Institutional Animal Care and Use Committee
ICAM-1: intercellular adhesion molecule -1
Ig: immunoglobulin
IHF: integration host factor
IL: interleukin
IN: intranasal
kDa: kilodalton
LOS: lipooligosaccharaide
LPS: lipopolysaccharide
LT(R192G-L211A): double mutant of *Escherichia coli* heat labile enterotoxin
MEF: middle ear fluid
MHC: major histocompatibility complex
MPL: monophosphoryl lipid A
NALT: nasal-associated lymphoid tissue
NETs: neutrophil extracellular traps
NP: nasopharynx or nasopharyngeal
NTHI: nontypeable *Haemophilus influenzae*
OCT: optimal cutting temperature medium
OM: otitis media
OME: otitis media with effusion
OMP: outer membrane protein
PAMP: pathogen-associated molecular pattern
PE: phycoerythrin
PAFr: platelet-activating factor receptor
POET: Pneumococcal Otitis media Efficacy trial
PRP: polyribosylribitol phosphate
rsPilA: recombinant soluble PilA
RSV: respiratory syncytial virus
RU: resonance unit
sBHI: supplemented Brain Heart Infusion broth
SLI:    sublingual immunization
SQ:     subcutaneous
SSC:    side scatter
TCI:    transcutaneous immunization
Tfp:    Type IV pilus
TFPQ3:  Type IV pilus quartile peptide #3
TLR:    Toll-like receptor
TM:     tympanic membrane
TMB:    3,3’,5,5’-tetramethylbenzidine
TNF:    tumor necrosis factor
U:      units
URTI:   upper respiratory tract infection
TVP:    *tensor veli palatini*
Chapter 1

Introduction

**Otitis media**

Otitis media (OM) is the most common disease of childhood. Defined as inflammation of the middle ear space regardless of viral or bacterial etiology, it can be both acute and chronic in nature. Acute OM (AOM) is disease with rapid onset and hallmark signs and symptoms of infection, including otalgia, fever and a bulging tympanic membrane due to accumulation of fluid within the middle ear space. The disease course of AOM is short in duration, lasting several weeks. Chronic OM, however, is disease that lasts beyond 3 months with the persistence of middle ear fluid and negative middle ear pressure. AOM affects 65-330 million people worldwide and while not associated with mortality in developed countries, both intratemporal and intracranial complications from chronic suppurative OM (CSOM; OM with discharge from the ear for at least 2 weeks) results in 50,000 deaths of children under the age of 5 years in developing countries (109, 167, 328). It is estimated that 83% of all children will experience an episode AOM by the age of three, with 46% of children experiencing three or more episodes by this age (320).

In contrast, otitis media with effusion (OME) or ‘silent OM’ lacks signs and symptoms of acute disease although fluid is present within the middle ear cavity. Approximately 90% of children between the ages of 6 months and 2 years will
experience OME (2) and while many episodes resolve within 3 months, recurrence and persistence of fluid within the middle ear can result in hearing loss and consequently behavioral, educational and developmental delays of this very young population (23, 138, 321).

**Economic burden**

With a significant incidence of disease, the costs associated with OM are considerable. The most recent statistics indicate that in the 1990s approximately 24.5 million visits were made to a physician in the United States with estimated costs including loss to the caretaker in wages and productivity of more than $5 billion annually (7, 93, 158, 231). The worldwide incidence and associated costs are assumed to be much greater.

**Risk factors for otitis media**

Factors that contribute to the development of OM include craniofacial abnormalities such as cleft palate (289) or due to Down’s syndrome (290). Exposure to smoke negatively impacts ciliary beat frequency and ciliogenesis (46, 115, 318); crowding living conditions and attendance at day care facilitates transmission of viral/bacterial agents of OM (276, 278); existence of allergies and ongoing infections predisposes to OM (259) and inadequate access to medical care also contributes to persistence of OM (243). Moreover, genetic susceptibility is a factor; in a twins and triplets study, monozygotic twins were shown to experience more episodes of OM and longer periods with middle ear fluid compared to dizygotic twins (40, 41).
Also important to the incidence of OM in the pediatric population is immunologic immaturity. Maternal antibody in a neonate is present transiently, with a half life of 21-30 days from birth (291). As children are colonized within one month of birth with the bacterial causative agents of OM (77), the waning of maternal antibodies and time necessary to develop a protective adaptive immune response leaves this population vulnerable to development of bacterial OM.

**Relevant anatomy**

The middle ear space is separated from the external auditory canal by the tympanic membrane and linked to the lateral wall of the nasopharynx by the Eustachian tube (ET). In healthy individuals, the middle ear is a sterile, air filled space which houses the three ossicles, the malleus, incus and stapes, and serves to transmit sound energy via the tympanic membrane and ossicles from the external auditory canal to the inner ear. In individuals with OM, fluid within the middle ear abrogates the movement of the tympanic membrane and ossicles and can result in conductive hearing loss of 20-30 decibels (265).

The ET is a tubal organ with three functions: protection, ventilation and drainage of the middle ear (30). Both goblet cells and ciliated cells line the ET, and are covered by a blanket of mucus and serous secretions (183, 185). Movement of the cilia in a metachronal wave serves to push the mucus blanket away from the opening of the middle ear and toward the pharyngeal opening of the ET to be swallowed. Particulate matter, including viruses or bacteria, can be trapped in the mucus, thus ET ciliary activity and mucus production are critical to maintain the sterility of the middle ear. Disruption of these functions is associated with development of OM.
Compared to healthy adults, in whom the incidence of OM is minimal, there exist anatomical and functional differences with the pediatric ET (30, 37, 280). In an adult, the length of the ET is 30-40 mm and sits 33-45° from horizontal, whereas in children <7 years of age, the ET is approximately 18 mm in length and lies 0-10° from a horizontal plane (30, 67, 280). It is proposed that the greater length of the ET and its angle of placement within the adult cranium are factors which restrict fluid or nasopharyngeal secretions from entering the middle ear (30). Additionally, the adult ET is functionally closed; upon contraction of the tensor veli palatini muscle (TVP), the ET is transiently opened to allow for equilibration of middle ear with atmospheric pressure (67). In a child, the TVP is not fully developed, imparting a semipatulous character to the ET. Thus, unlike an adult, the pediatric ET is a short and fairly open conduit into the middle ear, factors of which contribute to the incidence of OM in this population.

**Treatment of otitis media**

Treatment for bacterial OM has traditionally involved antibiotic therapy. With 20 million antibiotic prescriptions annually in the U.S., OM is the primary reason for a child to receive an antimicrobial (1, 109). Amoxicillin is the first-line therapeutic for AOM caused by *S. pneumoniae*, whereas amoxicillin-clavulanate may be prescribed against NTHI- and *M. catarrhalis*- induced OM to overcome β-lactamase production by the latter organisms (1). Due to the emergence of multiple antibiotic-resistant bacterial strains, the American Academy of Pediatrics and American Academy of Family Physicians have revised previous clinical practice guidelines to strongly recommend discrimination between AOM and OME at the time of diagnosis, in addition to judicious use of antibiotics (1, 2). Implementation of ‘watchful waiting’ for children with OME
versus immediate prescription of antibiotics to treat AOM is suggested for otherwise healthy children greater than 2 years of age.

Surgical intervention for OM involves myringotomy and placement of tympanostomy tubes into the tympanic membrane (TM). With the substantial incidence of OM in the pediatric population, tympanostomy tube insertion is the most common surgical procedure requiring anesthesia for children and while effective to relieve pressure and pain associated with accumulation of fluid from the middle ear, the procedure itself has risks including potential damage to the ear and those inherent with use of anesthesia (5). Moreover, chronic otorrhea, myringoclerosis and TM perforation upon extrusion or removal of the tubes are common complications (331).

**Role of respiratory viruses in OM**

OM can be of viral and/or bacterial etiology. A close association between viral upper respiratory tract infection (URTI) and OM is known, thus OM is considered a polymicrobial disease (13, 121, 219). Among respiratory tract viruses, respiratory syncytial virus (RSV) is the most common virus detected within nasopharyngeal secretions and middle ear fluids retrieved from children that develop AOM (44, 123). Other viral species frequently identified include rhinovirus, influenza A or B viruses, adenovirus (AV) serotype 1, 2, 3 or 5, and parainfluenza virus 1, 2 or 3.

Clinically, a seasonal pattern of viral URTI in children is noted, with an increase in incidence during spring and autumn (329). An outcome of viral URTI as it pertains to OM is ET dysfunction due to altered mucus production and abrogation of ciliary function (184). One technique to assess ET function is tympanometry to determine relative middle ear pressure. In a child, a negative middle ear pressure of >-100 daPa is
indicative of an ET dysfunction (250). A longitudinal study to examine ET function during viral URTI reports abnormal tympanograms and negative middle ear pressure in 55% of children during the first seven days of infection, which is most pronounced in children 6-11 months of age (268). Moreover, a peak in the incidence of viral URTI is observed immediately prior to, or concurrently with, an increase in episodes of bacterial OM (277). Thus, viral URTI is an important predisposing factor to bacterial OM.

In experimental models, in addition to negative middle ear pressure, reduction in ET mucocilliary clearance and dysregulated expression of host defense peptides is observed after inoculation with AV serotype 1, influenza A virus or RSV strain A2 (17, 198, 314). Moreover, in chinchilla models of experimental OM, a greater incidence of disease is observed upon viral-bacterial superinfection, compared to challenge with virus or bacterium alone (99, 314). Therefore, viral dysregulation of airway defenses is a key component toward the pathogenesis of OM.

**Bacterial agents of otitis media**

As mentioned previously, colonization by bacteria occurs soon after birth. A clinical study showed colonization of the pediatric nasopharynx (NP) with any of the bacterial species that cause OM within one month of birth, with 68% of children colonized by 6 months of age (77, 180). Three bacterial species are responsible for the majority of episodes of OM: *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* (NTHI) and *Moraxella catarrhalis*. The reported NP carriage rates for each species vary among published work, attributed to geographic location of sampling, culture methods, socioeconomic status of subjects and risk factors as described earlier (92). However, the overall burden of OM caused by each species is estimated to be 35-
40% due to *S. pneumoniae*; 30-35% by NTHI and 10-15% due to *M. catarrhalis* (350). While not strictly delineated, the pneumococcus is associated with AOM, while NTHI and *M. catarrhalis* are linked to chronic OM or OME. The work presented herein focuses on NTHI-induced OM.

**Nontypeable *Haemophilus influenzae***

NTHI belongs to the *Pasteurellaceae* family and similar to other members of the *Haemophilus* genus, has an absolute requirement for heme for growth (171). As heme is sequestered or bound to serum proteins in the human host, NTHI possess multiple mechanisms for uptake of iron (49, 110, 189, 207, 208). In vitro, NTHI are cultured on chocolate agar or on medium supplemented with heme and nicotinamide adenine dinucleotide, then incubated at 37°C with 5% CO₂ in a humidified atmosphere. On solid medium, NTHI colonies are round, flat and grey in color. Upon Gram stain, NTHI present as Gram-negative coccobacilli. NTHI are also shown to be motile in vitro when cultured on a chemically defined medium at an alkaline pH of 9.0, with movement facilitated by expression of Type IV pili (16, 48).

At present, the genomes for five NTHI strains are complete and demonstrate genome sizes from 1.81 to 1.91 Mb, (compare to 1.83 Mb for *H. influenzae* Rd KW20 and 1.98 Mb for *H. influenzae* type b) (86, 119, 228). Based on electrophoretic typing, NTHI are genetically distinct from encapsulated *H. influenzae* (226). Moreover, as its name implies, NTHI do not express a polysaccharide capsule, and while strains which harbor the capsule-associated insertion element IS1016 have been identified (159, 285, 307), NTHI do not possess the cap gene cluster required for capsule production (130, 172, 285).
Unlike strains of *Haemophilus influenzae* which may be grouped based on serotyping with capsule-reactive antisera or PCR-based capsule typing, a universal classification system is not established for NTHI. A number of techniques are employed to cluster NTHI strains, including biotyping based on enzyme activity, pulsed-field gel electrophoresis of genomic DNA, multi-locus sequence typing of several housekeeping genes and examination of protein and lipooligosaccharide (LOS) expression profiles or amino acid sequences (88, 223).

**Pathogenesis of NTHI**

NTHI persist asymptomatically within the NP of children and adults. Colonization is dynamic; one study reported carriage of at least one and up to nine NTHI strains in children up to three years of life and also observed frequent strain turnover (78, 278). In adults, an analysis of NTHI carriage detected two to five strains at one time and genetically different strains are detected over time (211). Whereas both children and adults are shown to possess antibodies against NTHI OMPs and LOS, this immune response is not effective to eradicate NTHI from the NP (76, 118, 165, 348). NTHI persistence and turnover is proposed to be due to production of strain-specific antibody, low serum or mucosal antibody titers, induction of antibody that targets immunologic decoys within OMPs expressed by NTHI or competition of NTHI with NP flora (76, 92).

Under appropriate conditions, such as predisposing viral URTI, NTHI can cause localized upper and lower respiratory tract infections, including OM, conjunctivitis, sinusitis, bronchitis, pneumonia, chronic obstructive pulmonary disease and cystic fibrosis (25, 74, 217, 287). The shift from NP commensal to respiratory pathogen is facilitated by dysregulation of airway homeostasis, such as dampened expression of
host defense peptides, epithelial cell damage, abrogated mucociliary clearance, altered
secretion of mucus and upregulated expression of host cell molecules to which NTHI
adhere (10, 148, 197, 198, 202, 203). Specific to OM, alteration of one or more host
defense mechanisms provides the opportunity for unrestricted growth and/or enhanced
adherence by NTHI within the NP. A well-established adenovirus-NTHI superinfection
model in the chinchilla demonstrates disease kinetics similar to natural OM development
in humans, wherein 7-10 days after viral infection, OM is observed (17, 202, 314, 329).
The release of immune pressure against NTHI allows the bacterium to gain access to
the middle ear by ‘growing up’ the compromised ET, which requires a period of 7-10
days (202). Thus, NTHI is an opportunistic commensal of the human NP.

**NTHI outer membrane proteins and lipooligosaccharide**

Adherence within the human respiratory tract is critical for NTHI colonization and
subsequent pathogenesis. NTHI express multiple outer membrane proteins (OMPs) and
LOS that can serve to bind the bacterium to host mucin, epithelial cells or specific
receptors expressed by host cells, including carcinoembryonic antigen-related cell
adhesion molecule (CEACAM)-1, human intercellular adhesion molecule (ICAM)-1,
platelet activating factor receptor (PAFr), Toll-like receptors (TLR)-2 or -4, or
extracellular matrix proteins (10, 32, 42, 85, 127, 148, 203, 267, 315). A number of
NTHI surface structures have been characterized in detail, and a brief summary of the
OMPs for which there is active interest follows.

a. **High molecular weight proteins (HMW) 1 and 2.** HMW1 and HMW2 exist as non-
covalently linked proteins in the outer membrane of NTHI and are shown to share
sequence similarity and antigenic cross-reactivity to the filamentous hemagglutinin of
Bortatella pertussus (308). The two proteins share 71% amino acid sequence identity and the predicted molecular masses range from 100-150 kDa (26, 308). Based on Southern blot analysis, 80% (47/ 59) of a panel of NTHI strains possessed either one or both hmw1A or hmw2A genes, and a majority of these strains exhibited adherence to epithelial cell lines (305, 306, 326). HMW1 and HMW2 exhibit conserved, but different binding specificities to epithelial cell lines in vitro (63, 107). HMW expression is phase-variable; observed in vitro as varied expression of hmw1A, one of the two genes which encode HMW1 (106). In vivo, niche-specific expression is shown as greater expression of HMW by NTHI strains isolated from the pediatric NP compared to paired isolates from the middle ear (62). Moreover, NTHI from patients with COPD exhibit reduced expression of HMW in serial cultures collected over time (43). Antibodies that target HMW mediate opsonophagocytosis of homologous and heterologous NTHI strains in vitro (345, 346).

b. Hia and Hap proteins. Of the NTHI strains that did not express HMW proteins described above, 15% expressed an autotransporter called Hia (306). Hia is a trimeric protein of 100-250 kDa. Unlike other NTHI autotransporters, during processing the Hia passenger domain remains associated to the β-domain of the molecule and it is this domain that facilitates adherence of NTHI to epithelial cell lines (304, 313). Similar to the HMW proteins, antibody against Hia facilitates opsonophagocytosis of homologous and heterologous NTHI strains in vitro (344).

Hap is 155 kDa autotransporter protein expressed by all NTHI strains tested thus far (27). Similar to Hia, the Hap passenger domain (Hapₚ) facilitates adherence to epithelial cell lines and extracellular matrix proteins, and promotes bacterial aggregation and formation of microcolonies in vitro (83, 85). However, unlike Hia,
Hap s is cleaved and released from the bacterium. It is suggested the proteolytic activity of Hap s acts on the host epithelium or matrix proteins to promote NTHI adherence or invasion (84).

c. **OMP P2.** OMP P2 is 36-42 kDa and is the most abundant protein in the outer membrane of NTHI. OMP P2 is a porin, comprised of a trimer of subunits. A topographical map of the deduced amino acid sequence of OMP P2 predicts a protein with eight surface-exposed loops, four of which [loops 2, 4, 5 and 7] demonstrate hypervariability among NTHI strains (28, 69, 71, 212, 292). Antibodies against OMP P2 are present within normal human serum and are bactericidal (220); however, mapping studies demonstrate that antibody is directed at loop 5 of the protein, and results in strain-specific activity (351).

d. **Protein D/ GlpQ.** Protein D/ GlpQ is a 42 kDa lipoprotein once thought to be an IgD-binding protein (142). However, it was later found that not all IgD myelomas bound to either NTHI or extracts of *E. coli* expressing a recombinant form of Protein D (283). Protein D/ GlpQ shares 67% sequence identity with an *Escherichia coli* glycerophosphodiester phosphodiesterase, GlpQ (215). Moreover, recombinant NTHI GlpQ exhibits activity similar to *E. coli* GlpQ by catalyzing the cleavage of L-α-glycerophosphocholine into choline and sn-glycerol-3-phosphate (178). Decoration of NTHI LOS with choline is a critical factor for NTHI to evade detection and clearance by the host (79, 151, 338, 339).

The *hpd* gene which encodes Protein D/ GlpQ shows 96%-99% identity among NTHI strains tested and further, despite immunologic pressure, no changes in this sequence was observed among sequential isolates collected from patients with COPD (70, 301). Protein D/ GlpQ is predicted to be surface-exposed in NTHI; the
deduced amino acid sequence consists of hydrophilic residues, moreover, enzymatic activity is localized within the membrane fraction of NTHI cell extracts (143, 215). Antiserum against Protein D/ GlpQ inhibits adherence of NTHI to primary respiratory epithelial cells and mutation of hpd results in fewer NTHI recovered in the NP and middle ear after challenge in a chinchilla model of experimental OM (151). As will be described later, NTHI Protein D serves as a carrier molecule for pneumococcal capsular polysaccharides in Synflorix™, a recently licensed pneumococcal conjugate vaccine (263).

e. **OMP26.** OMP26 is a 25-27 kDa protein with 98% amino acid sequence homology to *H. influenzae* Rd OmpH and 56% homology to *Pasteurella multocida* Skp chaperone protein (175). By Southern blotting, the gene which encodes OMP26 is detected in all NTHI strains tested (73). By electron microscopy, antibody directed against OMP26 labels NTHI, suggesting that this protein is present on the bacterial outer membrane (175). Both healthy children and children with AOM are shown to have antibody against OMP26, however this antibody is not bactericidal (258).

f. **OMP P5.** OMP P5 is a 36.5 kDa protein that is approximately 25% of the NTHI outer membrane protein profile (213). The gene which encodes OMP P5 shares 50% homology with *E. coli* ompA (213, 214, 296). Unlike OmpA proteins of Gram negative bacteria which are porins, OMP P5 is expressed as thin, peritrichously arranged fimbriae present on all NTHI strains isolated from the NP and middle ears of children with OM (20, 296, 335). It has been reported that OmpA proteins from *Klebsiella pneumonias*, *E. coli* and *Acinetobacter baumannii* induce dendritic cell (DC) maturation and activation, including expression of co-stimulatory molecules, cytokine secretion and stimulation of T-cell proliferation (144-147, 181, 256, 257,
These observations, among others, have led to the designation of this highly conserved family of bacterial proteins as a new pathogen-associated molecular pattern, or PAMP (145). NTHI OMP P5 is an OmpA protein homologue, and shown by several in vitro assays induces similar DC activation, suggesting that it, too, may be a PAMP (242, 296).

OMP P5 facilitates adherence to human oropharyngeal cells (20) and nasopharyngeal mucin (267), chinchilla ET mucin (203), RSV-infected A549 cells (148), CEACAM1 transfected HeLa (32) or CHO cells (127) and ICAM-1 (10). The N-terminus of OMP P5 is predicted to consist of four surface-exposed loops (68, 335). The first loop demonstrated the most variability among NTHI strains, the second and third loops are more conserved, and the fourth loop exhibits the greatest sequence conservation. Epitope mapping studies using serum from children with OM and chinchillas with experimental OM, in addition to chinchillas immunized with a loop 3-derived immunogen, revealed that the third loop of OMP P5 is an adhesin domain while the fourth loop is an immunological decoy (237, 240). Several NTHI-targeted vaccine candidates are in development, as will be described later.

g. **OMP P6.** OMP P6 is a 16 kDa lipoprotein that comprises between 1-5% of the total outer membrane protein profile of NTHI (213, 229). The P6 gene is highly conserved and the translated amino acid sequence is identical among NTHI strains (221, 230). Substitution among four specific amino acids in OMP P6 serves to distinguish *H. influenzae* from *Haemophilus haemolyticus*, and is utilized as a screening tool to confirm the identity of each bacterial species (222). OMP P6 binds to its own gene, and thus can modulate self-expression (293). Structurally, there are six predicted surface-exposed regions of OMP P6, regions that are recognized by antibody
present in serum from children and adults (31, 168, 186, 213, 223, 349). Antibody against OMP P6 is bactericidal and reacts against multiple strains of NTHI (225). Of note, two recent manuscripts seek to challenge the nucleotide conservation of \( p6 \) and present data from 14/151 strains with variable \( p6 \) gene sequences (161). Moreover, this group proposes that OMP P6 is not surface-exposed, rather a protein of comparable mass and with similar epitopes is instead the target of the observed antibody (200).

h. **Protein E.** Protein E is a 16 kDa protein that is a minor component in the NTHI outer membrane (274). The gene encoding Protein E shows 94%-100% nucleotide sequence identity among NTHI strains and the deduced amino acid sequence is 96%-100% identical (294). It is proposed that the housekeeping promoter, \( rpoD \), induces the constitutive expression of the gene for Protein E (294). Epitope mapping studies show that a central region of the protein is important for adherence of NTHI to epithelial cell lines and to bind to the extracellular matrix protein, vitronectin (117, 273).

i. **Type IV pilus (Tfp).** Until recently, *Haemophilus* was not considered motile. However, interrogation of the genome for NTHI strain 86-028NP revealed a gene cluster, \( pilABCD \), with homology to Tfp-related genes in other bacterial species (16, 119). Additionally, a competence gene cluster \( comABCDEF \) was also identified and included a secretin ComE, thus, NTHI possessed the components required to assemble and express a Tfp. Among a panel of 23 isolates, a single gene which encodes the majority subunit of Tfp, PilA, was detected (16). Moreover the deduced amino acid sequence for PilA from this panel of strains exhibits \( \geq 81\% \) identity to that of NTHI strain 86-028NP. Tfp are not produced constitutively, however expression of
this 14 kDa protein facilitates NTHI competence, adherence to primary respiratory epithelial cells, twitching motility and biofilm formation in vitro (16, 155). Moreover, in vivo, PilA expression is necessary for NTHI to form a robust biofilm in the middle ear and to maintain long term colonization within the NP of challenged chinchillas. Epitope mapping PilA identified that antibody in serum from NTHI-colonized children and adults, children with OM or adults with exacerbations of COPD react primarily to the N-terminus of the protein (236). In contrast, antibody in fluids collected from the site of infection (pediatric middle ear fluids and sputa from the adults) reacted primarily with the C-terminus of PilA. As NTHI are eventually cleared from the middle ear and lung, these data indicate that focusing the immune response on the C-terminus of the PilA could resolve or prevent NTHI-induced disease. Several NTHI-targeted vaccine candidates are in development, as will be described later.

j. Lipooligosaccharide (LOS). As with other Gram-negative bacteria found within the respiratory mucosa, NTHI express LOS or endotoxin, a 2-7 kDa glycolipid. LOS is comprised of a lipid A moiety coupled to a core oligosaccharide that consists of glucose, galactose, heptose and 2-keto-3-deoxyoctonic acid and is distinguished from lipopolysaccharide (LPS) by a lack of O-antigen repeats (97, 286, 337). While LOS is an abundant component on the outer surface of NTHI, differences exist in the relative quantity produced due to phase variable expression and in composition of LOS both among NTHI strains and within an individual isolate (38, 97, 253). LOS is shown to be both membrane-bound and released, with an increase in the quantity detected over time in NTHI culture supernatants (112). Exposure of host immune cells or epithelial cells to LOS results in release of pro-inflammatory mediators such as TNF-α, IL-8, IL-1β and IL-6 and cytotoxicity to ciliated epithelial cells, factors in
NTHI pathogenesis (45, 112, 150, 164). NTHI is also shown to decorate its LOS with host-derived sialic acid as a means to evade immune detection (33, 133, 249, 316).

**Biofilms and otitis media**

It is estimated that 80% of all infections have a biofilm component (120). A biofilm is defined as an organized bacterial community encased in an extracellular polymeric substance (EPS) and associated with a substrate (50). These bacterial communities are recalcitrant to antibiotic treatment, able to survive antibiotic concentrations 1000-times greater than planktonic bacteria (129). Antibiotic treatment, which often targets biosynthetic pathways, can fail due to the slowed metabolism of the bacteria within these structures (81). Moreover, the EPS can serve as a substantial barrier to restrict access of antimicrobials, host defense peptides and immune cells to the encased bacteria (87).

NTHI-induced diseases of the respiratory tract, including OM, can be chronic and/or recurrent in nature, a consequence of biofilms formed on the respiratory mucosae (12, 219). While once controversial (210), the localization of bacteria within a biofilm provides an explanation as to why middle ear fluids retrieved from children were often culture-negative, but PCR positive for bacterial DNA (21, 108, 261). Further support was shown by detection of mRNA corresponding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in 100% of middle ear fluids collected from children (266). As the half-life of this molecule is brief, these data demonstrated that metabolically active bacteria were present within the middle ear, despite culture-negative status of the middle ear fluids.
NTHI biofilms are present on the middle ear mucosa of children (116) and upon gross examination of the middle ear in animal models (72). In experimental models, NTHI biofilms are organized structures consisting of clusters or towers of viable bacteria, strands of bacterial and host DNA and intervening water channels, consistent with biofilms formed by the archetypical biofilm-forming *Pseudomonas* species (72, 132, 153). As revealed upon microscopic examination, the DNA is arranged in a mesh-like lattice and may serve as a scaffold for the biofilm structure (132, 153). Host DNA within the outermost edge of the biofilm EPS is a product of neutrophil extracellular traps (NETs) (132, 152, 154) and is hypothesized to sequester the biofilm and/or serve as a sink for host defense peptides.

NTHI LOS, IgA1 protease and OMPs, including HMW, Hia, OMP P5, OMP P6 and Tfp, in addition to a member of the DNABII family called integration host factor (IHF), are distributed within the biofilm EPS (91, 153, 154, 224, 336)(Goodman *et. al.*, submitted). As a means to reduce the host inflammatory response to the presence of LOS, NTHI masks the structure by decoration with host sialic acid or phosphorylcholine (111, 133, 134, 151). A novel and promising approach to eradication of established NTHI biofilms within the middle ear is to target proteins within the scaffold, such as Tfp or IHF and thus induce the collapse of the structure (238)(Goodman *et. al.*, submitted).

**Vaccine development**

In 1985, a vaccine against *H. influenzae* type b comprised of the capsular polysaccharide, polyribosylribitol phosphate (PRP) was made available. As children < 2 years of age responded poorly to the pure polysaccharide preparation, the next generations of vaccines incorporated a protein carrier such as diphtheria toxoid, tetanus
toxoid or meningococcal group B OMP, each conjugated to PRP (65). While successful in the prevention invasive disease due to *H. influenzae* even in the very young, these vaccines were ineffective against the acapsular NTHI. Recently, a 10-valent pneumococcal capsular conjugate vaccine [Synflorix™] was licensed, wherein NTHI Protein D serves as a carrier molecule for eight of the included pneumococcal serotypes (263). In a clinical trial, this formulation demonstrated 35.3% protective efficacy against NTHI-induced OM after parenteral immunization of children (262). Although shown as a secondary outcome measure, this trial nonetheless demonstrated, for the first time, that parenteral delivery of a NTHI-derived antigen could provide protection against OM due to NTHI. Current work aspires to enhance the modest protection observed.

Adherence to the respiratory mucosa is a critical first step toward NTHI-induced disease, thus many efforts have focused on the development of vaccines which target these structures. As described previously, NTHI express multiple adhesins on the outer membrane. Purified forms of OMPs, or derivatives thereof, have been tested in experimental models of NTHI-induced OM. Protection against NTHI-induced OM is primarily antibody-mediated (260), thus characteristics for an ideal OM vaccine require that the vaccine must be immunogenic, especially in the very young; target a conserved antigen on the bacterium; maintain the immunogenicity of each component if multi-subunit; induce a durable immune response; and not promote replacement by other non-vaccine bacterial species (11).

Several immunogens considered to be leading candidates for the prevention of NTHI-induced OM include Protein D, OMP P5-derived peptides, OMP26, OMP P6 and Tfp PilA (11, 51, 216, 260). A summary of the findings with these immunogens in experimental models is presented.
a. **Protein D.** Protein D is the most advanced, in terms of clinical application. A rationale for inclusion in Synflorix™ is its demonstrated homology among NTHI strains, thus antibody directed against this molecule should target all strains encountered in the pediatric NP. As demonstrated clinically, 35% protective efficacy was achieved against NTHI- induced OM (262). This result was predicted pre-clinically; wherein passive transfer of pediatric serum from children that received the Protein D-pneumococcal conjugate vaccine to chinchillas provided 34% protection against experimental NTHI-induced OM (239). The results from this study also provided strong support of the chinchilla passive transfer-superinfection model as a pre-clinical predictor of a clinical outcome.

b. **OMP P5-derived peptides.** OMP P5, although expressed by all NTHI, demonstrates heterogeneity among strains and provides partial protection upon NTHI challenge in chinchilla models (296). Multiple algorithmic analyses of the deduced amino acid sequence of OMP P5 predict four surface-exposed loops in the N-terminal half of this protein and within the third surface-exposed loop is a 19-mer B-cell epitope incorporated into an immunogen called ‘LB1’ (19). Among NTHI strains tested, there is limited amino acid sequence diversity within this moiety which allows for segregation of isolates into three groups, 76% of which cluster into one majority group (18). The immunogen LB1 is a 40-mer synthetic chimeric peptide comprised of the aforementioned 19-mer B-cell epitope from OMP P5 that is co-linearly synthesized with a T-cell promiscuous epitope from measles virus fusion protein, incorporated to be broadly permissive in binding to MHC class II molecules (19, 160). After parenteral administration with LB1 or delivery of antiserum against LB1 by passive transfer, significant protection against homologous and heterologous NTHI
challenge in chinchilla and rat models of OM is observed (18, 19, 162, 176, 238). Moreover, transcutaneous immunization of chinchillas with LB1 results in significantly earlier clearance of NTHI from directly challenged NP and middle ears and significantly earlier resolution of established middle ear biofilms compared to controls (238). The mechanism for the observed protection is attributed to the activation and migration of dermal dendritic cells.

c. **OMP26.** In a rat model, immunization with native OMP26 into Peyer’s patches followed by tracheal boost results in significantly fewer NTHI in bronchoalveolar fluids after homologous or heterologous bacterial challenge (175). Following the same immunization regimen, enhanced clearance of NTHI from the rat lungs is achieved with an recombinant OMP26 that incorporates its leader sequence, compared to recombinant OMP26 without the leader sequence (73). Protection was attributed to greater serum and mucosal antibody titers and enhanced lymphocyte proliferation upon receipt of the former immunogen. In a chinchilla model, recombinant OMP26 incorporating the leader sequence also demonstrates significantly earlier clearance of NTHI from the NP, but not middle ear, compared to the cohort that received only the adjuvant (176).

d. **OMP P6.** Using an immunization regimen of Peyer’s patch-prime followed by tracheal boost with native OMP P6, significantly fewer NTHI are retrieved from bronchoalveolar fluids and lung homogenates after pulmonary challenge of rats with a homologous, but not heterologous, NTHI strains (177). In murine models of intranasal (IN) immunization with native or recombinant OMP P6, a significant reduction in NP colonization is observed after IN challenge with a homologous NTHI strain, compared to sham-immunized cohorts (135, 136). Protection is attributed to
the production of immunogen-specific IgG and IgA in serum and mucosal secretions (136, 169, 279). In an effort to focus the induced immune response, several groups have identified immunodominant T-cell epitopes in the central part of OMP P6 and show that immunization with representative peptides induces a protective immune response in mouse models (140, 199, 233).

e. **Tfp PilA.** An immunogen to target Tfp was designed that focuses on the majority subunit of this structure, PilA. To promote the production of a soluble immunogen, the hydrophobic first 27 amino acids of mature NTHI Tfp PilA are excluded from the design yielding a 114 residue recombinant protein called ‘rsPilA’ (236). Passive transfer of antiserum against PilA provides significant protection against experimental NTHI-induced OM in a chinchilla model (236). Moreover, transcutaneous immunization of chinchillas with PilA results in significantly earlier clearance of NTHI from the NP and directly challenged middle ears and significantly earlier resolution of established middle ear biofilms compared to controls (238).

f. **chimV4.** In an effort to target two NTHI adhesins by delivery of one immunogen, a chimeric immunogen called ‘chimV4’ was designed. A modified rsPilA serves as an immunogenic carrier for a slightly larger (24 amino acids) variant of the OMP P5 B-cell epitope described within the immunogen LB1 (236). Epitope mapping studies revealed that the N-terminus of the OMP P5 B-cell epitope and the C-terminus of PilA are immunodominant epitopes within the respective proteins (236, 240). Thus, chimV4 was designed such that OMP P5 epitope is situated at the N-terminus of PilA. Passive transfer of antiserum against chimV4 demonstrates significant protection against experimental NTHI-induced OM in a chinchilla model (236). Moreover, transcutaneous immunization with chimV4 following preventative or
therapeutic regimens results in rapid eradication of NTHI from the NP and middle ear (238).

**Noninvasive immunization strategies**

Currently, children in the U.S. receive up to 25 vaccines by injection during the first two years of life (170). This can be cause for concern by parents who may ultimately delay or refuse immunization of their child. Also, while not of considerable concern in developed countries, the reuse of needles in developing countries poses serious risk for transmission of blood-borne diseases (149, 244). Thus, while vaccination by injection is proven to be extremely effective as a preventative intervention, development of alternative delivery strategies for current or future formulations could potentially serve as equally effective means to induce protective immunity while simultaneously addressing bottlenecks associated with injectable vaccines.

a. **Intranasal immunization.** One noninvasive immunization method is delivery via an intranasal (IN) route. This delivery method takes advantage of the high permeability, low enzymatic activity and presence of immunocompetent cells within the nasal cavity (251). The mechanism for immune induction after IN immunization is attributed to activation of DCs which are abundant throughout the nasal mucosa (141, 333). The DCs engage and process antigens, migrate to lymphoid tissues and prime naive T-cells, a process which initiates humoral and cellular immune responses both locally and at sites distal to administration (61, 64, 131). Further, IN immunization can induce both systemic and mucosal antibody (252).
IN immunization by nasal spray or droplet is a simple means to administer a vaccine. However, this route of delivery was once not favored due to diagnosis of Bell’s palsy in 46 individuals (27%) after receipt of Berna Biotech’s IN trivalent influenza vaccine, Nasalflu™ in 2000-2001 (227). Facial paralysis was attributed to the adjuvant, an enzymatically active *E. coli* heat labile toxin that binds to GM1-gangliosides and likely trafficked along the olfactory neurons and entered the olfactory bulbs (325). However, the recent Influenza A pandemics have resulted in re-introduction of IN vaccination strategy by MedImmune’s FluMist®, a formulation not adjuvanted with a toxin, which is widely available and appears to have gained public acceptance.

b. Transcutaneous immunization. A second noninvasive strategy is transcutaneous immunization (TCI), the application of a vaccine onto intact skin. TCI induces an immune response by engaging antigen-presenting cells present within the epidermis and dermis, the Langerhan’s cells and dermal DCs, respectively (334). There are multiple benefits to this immunization route, which include the simplicity and noninvasive nature of delivery, reduced cost as syringes, needles and trained medical professionals are not required to deliver the vaccine and the prospect for greater vaccine distribution beyond developed countries due to typically cheaper production costs (105). Previous studies show that TCI with bacterial or viral proteins and other peptide antigens induces an immune response in both animals and humans (29, 190, 332). Furthermore, via use of animal models, there is evidence of protection against subsequent bacterial, viral or toxin challenge (96, 270, 297, 311). Whereas parenteral immunization elicits primarily a systemic immune response, TCI induces both systemic and mucosal immunity (201).
c. **Sublingual immunization.** Sublingual immunization (SLI) is the application of vaccines underneath the tongue. This method targets the dense DC network within the sublingual caruncle (235, 299) and affords rapid absorption and entry of molecules into the blood stream. Sublingual immunotherapy is employed for treatment of food or environmental allergies, asthma and rhinosinusitis (209, 234). SLI against infectious diseases induces mucosal and systemic immune responses both locally and distally (54, 55, 126).

Advantages to each immunization route include the induction of both mucosal and systemic immunity, in addition to development of mucosal immunity at sites distal to administration (57, 131). Both IN and TCI regimens have demonstrated efficacy in experimental models of OM (4, 56, 128, 136, 169, 238, 279) and while in its infancy, there is potential for application of SLI as an additional vaccination strategy. OM is a disease of the uppermost respiratory tract, therefore the ability to induce immunity at the mucosae of this anatomical region has the potential to reduce, or preferably prevent, the onset of disease in the middle ear. Induction of antibody at the mucosal surface is optimal to target an organism such as NTHI whose only niche is the human nasopharynx.

**Dendritic cells**

An important constituent of the innate immune system is the DC, a potent antigen presenting cell found in blood and many tissues and of the body capable of initiation and modulation of primary immune responses (24, 247, 309). In both humans and mice, there are two broad classes of DCs: myeloid or conventional DCs and plasmacytoid or blood DCs. While there is discussion regarding discrimination of DCs from
macrophages (137), it is generally accepted that high expression of the integrin CD11c and the ability to migrate to lymphoid tissues and interact with T-cells are attributes specific to the DC cell type (95).

Immature conventional DCs exist within all tissues and function as sentinels where by sampling the environment, distinguish ‘self’ versus ‘nonself’ antigens, to appropriately initiate, or not, immune responses (309). Thus, these cells are optimally located at sites of constant antigen insult. Immature DCs express low levels of co-stimulatory molecules CD40, CD80 and CD86, compared to mature DCs and are highly phagocytic, capable of phagocytosis, endocytosis, pinocytosis and receptor-mediated uptake mechanisms (24, 275, 309). Upon processing of internalized antigen, peptide fragments are presented in the context of major histocompatibility (MHC) class II molecules on the cell surface. Maturation of DCs is a terminal event; cells in this state lose phagocytic function, however, upregulate expression of the aforementioned co-stimulatory molecules and the chemokine receptor CCR7, which mediates trafficking via CCL19 and CCL21 into lymphoid tissues (196, 264). Once there, mature DCs can interact with the appropriate T-cell clone.

Targeting DCs by vaccination has the potential to impact the resultant immune response. DCs express multiple receptors, including pattern recognition receptors that could be exploited by appropriate vaccine formulation (66 439, 248, 298 689). It is shown that DCs that originate from a specific tissue can influence T-cells to home to that site, a process called selective imprinting (205, 341). Thus, the selection of antigen or adjuvant could influence or elicit a desired immune response.
Specific Aims

The specific aims for this research included:

1. Identification of optimal conditions to induce the differentiation of chinchilla bone marrow-derived precursor cells to dendritic cells and examine the phenotype and function of the resultant chinchilla DCs.

2. Examination of OMP P5 as a potential pathogen-associated molecular pattern (PAMP), to begin to explain the efficacy afforded by OMP P5-derived vaccine candidates against experimental NTHI-induced OM.

3. Assessment of the efficacy of two noninvasive immunization strategies, intranasal and transcutaneous, to prevent the development of experimental otitis media in a chinchilla model.

4. Examination of the role for DCs in the induction of the immune response induced by intranasal or transcutaneous immunization strategies.
Chapter 2
Differential uptake and processing of a *Haemophilus influenzae* P5-derived immunogen by chinchilla dendritic cells

**Introduction**

Nontypeable *Haemophilus influenzae* (NTHI) is a commensal of the human nasopharyngeal flora, yet is also responsible for diseases of the upper and lower airway, including otitis media (OM), sinusitis, bronchitis and exacerbations of chronic obstructive pulmonary disease (74, 217). Adherence of this bacterium at mucosal sites is a critical first step toward NTHI pathogenesis and as a result, there is great interest in the development of vaccines that target NTHI surface-exposed adhesins (52, 102, 218, 260). The outer membrane protein P5-homologous adhesin (OMP P5) is one of several adhesins expressed by NTHI, and has been shown to facilitate adherence of this bacterium to human oropharyngeal cells (20) and nasopharyngeal mucin (267), chinchilla Eustachian tube mucin (203), respiratory syncytial virus-infected A549 cells (148), carcinoembryonic antigen-related cell adhesion molecule (CEACAM)-1 transfected HeLa (32) or CHO cells (127) and human intercellular adhesion molecule (ICAM)-1 (10).

A synthetic, chimeric peptide immunogen, called LB1, is derived from a surface-exposed region of OMP P5 (19). In rat and chinchilla models of OM, LB1 has
demonstrated significant protective efficacy against both homologous and heterologous NTHI challenge when delivered parenterally (162, 176). Furthermore, significant efficacy was observed when LB1 was administered intranasally (IN) to chinchillas, despite the induction of relatively low levels of serum and mucosal antibodies, compared to that induced by immunization via a parenteral route (127, 237). It has been shown that antigen processing by dendritic cells (DCs) can influence vaccine efficacy (53, 66), as upon migration to lymphoid tissues, DCs present antigen in the context of MHC molecules to naive T-cells, serving to either initiate an immune response or tolerize T-cells (24, 271, 310, 343). Therefore, characteristics of the antigen itself, and its processing by the DC could influence outcome of this interaction (immune induction vs. tolerance).

Recently, it was reported that recombinant OmpA from *Escherichia coli* O157:H7, *Klebsiella pneumoniae* and *Acinetobacter baumannii* interacts with and induces maturation of DCs, an observation that resulted in the designation of the OmpA family of bacterial proteins as a new pathogen-associated molecular pattern, or PAMP (144, 145, 181, 323). As OMP P5 is member of the OmpA family of proteins (296), we also wanted to determine whether LB1, which is derived from OMP P5, shared similar properties of a PAMP (i.e. ability to interact with and induce the maturation of DCs) among others.

Herein, we identified optimal conditions to culture chinchilla bone marrow-derived precursor cells to promote their differentiation to DCs, and characterized the differential uptake and presentation of LB1 by these cells *in vitro*. Additionally, we began to identify sites of immune induction by tracking the migration of DCs to lymphoid tissues *in vivo*. To do so, we labeled DCs resident within the chinchilla nasal cavity by IN delivery of the dye carboxyfluoroscein succinimidy l ester (CFSE), followed by IN administration of LB1.
We subsequently confirmed our observations by specifically tracking LB1-activated, bone marrow-derived DCs that had been labeled with CFSE in vitro prior to their administration IN. These data are the first to characterize chinchilla DCs. Moreover, our data demonstrated a role for DCs in the processing and presentation of the peptide immunogen LB1 when delivered to the chinchilla nasal cavity, which likely underlies the observed preclinical vaccine efficacy obtained upon IN immunization.

**Materials and Methods**

**Animals.** Adult chinchillas (*Chinchilla lanigera*) were obtained from Rauscher’s Chinchilla Ranch (LaRue, OH). Animal care and all related procedures were performed in concordance with institutional and federal guidelines, and were conducted under an IACUC- approved protocol.

**Generation of chinchilla DCs.** Bone marrow cells were isolated from the femurs of adult chinchillas and cultured in RPMI 1640 medium containing 2 mM L-glutamine, 100 U penicillin/ ml, 100 µg streptomycin/ ml, 1 mM pyruvate (Mediatech), 5 mM HEPES (Acros), 0.75 g NaHCO₃/ ml (Fisher Scientific) and 0.05 mM β-mercaptoethanol (Sigma). DCs were induced by culturing 2 x 10⁶ bone marrow cells in 8 ml medium supplemented with 40 ng recombinant human GM-CSF/ ml and 40 ng recombinant human IL-4/ ml (R&D Systems) in 100 mm tissue culture dishes. Medium was also supplemented with either 5% fetal bovine serum (FBS; Mediatech) or 5% naive chinchilla serum. On day 4 of culture, an additional 4 ml of fresh medium was added to the cultures and on day 6, non-adherent cells were collected to assay as immature cells.
**Antibodies and antigens.** We used the following fluorochrome-conjugated, commercially available antisera to detect cell surface molecules expressed by chinchilla bone marrow-derived DCs: mouse anti-rat CD11c-FITC, mouse anti-rat CD80–PE and mouse anti-guinea pig MHC class II revealed with goat anti-mouse IgG-PE (AbD Serotec). Appropriate isotype-matched antibody controls were included in all assays. To induce maturation of chinchilla DC-like cells *in vitro*, cells were stimulated with either 10 ng *E. coli* lipopolysaccharide (LPS; Sigma)/ml or 10 ng recombinant human TNF-α (Sigma)/ml for 24 hours prior to labeling the cells for flow cytometry.

Native P5 protein is an approximately 36 kDa outer membrane protein (OMP) and was isolated and purified from NTHI strain 86-028NP, as previously described (296). LB1 is a 4.6 kDa, 40-mer synthetic, chimeric peptide vaccinogen derived from a surface-exposed loop of OMP P5 and has also been described (19). To manage potential differences in binding and uptake of antigens by the chinchilla DCs due to the substantial difference in relative molecular mass of OMP P5 compared to LB1, a 26 kDa NTHI protein (175) called ‘control protein’ and a 3.5 kDa, 34-mer synthetic peptide called ‘TFPQ3’ (236) were used as size control antigens. These ‘control’ antigens were selected exclusively based on molecular mass and not on potential known immunoreactivity or lack thereof. Endotoxin contamination of the proteins and synthetic peptides was determined to be ≤ 0.25 EU/ml by Pyrosate agglutination assay (Associates of Cape Cod, Inc.). Peptides and proteins were coupled to FITC via fluorescein protein labeling kit according to manufacturer’s instructions (Pierce Biotechnology). Based on the coupling chemistry and amino acid composition of LB1 and TFPQ3, the peptides were estimated to be comparably labeled with FITC.
**Chemotaxis.** To demonstrate the ability of immature, LB1-activated or TFPQ3-activated chinchilla DCs to sense and respond to a stimulus, a chemotaxis assay was performed. Immature DCs were induced to mature by incubation overnight with either 1 µg TFPQ3 or LB1/ ml culture medium supplemented with 5% naive chinchilla serum, but without cytokines. Cells were washed and adjusted to a concentration of $10^6$ DCs/ ml and 12 µl of unstimulated or stimulated DCs was added to the one of two horizontally positioned compartments that were separated by a 260 µm gradient channel in a TAXIScan instrument (Effector Cell Institute, Inc.) (157, 232). To align DCs with the edge of the channel, buffer was aspirated from the second compartment. A volume of 15 µl of 1µM recombinant human C5a (Calbiochem) or buffer was then added to the second compartment. After 60 min., the cumulative number of DCs that had entered the chemotactic gradient in the channel toward C5a or buffer was calculated for each of three independent experiments.

**Antigen binding and internalization by chinchilla DCs.** Immature DCs ($1 \times 10^5$) were incubated with 1 µg FITC-labeled peptide or protein/ ml medium for 1 hour at 4°C (to assay surface binding of antigen) prior to shifting the cultures to 37°C (to assay internalization of bound antigen). Cells were washed, fixed with 2% paraformaldehyde in 0.1 M phosphate buffer and assayed immediately by flow cytometry with a BD FACSCalibur. A total of 10,000 viable events were collected for three independent assays and data were analyzed with FloJo software (Tree Star, Inc.).

**Antigen endocytosis.** Immature DCs were mixed with 1 µg FITC-labeled peptide or protein/ ml medium and allowed to adhere to poly L-lysine (Sigma) - coated
coverslips for 30 min at 37°C. Alternatively, to block endocytosis, immature DCs were pre-incubated with 10 nM cytochalasin D (Sigma) for 20 min prior to and during incubation with FITC-labeled peptides. Cells were then fixed and nonspecific binding sites were blocked with either 2% bovine serum albumin in Dulbecco’s phosphate buffered saline (DPBS; Mediatech) or 10% normal goat serum in DPBS (Zymed). To label F-actin, cells were incubated with rhodamine-phalloidin (Molecular Probes). To label MHC class II molecules, DCs were incubated with mouse anti-guinea pig MHC class II (AbD Serotec) and detected with goat anti-mouse IgG conjugated to Texas Red (Invitrogen). To inhibit endosome acidification and MHC class II antigen presentation, cells were treated with 10 nM chloroquine (MP Biologicals) for 20 min prior to and during incubation with FITC-labeled peptides. An appropriate isotype- matched antibody control was included in each assay. To inhibit fluorescent quenching, Prolong Anti-fade Gold (Invitrogen) was added to each slide prior to sealing. Slides were examined with a Zeiss LSM 510 Meta confocal system attached to a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Inc.).

**Antigen presentation by chinchilla DCs.** For immunization, three doses of 10 µg LB1 plus 10 µg monophosphoryl lipid A (MPL; Corixa) or 10 µg MPL alone were administered to alert chinchillas in a volume of 100 µl via subcutaneous injection at weekly intervals. One week after receipt of the third dose, chinchilla splenocytes were isolated by crushing the spleen and passing the cells through a 40 µm cell strainer. Erythrocytes were removed and suspensions were enriched for viable cells by centrifugation with Ficoll Paque (GE Healthcare). Resultant cells were labeled with mouse anti-rat CD3 conjugated to biotin (AbD Serotec) for enrichment of CD3⁺ T-cells by
streptavidin-conjugated magnetic bead separation (MACS; Miltenyi). CD3+ T-cells were then cultured in 100 mm² petri dishes in culture medium supplemented with 5% naive chinchilla serum, but without cytokines. As chinchillas are outbred and thus not genetically identical animals, we also cultured bone marrow cells from the same animal for 3 days to allow for differentiation of precursor cells to DCs in order to demonstrate that the observed T-cell proliferation was due to the presentation of antigen by DCs, and not due to an allogeneic MHC molecule response. After 3 days in culture, the CD3+ T-cells were seeded into wells of 96-well round-bottom plates at a density of 2 x 10⁵ cells per well in triplicate. The autologous bone marrow-derived DCs were incubated with 1 µg or 10 µg LB1 or control peptide/ml for 2 hours to allow for uptake of antigen, washed, and then added to wells which contained the CD3+ T-cells at a density of 10⁴ cells per well in medium without cytokines. Controls consisted of wells that contained 1 µg or 10 µg concanavalin (Sigma)/ml plus CD3+ T-cells and DCs, wells without an antigen (unstimulated) or CD3+ T-cells only. After 72 hours, 20 µl of Alamar Blue (AbD Serotec) were added to each well and the cultures were incubated for an additional 24 hours. Proliferation was assessed as the chemical reduction of Alamar blue by the cells in culture and detected as the fluorescence of each well at 530 nm excitation and 590 nm emission wavelengths with a Bio-Tek Synergy HT microplate reader. Statistical significance of three independent assays was determined by Tukey’s honestly significant difference (HSD) test for analysis of variance (ANOVA) pair-wise comparisons.

**In vivo migration of chinchilla respiratory DCs following IN immunization.**

To track the migration of chinchilla nasal mucosa DCs, we adapted the technique described by Legge *et. al.* (182) for use in the chinchilla host. Carboxyfluorescein
succinimidyl ester (CFSE; Invitrogen) was dissolved to 100 µM in dimethyl sulfoxide and further diluted in pyrogen-free saline to 8 µM prior to administration via microaerosol sprayer (Wolfe Tory Medical, Inc.) in a volume of 40 µl/ naris to alert, prone chinchillas. Fluid administered via this regimen is retained exclusively within the chinchilla nasal cavity, with no dose-loss to the respiratory or gastrointestinal tracts (330). Six hours later, chinchillas received either 10 µg LB1 + 10 µg MPL or 10 µg MPL alone via microaerosol spray as before. At 1, 3 and 12 hours later, animals were sacrificed and the cervical, brachial, axillary and mediastinal lymph nodes were removed in addition to the spleen. The nasal-associate lymphoid tissue (NALT) was also removed (156). Tissues were crushed into DPBS and cells passed through a 40 µm cell strainer. The spleen was also crushed, strained and the cells centrifuged with Ficoll-Paque. DCs were labeled with mouse anti-rat CD11c-Alexa fluor 647 (AbD Serotec) and CD11c⁺ CFSE⁺ cells were detected by flow cytometry. A total of 5000 viable events were collected for each of three independent assays.

To investigate whether LB1-activated, bone marrow- derived DC would similarly follow homing signals within the nasal cavity to the NALT, in vitro- derived DCs were labeled with 10 µM CFSE prior to incubation with either 10 µg LB1 + 10 µg MPL or 10 µg MPL alone for 15 min. These treated DCs were washed to remove unbound peptide, adjusted to 10⁶ cells/ 80 µl and instilled drop-wise by micropipet to alert, prone chinchillas in a volume of 40 µl per naris. Animals were sacrificed 1 hour later and the lymphoid tissues processed as described above. DCs were labeled with mouse anti-rat CD11c-Alexa fluor 647 and CD11c⁺ CFSE⁺ cells were detected by flow cytometry. A total of 5000 viable events were collected for each of three independent assays.
Aliquots of the NALT cell suspension retrieved 1 hour after IN administration of LB1 + MPL were incubated for one hour at 37°C, 5% CO₂ on poly-L lysine-coated coverslips. Cells were fixed, blocked with 10% normal goat serum and incubated with mouse anti-rat CD11c conjugated to Alexa fluor 647, mouse anti-guinea pig MHC class II revealed with goat anti-mouse IgG- conjugated to Texas Red and mouse anti-rat CD3 conjugated to biotin revealed with streptavidin conjugated to Alexa fluor 405 (Invitrogen). Slides were sealed after addition of Prolong AntiFade Gold with DAPI, or without DAPI, prior to viewing via confocal microscopy.

Results

Cytokine and serum source influenced the differentiation of chinchilla bone marrow-derived cells in culture. To establish in vitro cultures of chinchilla DCs, we isolated bone marrow cells from the femurs of chinchillas and cultured these cells in medium supplemented with recombinant human cytokines (40 ng GM-CSF/ ml and 40 ng IL-4/ ml), which resulted in viable cultures. We further assessed whether the serum source in the medium influenced the development of these cells. After one day, cells in the medium supplemented with a homologous serum source (naive chinchilla serum) remained in suspension as individual cells (Fig. 2.1A), which in time, developed into a mixture of floating and adherent cells (Fig. 2.1C). After six days, we observed numerous floating aggregates of cells (Fig. 2.1E) within this culture. Chinchilla bone marrow cells cultured in medium supplemented with FBS, a serum source widely used in cell culture systems, formed aggregates suggestive of rapidly proliferating cells that had attached to the bottom of the culture dish (Fig. 2.1B) which appeared larger in size after four days in culture (Fig. 2.1D) and finally dispersed after six days (Fig. 2.1F).
By flow cytometry, the viable cells cultured in medium supplemented with naive chinchilla serum were comparable in cell size to those within the medium containing FBS, as demonstrated by the forward scatter profile (Fig. 2.1G & H, respectively). However, the cells cultured in medium supplemented with chinchilla serum appeared more complex (as demonstrated by the side scatter profile), compared to cells within the medium containing FBS. This latter observation suggested differences in the inner complexity of the cells (i.e. shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). As the development of cells within the medium supplemented with naive chinchilla serum closely followed that reported for murine and rat bone marrow cultures (139, 317), we concluded that optimal medium supplements required to culture chinchilla bone marrow cells included the use of recombinant human cytokines and a homologous serum source.
Figure 2. Influence of serum source on survival and differentiation of chinchilla bone marrow-derived cells in culture.

Light microscopy images of chinchilla bone marrow-derived cells cultured in medium supplemented with 5% naive chinchilla serum (panels A, C & E) or fetal bovine serum (panels B, D & F). Arrows in E and F indicate floating clusters of cells. Representative dot plots demonstrating forward scatter and side scatter profiles of bone marrow-derived cells cultured for 6 days in medium supplemented with either naive chinchilla serum (panel G) or FBS (panel H).
**Chinchilla bone marrow-derived cells exhibited classic DC morphology and phenotype after culture with GM-CSF and IL-4.** In order to examine the expression of classical DC surface markers by the chinchilla DC-like cells, we tested antibodies raised against molecules expressed by other species, including human, mouse and rat, for reactivity with the chinchilla bone marrow-derived cells, as no chinchilla-specific reagents are available commercially. Of the panel tested, we observed the greatest positive result with mouse anti-rat CD11c (Fig. 2.2A). It was not unprecedented that a rat-specific reagent recognized a chinchilla homologue, as we and others routinely use rat- (and human-) specific reagents for immunodetection in the chinchilla model (82, 133, 163, 237, 284). Greater than 75% of cells within each culture were CD11c+, as determined by flow cytometry, also comparable to reports of cells cultured from other species. We further examined the morphology and phenotype of the DC-like cells by inducing the maturation of the bone marrow-derived cells with 10 ng *E. coli* LPS/ ml. Immature (unstimulated) cells in culture expressed fine processes that extended from the cell surface (Fig. 2.2B), and had a large, indented nucleus, which is commonly described for DCs (Fig. 2.2B, inset), whereas mature or LPS-stimulated DC-like cells expressed much larger dendrites (Fig. 2.2C and inset). CD11c+ DC-like cells stimulated with either LPS or recombinant human TNF-α upregulated expression of both MHC class II molecules (Fig. 2.2D) and CD80 (Fig. 2.2E), compared to immature cells. Therefore, as the chinchilla DC-like cells were both morphologically and phenotypically comparable to that described for murine and rat bone marrow-derived DCs, we were confident we had identified conditions to promote the differentiation of precursor cells within the chinchilla bone marrow to DCs.
Figure 2. Chinchilla bone marrow-derived cells exhibited DC-like phenotype and morphology.

Panel A, representative histogram demonstrating expression of CD11c by chinchilla bone marrow-derived cells as determined by flow cytometry. Black histogram, isotype control; red histogram anti-CD11c. Light microscopic images of immature (panel B) or LPS-matured (panel C) chinchilla bone marrow-derived cells in culture. Arrows indicate surface expression of fine processes by immature cells, or larger dendrites by LPS-matured DCs. Insets, morphology of immature (panel B) or LPS-matured (panel C) cells after staining with Giemsa. Histograms demonstrated expression of MHC class II (panel D) and CD80 (panel E) by immature chinchilla bone marrow derived cells (black histograms), TNF-α-matured cells (red histograms) and LPS-matured cells (blue histograms) as determined by flow cytometry.
**Chinchilla DCs exhibited chemotaxis.** One of many functions of DCs is the ability to sense a chemokine gradient and respond by directional movement. We therefore assessed chinchilla DC chemotaxis toward a classic chemotactic agonist, C5a, *in vitro* (206). As demonstrated by the pseudocolored images captured with the TAXIScan system after 30 min, a greater number of LB1-activated DCs were present within the channel, compared to the DCs that had been activated with another synthetic peptide of similar mass (TFPQ3) (Fig. 2.3A & B, respectively). Furthermore, the chemotactic response was specifically directed toward compartments that contained C5a, as minimal spontaneous migration of cells was observed towards compartments that contained only buffer (Fig. 2.3C), regardless of maturation status. When plotted as the cumulative number of DCs that entered the chemotactic gradient in the channel toward C5a, the number of TFPQ3-activated DCs was marginally greater than immature DCs. In contrast, LB1-stimulated DCs continually entered the chamber when C5a was present. These data demonstrated that DCs stimulated with LB1 were induced to mature and responded to the presence of a chemoattractant.
Figure 2. Chemotaxis of chinchilla DCs toward C5a.

Representative images captured with the TAXIScan instrument after 30 min. of chemotaxis toward C5a. Pseudocolored images show TFPQ3-activated DCs (panel A) and LB1-activated DCs (panel B). Panel C, Plot demonstrating the cumulative number of DCs that entered the channel over time. Solid lines depict DC chemotaxis toward C5a while dashed lines demonstrate spontaneous migration of DCs in buffer. Red lines, LB1-activated DCs; green lines, TFPQ3-activated DCs and grey lines, immature/unstimulated DCs.
**Chinchilla DCs preferentially bound and internalized LB1.** To examine the relative ability of immature chinchilla DCs to bind and internalize NTHI OMP P5 and LB1, we utilized a panel of both native proteins and synthetic peptides. FITC-labeled proteins or peptides were incubated with immature chinchilla DCs at 4°C to examine antigen surface binding, then cultures were shifted to 37°C to promote internalization of the bound antigen. At 4°C, all cultures demonstrated binding of protein or peptide, although to varying magnitudes (Fig. 2.4A). Whereas DCs incubated with FITC-labeled OMP P5 demonstrated greater binding compared to the 26 kDa control peptide, the culture of DCs incubated with FITC-labeled LB1 was most fluorescent of all cultures, with approximately 8-fold greater relative fluorescence compared to DCs incubated with a peptide of similar molecular mass (TFPQ3).

Upon temperature shift to 37°C, the trend of relative fluorescence remained the same as that observed at 4°C; however the mean fluorescence of each culture increased (Fig. 2.4B). At either temperature, DC cultures incubated with the peptides were more fluorescent than cultures incubated with either protein, which suggested that the smaller molecular size of the peptides compared to the native proteins may facilitate antigen binding. Furthermore, the greater binding and uptake of LB1 compared to the peptide of comparable size suggested a unique characteristic specific to this immunogen, for example, the presence of a PAMP-like motif.

To further investigate this theory and assess whether the preferential binding of LB1 by the chinchilla DCs was also receptor-mediated, we examined the localization of the FITC-labeled proteins and peptides within DCs by confocal microscopy after the temperature shift to 37°C. In agreement with the flow cytometry data, immature chinchilla DCs internalized the FITC-labeled proteins and peptides, although to differing
magnitudes. Whereas DCs incubated with the FITC-labeled 26 kDa control protein (Fig. 2.4C) or FITC-labeled OMP P5 (Fig. 2.4D) contained a few areas of punctate green fluorescence, greater internal fluorescence was observed by DCs incubated with either FITC-labeled TFPQ3 (Fig. 2.4E) or FITC-labeled LB1 (Fig. 2.4F). The greatest internal fluorescence was clearly visible within the cells incubated with LB1, as numerous punctate signals were observed (Fig. 2.4F). Furthermore, co-localization of actin and LB1 was observed (see Fig. 2.4F, yellow signal), which suggested that uptake of the FITC-labeled LB1 likely occurred via endocytosis. To confirm this, DCs were treated with cytochalasin D prior to and during incubation with FITC-labeled LB1 to inhibit actin polymerization and formation of endocytic vesicles. Whereas cytochalasin D treatment did not prevent binding of LB1 by the chinchilla DCs, as demonstrated by the punctate green signals in Fig. 2.4G, internalization of the peptide was inhibited as evidenced by the fact that no co-localization of actin and LB1 was observed. These data suggested that binding and internalization of LB1 was potentially mediated by receptor-mediated endocytosis and further, that a motif might be present within the peptide immunogen LB1 itself which facilitated this interaction.
Figure 2. 4. Chinchilla DCs differentially bound and internalized FITC-labeled proteins and peptides.

Representative FACS histograms demonstrating mean fluorescence of chinchilla DC cultures incubated at 4°C to assess cell surface binding of antigens (panel A), followed by temperature shift to 37°C to assess internalization of bound antigens (panel B). Representative histograms demonstrating the relative fluorescence of immature DCs (black histograms) or DCs incubated with FITC-labeled antigens: control protein (blue histograms), OMP P5 (purple histograms), TFPQ3 (green histograms) or LB1 (red histograms). Confocal microscopy images demonstrated the localization of FITC-labeled proteins and peptides within chinchilla DCs (green fluorescence) after the temperature shift to 37°C. DCs were incubated with either control protein (panel C), OMP P5 (panel D), TFPQ3 (panel E) or LB1 (panel F). F-actin was labeled with rhodamine-phalloidin (red fluorescence). Note yellow fluorescence in panel F which demonstrated co-localization of FITC-labeled LB1 with actin which was abrogated upon treatment with cytochalasin D (panel G).
**DCs upregulated MHC class II molecules and induced T-cell proliferation**

**upon activation.** To more closely examine the specific interaction between the chinchilla DCs and LB1, we examined whether immature chinchilla DCs stimulated with either LB1 or a peptide of similar size subsequently upregulated expression of MHC class II molecules. As detected by flow cytometry (Fig 2.5A, green histogram) and confocal microscopy (Fig 2.5B, red fluorescence), chinchilla DCs incubated with TFPQ3 were labeled with a MHC class II-specific antibody, although the DCs exhibited only relatively moderate upregulation of MHC class II molecules over unstimulated DCs (Fig. 2.5A, black histogram). Upon incubation of DCs with LB1, however, notably greater MHC class II-specific fluorescence was observed (Fig. 2.5A, red histogram and Fig. 2.5C). Treatment of the DCs with chloroquine to inhibit endosome acidification and MHC class II antigen presentation abrogated the MHC class II-specific fluorescent signal (Fig. 2.5D). These data further demonstrated that internalization of LB1 induced DC maturation, as evidenced here by upregulated expression of MHC class II molecules.

We next examined the consequence of LB1 internalization and upregulation of MHC class II molecules by measuring the induction of CD3$^+$ T-cell proliferation *in vitro*. We first confirmed that chinchilla T-cells could be induced to proliferate as there have been no reports to our knowledge of chinchilla T-cell proliferation assays. Using a non-specific stimulus, we observed dose-dependent proliferation upon *in vitro* stimulation of chinchilla CD3$^+$ T-cells with 10 $\mu$g or 1 $\mu$g concanavalin A/ml in the presence of DCs (Fig. 2.5E). Furthermore, CD3$^+$ T-cells isolated from chinchillas immunized with MPL exhibited comparable proliferation at the 10 $\mu$g concanavalin A/ml dose. These responses were statistically significant when compared to cultures with no stimulus ($p$≤
0.05) and thus confirmed the utility of this assay system to measure chinchilla T-cell proliferation.

To examine the proliferation induced by a specific stimulus, DCs were incubated with LB1 prior to culture with CD3⁺ T-cells isolated from chinchillas immunized with LB1 + MPL. Dose dependent proliferation resulted which was significantly greater than the proliferation induced by DCs incubated with cells with no stimulus or by CD3⁺ T-cells cells isolated from animals immunized with MPL only (p ≤ 0.05). These data indicated that upon uptake of LB1, chinchilla DCs effectively processed the immunogen and presented peptide fragments, likely in the context of MHC class II and co-stimulatory molecules, to T-cells thereby inducing their proliferation. Moreover, our data suggested that T-cells recovered from chinchillas immunized with LB1 demonstrated a memory response, as significantly greater proliferation of T-cells isolated from chinchillas immunized with LB1 was induced by DCs presenting the same antigen, compared to DC presentation of an unrelated peptide of comparable size (p ≤ 0.05).
Figure 2. Chinchilla DCs expressed MHC class II molecules and effectively presented antigen to CD3+ T-cells in vitro.

Panel A, upregulation of MHC class II expression as demonstrated by flow cytometry. Black histogram, no stimulus; green histogram, DCs incubated with TFPQ3; red histogram, DCs incubated with LB1. Panels B & C, confocal microscopy images demonstrating MHC class II molecules (red fluorescence) on the surface of chinchilla DCs incubated with either TFPQ3 (panel B) or LB1 (panel C). Chloroquine treatment of DCs incubated with LB1 abrogated the MHC class II-specific fluorescent signal (panel D). Panel E, chinchilla CD3+ T-cells primed in vivo with LB1 proliferated upon DC presentation of LB1 in vitro. Results are shown as the mean ± standard deviation of three independent experiments. *, $p \leq 0.05$ compared to cells with no stimulus. †, $p \leq 0.05$ compared to CD3+ T cells isolated from chinchillas immunized with MPL only, °, $p \leq 0.05$ compared to cells stimulated with TFPQ3. Significance was determined by Tukey HSD test for ANOVA pair-wise comparisons.
Chinchilla respiratory DCs migrated to local lymphoid tissues following intranasal administration of LB1. We next examined the migration of DCs resident within the chinchilla nasal cavity following IN administration of LB1 plus the adjuvant, MPL. To identify potential sites of immune induction, we administered the dye CFSE via microaerosol spray to chinchillas prior to administration of the immunogen. CFSE labeling allows for the discrimination between cells resident within lymphoid tissue and DCs that had migrated to these tissues from the nasopharyngeal mucosa and has been shown not to disturb DC homeostasis (182). By this method, at the one and three hour time points after IN administration, the greatest mean fluorescence of CD11c⁺CFSE⁺ cells was detected within the NALT of the animals that received LB1+ MPL (Fig. 2.6A), compared to all other tissues examined (Fig 2.6B-F), suggesting that the NALT could serve as a primary inductive site following IN immunization of the chinchilla host. Moreover, in addition to the NALT, greater mean CFSE⁺ fluorescence was detected from CD11c⁺ cells within the brachial and axillary lymph nodes from the animals that received LB1+ MPL, compared to animals that received only MPL (Fig. 2.6B & C). These data demonstrated that the LB1 delivered with MPL influenced greater DC activation and subsequent migration to these lymphoid tissues than did the adjuvant alone.

Over a 12 hour period, a decrease in CFSE⁺ fluorescence was observed within the NALT, brachial and axillary lymph nodes, which indicated that IN delivery of LB1+ MPL triggered rapid and transient influx of DCs from the nasal cavity. In contrast, the CFSE⁺ signal detected in cervical and mediastinal lymph nodes as well as the spleens from the animals that received LB1 + MPL was comparable to animals that received MPL alone (Fig. 2.6D- 6F), which suggested that these lymphoid tissues likely were not primary inductive sites following IN immunization of this host. Thus, DCs within the
chinchilla nasal cavity were induced to mature following IN delivery of LB1 + MPL, which resulted in migration of these activated cells to, primarily the NALT, but also to the axillary and brachial lymph nodes.
Figure 2.6. DCs resident within the nasal mucosa migrated to the local lymphoid tissues after IN administration with LB1+MPL.

Scatter plots depict cells isolated 1 hour after receipt of LB1 + MPL (top plot) or MPL alone (bottom plot) for each lymphoid tissue examined. The shaded region specifies CD11c+CFSE+ cells. Bar graphs demonstrate the corresponding mean CFSE+ fluorescence of the CD11c+CFSE+ DCs 1, 3 or 12 hours post IN administration of LB1+MPL or MPL alone. Note difference in scale between NALT and other lymphoid tissues.
To confirm that the positive signal we described above was specifically due to an influx of DCs from the nasal cavity and not diffusion of dye from that site, we labeled DCs derived from culture of bone marrow cells with CFSE, then activated these cells with LB1+MPL or MPL alone \textit{in vitro}. These CFSE-labeled and activated cells were then instilled into the chinchilla nasal cavity. One hour later, a distinct population of CD11c$^+$ CFSE$^+$ cells was detected within the NALT after receipt of DCs activated with LB1+ MPL (Fig. 2.7A) but not when activated with MPL alone (Fig. 2.7B). Further, 11.5% of the cells isolated from the NALT of the animal administered LB1-activated DCs were CD11c$^-$CFSE$^-$, whereas a population of $\leq$1% was detected within the brachial, axillary, cervical and mediastinal lymph nodes and the spleen (data not shown). Whereas homing of nasal mucosa DCs to the axillary and brachial lymph nodes was shown in Fig. 2.6B & C, few of the instilled bone marrow-derived DCs were detected within these tissues, suggesting subtle differences between the DC cell types (\textit{i.e.} those derived from culture \textit{in vitro} versus those cells which develop \textit{in vivo}). Nevertheless, the migration of the instilled DCs from the nasal cavity to primarily the NALT correlated well with the study wherein CFSE had been delivered directly to the nasal cavity (see Fig. 2.6A). The similarity of the results obtained using these two distinct but complimentary \textit{in vivo} assays strongly indicated that local cytokine/chemokine signals likely guided the LB1-activated DCs from the chinchilla nasal cavity to the NALT.
Figure 2. CFSE-labeled, LB1-activated bone marrow-derived DCs migrated to the NALT upon IN instillation.

Scatter plots represent cells isolated from the NALT of animals that received DCs activated in vitro with LB1 + MPL (panel A) or MPL (panel B) 1 hour post-IN administration.
By confocal microscopy, we further verified that DCs resident within the nasal cavity migrated to the NALT after IN administration of LB1 + MPL. CFSE\textsuperscript{+} cells were easily distinguished from the other cells isolated from this lymphoid aggregate due to their intense green fluorescence and unique morphology (i.e. expression of extensive dendrites; Fig. 2.8A). Furthermore, the CFSE\textsuperscript{+} cells were labeled with an anti-CD11c antibody, thus confirming that these cells were DCs (Fig. 2.8B, red fluorescence). We also observed CD3\textsuperscript{+} T-cells in close proximity to the DCs within this sample (Fig. 2.8C, blue fluorescence), suggesting potential interaction between the two cell types. Upon closer examination, MHC class II molecule expression on the DCs was localized directly under the CD3\textsuperscript{+} T-cell fluorescent signal (Fig. 2.8D and inset; red and blue fluorescence, respectively). These data suggested that delivery of LB1 to the nasal cavity of chinchillas by microaerosol spray induced DCs resident within the nasal cavity to take up this antigen, mature and migrate to local lymphoid tissues where they appeared to interact with T-cells within the NALT.
Figure 2. Interaction of respiratory CFSE+ DCs with CD3+ T-cells upon migration to the NALT.

Panel A, confocal microscopy images of cells isolated from the NALT one hour after IN administration of LB1+MPL demonstrating the presence of CFSE+ cells (green) within the cell suspension (nuclei stained blue). Panel B, CFSE+ cell expressing the DC marker, CD11c (fuchsia). Panel C, detection of CD3+ fluorescent signals (blue) in close proximity to a CFSE+ DC (green, see arrows). Expression of MHC class II (red) by the CFSE+ cell in panel C is localized directly under the CD3+ T-cells (blue).
Discussion

Despite the great utility of the chinchilla for studies of viral and bacterial pathogenesis in OM (19, 33, 60, 98, 104, 133, 155, 193, 207, 241, 314), as well as for preclinical vaccine efficacy studies (18, 101, 103, 162, 260, 312), the cellular mechanisms for the induction of a protective immune response in this rodent host have yet to be fully characterized. One component of the innate immune system is the DC, a potent antigen presenting cell found in many tissues of the body which is capable of initiation and modulation of primary immune responses (24, 247, 309). As we have observed significant efficacy against the development of NTHI-induced OM after IN immunization of the chinchilla with LB1, we were interested to determine whether processing and presentation of the peptide immunogen by nasal cavity DCs likely participated in the observed protective response. We therefore examined the capture and presentation of LB1 by chinchilla bone marrow-derived DCs in vitro and by DCs within the chinchilla nasal cavity in vivo.

It is well established that precursor cells within the bone marrow of mice and rats can be induced to differentiate to DCs by the inclusion of GM-CSF and IL-4 in the culture medium (139, 187, 272, 281). We therefore sought to adapt this methodology to generate DCs from chinchilla bone marrow cells. Two factors impacted our ability to culture the chinchilla-derived cells: cytokine specificity and serum source. First, human-specific recombinant GM-CSF and IL-4, but not murine-specific cytokines supported the viability of these cells in culture. This observation was not without precedence, as the use of human- (and rat-) specific reagents for immunodetection is more successful than when one tries to use mouse-specific reagents (133, 284), which indirectly suggests that the chinchilla is closer to the rat and human than to mice in terms of their similarity of
immune molecules. Second, the serum source used to supplement the medium influenced the development of the culture, an observation similarly reported for murine DCs (114, 282). Therefore, whereas it is not surprising that chinchillas possess DCs, our data are the first to describe their culture.

It has been reported that DCs bind to OmpA proteins from *K. pneumoniae*, *E. coli* and *A. baumannii*, inducing DC maturation and activation, including expression of co-stimulatory molecules, cytokine secretion and stimulation of T-cell proliferation (147, 181, 323). These observations, among others, have led to the designation of this highly conserved family of bacterial proteins as a new PAMP (145). NTHI OMP P5 is an OmpA protein homologue (296). As shown by flow cytometry and confocal microscopy, chinchilla DCs bound and internalized OMP P5 *in vitro*. However, this result was overshadowed by far greater binding and internalization of LB1 by the DCs. This may have been due to the smaller molecular size of LB1 compared to the native protein (4.6 kDa vs. 36.4 kDa). Alternatively, a motif from OMP P5 that is included in the sequence of LB1 to which the DCs bind may be more optimally presented compared to the native protein. It is interesting to speculate that this 19-mer motif (known to be a B-cell epitope) is a PAMP, which would allow for DC binding to the immunogen via a pattern recognition receptor (6). We continue to investigate this hypothesis.

*In vivo*, by two techniques, we tracked the migration of DCs from chinchilla nasal cavity to the NALT. These data demonstrated that LB1 induced DC maturation which resulted in the ability of these cells to sense and respond to local homing signals within the nasal cavity, as similar migration was not detected when only MPL was administered. Our data further demonstrated that a single dose of LB1 delivered IN was sufficient to induce the maturation and migration of the DCs. Moreover, the NALT may
serve as a primary inductive site following IN immunization in this host. Rodent NALT has been reported to have functional similarities to the Waldeyer’s lymphoid ring, the lymphatic tissue of the pharynx, palatine tonsils and lingual tonsils, as well as other lymphoid tissue in the area which encircles the human nasopharynx and oropharynx (125, 302, 347). As such, the ability to target the lymphoid tissues proximal to the anatomical site of infection should be an important consideration when developing a vaccine and a vaccine delivery system.

In summary, our data are the first to describe the in vitro culture, functional characterization and in vivo tracking of chinchilla DCs. We continue to examine the role of DCs in the development of a protective immune response following intranasal immunization with LB1 and several other NTHI adhesin-derived or surface accessible antigens, for the prevention of NTHI-induced OM. Moreover, the chinchilla serves as a relevant animal in which to model multiple viral and bacterial diseases of the human respiratory tract; therefore the potential to apply the methods described herein need not be limited to examination of vaccine efficacy for NTHI-induced OM only. Components of both innate and adaptive immunity can influence DC functions. Therefore, the data presented here will be useful as we continue to examine the role of DCs in the initiation and modulation of the immune response in the chinchilla model.
Chapter 3

Intranasal immunization with an OMP P5-derived immunogen induces rapid clearance of nontypeable *Haemophilus influenzae* from the chinchilla nasopharynx and middle ear

Introduction

Otitis media (OM) is a common pediatric disease caused by bacteria which normally reside within the nasopharynx (NP) (*i.e.* nontypeable *Haemophilus influenzae* [NTHI], *Streptococcus pneumoniae* and *Moraxella catarrhalis*). OM is also a polymicrobial disease, wherein upper respiratory tract viral infection dysregulates aspects of the physical and innate immune defenses of the middle ear, thus facilitating bacterial ascension of the Eustachian tube and entry into the middle ear with subsequent disease development (15, 122, 124, 198, 255). NTHI is also responsible for additional diseases of the upper and lower airways, including sinusitis, bronchitis and exacerbations of chronic obstructive pulmonary disease (74, 287, 288). Thus, there is significant interest in the development of vaccines to prevent NTHI-mediated respiratory tract infection. Many of these vaccine candidates target bacterial outer membrane proteins (OMPs), other surface proteins and lipooligosaccharide expressed by NTHI (52, 218, 254, 260).

As adherence to the respiratory tract mucosae is a critical first step for NTHI colonization and subsequent pathogenesis, we have focused vaccine development...
efforts on one of many adhesins expressed by NTHI, the outer membrane protein (OMP) P5-homologous adhesin, or OMP P5 (296). This adhesin facilitates adherence of NTHI to human oropharyngeal cells (20) and nasopharyngeal mucin (267), carcinoembryonic-related cell adhesin molecule 1-transfected HeLa (32) or CHO (127) cells and human intercellular adhesin molecule 1 (10). Epitope mapping efforts using serum and middle ear fluids from both chinchillas with experimental OM and children with OM due to NTHI allowed us to characterize the third of four predicted surface-exposed regions of OMP P5 as an adhesin binding domain (240).

With this knowledge, and a goal to limit or inhibit NTHI adherence to the respiratory mucosa as a means to prevent the development of OM, a synthetic, chimeric peptide immunogen was developed. Called LB1, this immunogen is comprised of a 19-mer B-cell epitope located within the third surface-exposed region of OMP P5 that is co-linearly synthesized with a T-cell promiscuous epitope from measles virus fusion protein (19). Antibodies induced after parenteral immunization with the resulting 40-mer peptide immunogen consistently provide significant protective efficacy against both homologous and heterologous NTHI challenge in chinchilla models of experimental OM (18, 162) and also in a rat model of pulmonary and middle ear clearance (176). These data, among others, established LB1 as an extremely efficacious immunogen when administered parenterally in preclinical evaluation. As OM is a mucosal disease, we wanted to now expand our immunization strategy to both target inductive sites within the uppermost airway and also examine noninvasive approaches to vaccinate against OM.

One noninvasive immunization method is delivery via an intranasal (IN) route. This delivery method takes advantage of the high permeability, low enzymatic activity and presence of immunocompetent cells within the nasal cavity (251). The mechanism
for immune induction after IN immunization is attributed to activation of dendritic cells (DCs) which are abundant throughout the nasal mucosa (141, 333). The DCs engage and process antigens, migrate to lymphoid tissues and prime naive T-cells, a process which initiates humoral and cellular immune responses both locally and at sites distal to administration (61, 64, 131). Further, IN immunization can induce both systemic and mucosal antibody (252). Induction of antibody at the mucosal surface is optimal to target an organism such as NTHI whose only niche is the human nasopharynx.

Several groups have tested the outcome of IN immunization with NTHI-derived antigens including OMPs P2, P4, P6, Hap and detoxified lipooligosaccharide (4, 56, 128, 169, 194, 246) and demonstrated, using murine models, the production of immunogen-specific IgG and IgA in both serum and mucosal secretions. An additional rodent in which to model this human disease is the chinchilla. The chinchilla serves as a robust and highly reproducible animal model of both viral and bacterial pathogenesis of OM (33, 60, 98, 104, 133, 154, 193), as well as a rodent host in which to perform preclinical vaccine efficacy trials (18, 162, 176, 312). Moreover, a chinchilla model of viral upper respiratory tract infection-predisposed bacterial OM is shown to be predictive of a clinical trial outcome as the efficacy against NTHI-induced OM achieved in a pre-clinical study was highly similar to that achieved in the POET study, a pediatric clinical vaccine trial conducted in the Czech Republic and Slovakia (34% or 35.3% efficacy, respectively) (239, 262). Herein, we adapted our current parenteral immunization strategy to a less invasive, but potentially equally efficacious IN regimen. Using this delivery route in a chinchilla model of experimental NTHI-induced OM, we examined the immune response elicited and the protection afforded by an exclusively IN immunization regimen versus one of parenteral priming followed by IN boosts using LB1 as the immunogen.
Materials and Methods

Animals. Forty-two healthy adult chinchillas (*Chinchilla lanigera*; Rauscher’s Chinchilla Ranch, LaRue, Ohio; mean mass 461 ± 162 g) with no evidence of middle ear disease as determined by video otoscopy (MedRx, Largo, FL) and tympanometry (EarScan, Murphy, N.C.) were enrolled and divided into 6 cohorts of 7 animals each. Animal care and all procedures were performed in concordance with institutional and federal guidelines, and were conducted under an approved protocol.

Immunogen and adjuvant. LB1 is a synthetic chimeric peptide immunogen comprised of a 19-mer B-cell epitope derived from surface-exposed region 3 of NTHI OMP P5 that was co-linearly synthesized with a T-cell promiscuous epitope from measles virus fusion protein and has been extensively assayed in models of OM (18, 19, 162, 176). Monophosphoryl lipid A (MPL) plus trehalose dimycolate (Corixa, Seattle, WA) served as the adjuvant.

Adenovirus and NTHI isolates. Adenovirus serotype I was recovered from a pediatric patient at Nationwide Children’s Hospital and has been used in chinchilla models (314). NTHI strain 86-028NP was isolated from the nasopharynx of a child undergoing tympanostomy and tube insertion for chronic OM at Nationwide Children’s Hospital. This strain has been extensively assayed in chinchilla models (8, 18, 133, 162, 194, 208, 239).

Immunization and challenge. Alert, prone chinchillas were immunized by receipt of either three IN doses of 40 μl immunogen per naris (IN/IN/IN) via pediatric
microaerosol sprayer or one subcutaneous injection of 100 µl along the rear flanks followed by two IN doses of 40 µl per naris (SQ/IN/IN), with each dose administered at two week intervals (Table 3.1). The volume administered IN in this manner is retained within the chinchilla nasal cavity without loss to the respiratory or gastrointestinal tracts (330).

Adenovirus and NTHI challenges were performed as previously described (18). Briefly, one week after receipt of the third immunizing dose, all chinchillas were inoculated IN with adenovirus serotype 1 via passive inhalation of $1.9 \times 10^7$ TCID$_{50}$ delivered in 0.2 ml (0.1 ml per naris). Seven days later, chinchillas were challenged IN with $10^8$ CFU NTHI strain 86-028NP delivered in 0.2 ml pyrogen-free saline divided equally between the nares, as well as transbullarly with 2500 CFU NTHI delivered in 0.3 ml sterile pyrogen-free saline per bulla. Challenge doses were confirmed by plate count.

**Assessment of experimental disease.** Animals were evaluated daily for signs of development of OM. By video otoscopy (using a 0˚, 3-inch probe connected to a digital camera system), signs of tympanic membrane inflammation and/or presence of middle ear fluids (MEF) within the middle ear space were recorded, and overall signs of OM were rated on a scale of 0 to 4+ (18, 162, 239). Tympanometry was also performed to monitor changes in middle ear pressure (MEP), tympanic membrane compliance and tympanic width (Tw). Abnormal tympanograms were considered those with MEP values $\geq -100$ daPa or $> +60$ daPa, compliance values $\leq 0.5$ or $\geq 1.2$ ml, and/or Tw values $\geq 150$ daPa (100, 192, 322). A score of $\geq 2.0$ indicated the presence of a MEF, and hence OM. The presence of a MEF was always considered abnormal.
Table 3.1. Immunization dose and route for chinchilla cohorts.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Route</th>
<th>Total dose LB1 delivered</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 µg LB1 + 10 µg MPL</td>
<td>IN/IN/IN&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90 µg</td>
</tr>
<tr>
<td>10 µg LB1 + 10 µg MPL</td>
<td>IN/IN/IN</td>
<td>30 µg</td>
</tr>
<tr>
<td>10 µg MPL</td>
<td>IN/IN/IN</td>
<td>0 µg</td>
</tr>
<tr>
<td>30 µg LB1 + 10 µg MPL</td>
<td>SQ/IN/IN&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90 µg</td>
</tr>
<tr>
<td>10 µg LB1 + 10 µg MPL</td>
<td>SQ/IN/IN</td>
<td>30 µg</td>
</tr>
<tr>
<td>10 µg MPL</td>
<td>SQ/IN/IN</td>
<td>0 µg</td>
</tr>
</tbody>
</table>

<sup>a</sup> Three IN doses

<sup>b</sup> One SQ priming dose, followed by two IN doses
Collection of serum, nasopharyngeal lavage fluids and middle ear fluids.

Blood for serum and NP lavage fluids were collected prior to and one week after each immunization. NP lavages were also performed one day prior to, and on days 3, 6, 9, 12 and 15 after, bacterial challenge. Epitympanic taps to retrieve MEF were performed on any chinchilla whose tympanic membrane was rated as ≥ 2.5 on a scale of 0 to +4.0, as determined by video otoscopy and tympanometry. NP lavage and middle ear fluids were serially diluted and plated onto chocolate agar supplemented with 15 µg ampicillin/ ml medium or chocolate agar, respectively, to semi-quantitate CFU NTHI per ml fluid type.

Immunohistochemistry. To visualize the distribution of LB1 throughout the nasal cavity after administration by microaerosol nasal spray, after sacrifice, the nasal cavity of each chinchilla was instilled with 2% w/v paraformaldehyde in 0.1 M phosphate buffer and the specimens decalcified (156). The skulls were split along the sagittal plane and embedded in OCT. Serial sections of 5 µm thickness were cut and sections incubated with Avidin/ Biotin (Vector, Burlingame, CA) and the blocking reagent Background Sniper (Biocare Medical, Concord, CA) prior to incubation with either rabbit anti-LB1 serum or naive rabbit serum. Biotinylated goat anti-rabbit IgG (Dako, Glostrup, Denmark) was added next, followed by HRP-conjugated streptavidin (Biocare Medical). Color was developed with DAB.

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed on serum, NP lavage fluids and MEF to detect LB1-specific antibody. Pooled NP lavage fluids and MEFs were incubated in LB1-coated wells for 3 hr at 25°C to detect IgG or IgA, or serum was incubated in LB1-coated wells for 1 hr at 37°C for assessment of total
immunoglobulin. Antibody was detected with HRP-conjugated goat anti-rat IgG, IgA (Bethyl Laboratories, Montgomery, TX) or HRP-conjugated protein A (Zymed, S. San Francisco, CA). Reciprocal titers were defined as the dilution that yielded an OD$_{450}$ value of 0.1 above control wells that were incubated without sample fluids. Assays were performed a minimum of three times and reciprocal titers reported as the geometric mean (GMT).

**Biosensor assay.** To determine the relative amount of LB1-specific immunoglobulin in sera, real-time biosensor assays were performed using a Biacore 3000 (Biacore, Uppsala, Sweden). All reagents were purchased from Biacore. LB1 was suspended in sodium acetate buffer, pH 4.5, prior to immobilization to the surface of an activated reagent grade CM5 sensor chip using amine coupling chemistry. A control reference surface, to which no peptide was bound, was also created. For interaction analysis, 15 µl of undilute serum or MEF diluted 1:5 in HBS-EP running buffer were injected across the sensor chip surface at a flow rate of 5 µl/ min. The relative amount of LB1-specific antibody in each sample was calculated as the difference in resonance units (RU) 5 seconds before and 45 seconds after each sample was injected.

**T-cell proliferation assay.** To identify the site(s) of immune induction following IN or SQ immunization, a cell proliferation assay was performed using unfractionated cells isolated from chinchilla lymphoid tissues. Three cohorts of three adult chinchillas each were immunized three times IN/IN/IN or SQ/IN/IN with either: 10 µg LB1 plus 10 µg MPL; 10 µg of a ‘control peptide’ plus 10 µg MPL; or 10 µg MPL alone. The control peptide was a 34-mer synthetic peptide derived from the PiiA protein of the NTHI type IV
pilus (Tfp) and was selected because Tfp are also NTHI adhesins and due to the fact that this peptide was comparable in size to LB1 (236). One week following receipt of the third and final immunization, chinchillas were sacrificed and the cervical, brachial, axillary and mediastinal lymph nodes, as well as the nasal associated lymphoid tissue (NALT) were isolated. Tissues were crushed into complete RPMI medium [cRPMI, RPMI 1640 medium (Mediatech, Manassas, VA) containing 2 mM L-glutamine, 100 U penicillin/ml, 100 µg streptomycin/ml, 1 mM pyruvate (Mediatech), 5 mM HEPES (Acros, Geel, Belgium), 0.75 g NaHCO₃/ml (Fisher Scientific, Pittsburgh, PA) and 0.05 mM β-mercaptoethanol (Sigma, St. Louis, MO)] and the cells passed through a 40 µm cell strainer. The spleen was also isolated, crushed, the resultant cells layered onto Ficoll-Paque (GE Healthcare, Piscataway, N.J.) and centrifuged at 400 x g for 30 min at 18°C. Lymphocytes were collected at the interface. The isolated cells were then washed with phosphate buffered saline plus 0.1% bovine serum albumin by centrifugation at 300 x g for 5 min. at 25°C and labeled with 8 mM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. A total of 10⁶ cells was seeded into each well of round-bottom 96-well microtiter plates. To stimulate proliferation, 10 µg of LB1, the control peptide or concanavalin A (Sigma) was added to wells in triplicate and the cells were incubated for 6 days at 37°C, 5% CO₂. Cells were collected, T-cells labeled with mouse anti-rat CD3-PE (AbD Serotec, Raleigh, N.C.) and a total of 5000 live events was collected with a BD FACSCalibur (Becton Dickinson, Franklin Lakes, N.J.). Data were analyzed with FloJo software (Tree Star, Inc., Ashland, OR). Proliferation was demonstrated as a reduction in CFSE- specific fluorescence of CD3⁺ cells compared to unstimulated CD3⁺ cells (188) due to division of stimulated T-cells.
**Statistical analysis.** The Biometrics Laboratory at The Ohio State University College of Medicine conducted all statistical analysis. Relative clearance of NTHI from middle ears and nasopharynges was analyzed using Fisher’s exact test. Significance was accepted at a $p$-value of $\leq 0.01$. 
Results

**Histological analysis.** To first determine how far retrograde into the nasal cavity LB1 was delivered when administered via microaerosol sprayer, as well as how long after IN delivery we could detect the presence of LB1 within the nasal cavity, we performed immunohistochemical analysis. We saw no evidence of LB1 in tissues recovered from the dorsal or ventral surfaces of the nasopharynx (NP), or in the mucosa that lined the ethmoid turbinates after immunization with the adjuvant MPL alone (Fig. 3.1A, C & E, respectively). In contrast, in tissues recovered from animals that received LB1 plus MPL, strong positive labeling for LB1 was evident as far retrograde in the NP as the ethmoid turbinates (Fig. 3.1B, D & F). This labeling was positive for LB1 41 days after receipt of the final immunizing dose. No labeling of any tissue was observed when naive rabbit serum served as the primary antibody (data not shown). Therefore, IN immunization via microaerosol sprayer facilitated delivery of LB1 rostrally throughout the chinchilla nasal cavity. Further, these data demonstrated a prolonged presence of this immunogen in the NP.
Tissues were procured from the dorsal (panels A & B) and ventral (panels C & D) surfaces of the NP in addition to the ethmoid turbinates (panels E & F) of chinchillas immunized IN with either MPL alone (panels A, C & E) or 10 µg LB1 plus MPL (panels B, D, F). Note positive labeling for LB1 (black) within each nasal cavity tissue examined 41 days after receipt of LB1, but not within tissues retrieved from an animal administered MPL alone. Thus, delivery by microaerosol sprayer facilitated dispersal of the immunogen throughout the nasal cavity where it was maintained for 41 days. Goblet cells were labeled with methyl green in all panels.
**Nasopharyngeal colonization.** Of the animals enrolled, 32 of 42 (76%) chinchillas completed the study. Attrition was due to technical difficulty associated with cardiac puncture for blood collection. As a result, 7 chinchillas comprised the cohort that received 30 µg LB1 per dose SQ/IN/IN; 6 chinchillas in the cohort administered 30 µg LB1 per dose IN/IN/IN; five animals each comprised the cohorts delivered either 10 µg LB1 per dose IN/IN/IN, the adjuvant MPL IN/IN/IN or the adjuvant MPL SQ/IN/IN. Four chinchillas completed the study within the cohort that received 10 µg LB1 per dose SQ/IN/IN.

To examine the ability of each immunization regimen to induce the clearance of NTHI from the NP of chinchillas after bacterial challenge, we performed periodic NP lavages and cultured the recovered fluids to confirm the presence of NTHI at this anatomical site. Among the cohorts immunized IN/IN/IN, only the cohort that received 10 µg LB1 per dose completely eradicated culturable NTHI from the NP of all animals within the 15-day observation period (Fig. 3.2). While not statistically significant when compared to the cohort that received only MPL, this result nonetheless demonstrated that an exclusively IN immunization regimen could elicit an effective immune response. Receipt of 30 µg LB1 per dose, in contrast, resulted in an outcome that was similar to the cohort administered MPL alone.

Within cohorts immunized SQ/IN/IN, again receipt of 10 µg LB1 per dose induced an approximately 25% reduction in the percentage of animals colonized with NTHI within 6 days after challenge (Fig. 3.2). Moreover, only one of four chinchillas in this cohort remained culture-positive for NTHI 12 and 15 days after bacterial challenge. However, delivery of 30 µg LB1 per dose SQ/IN/IN did not augment clearance of NTHI from the NP compared to receipt of either 10 µg LB1 per dose or MPL alone. Collectively, these data
demonstrated that receipt of 10 µg LB1 per dose by either IN/IN/IN or SQ/IN/IN
immunization regimen were the most effective of the doses tested in terms of eliciting
clearance of NTHI from the NP.
Figure 3.2. Relative clearance of NTHI from the NP of chinchillas immunized with LB1+MPL or MPL alone by an IN/IN/IN or SQ/IN/IN regimen.

While not statistically significant, administration of 10 µg LB1 IN/IN/IN induced rapid eradication of NTHI from the chinchilla NP, a result that was likely biologically relevant.
Resolution of OM after direct challenge of the middle ear. To test the ability of each regimen to induce resolution of experimental OM in these same cohorts of animals, we also challenged immunized chinchillas by direct inoculation of NTHI into the middle ear cavity to initiate active disease. To calculate the percentage of middle ears with OM per cohort, each tympanic membrane was blindly evaluated by video otoscopy and scored on a 0 to 4+ scale which ranked inflammation along the tympanic membrane and presence or absence of a MEF behind the tympanic membrane. A score of ≥ 2.0 was always considered evidence of OM. We observed that among cohorts immunized IN/IN/IN, receipt of either 10 µg or 30 µg LB1 per dose induced a 40% or 33% reduction in the percentage of middle ears with OM in each cohort, respectively, within 3 days after NTHI challenge (Fig. 3.3) compared to only 20% in the cohort immunized with MPL alone via this exclusively mucosal regime. Moreover, 92% (11/12) of middle ears in the cohort administered 30 µg LB1 per dose IN/IN/IN resolved OM within twelve days after NTHI challenge. Chinchillas immunized with 10 µg LB1 per dose IN/IN/IN completely resolved OM within six days after NTHI challenge, a result which was statistically significant compared to the cohort administered only MPL by the same regimen (p< 0.01).

Among the cohorts immunized SQ/IN/IN, 6 days after NTHI challenge, 50% of middle ears of animals which received 10 µg LB1 per dose and 83% of middle ears in the cohort that received 30 µg per dose had resolved OM, compared to only 22% of middle ears in the cohort which received the adjuvant alone (Fig. 3.3). Moreover, the cohort immunized with 30 µg LB1 per dose resolved OM significantly earlier than the cohort which received MPL alone (p< 0.001). Chinchillas administered LB1 by either regimen spent an average of 4.5 days (range, 3 to 8 days) with experimental OM,
compared to 10.5 or 13.5 days for the animals which received only MPL by an IN/IN/IN or SQ/IN/IN regimen, respectively.
Figure 3. Relative resolution of OM after direct challenge of middle ears.

Immunization with 10 µg LB1 per dose IN/IN/IN or 30 µg LB1 per dose SQ/IN/IN induced clearance of NTHI from middle ears significantly earlier than cohorts immunized with MPL by the same regimen. *, $p \leq 0.01$ compared to the cohort that received MPL only IN/IN/IN. **, $p \leq 0.001$ compared to the cohort administered MPL alone SQ/IN/IN.
Representative images of chinchilla tympanic membranes obtained by video otoscopy, and corresponding tympanometric plots, revealed resolution of MEF after direct middle ear challenge in cohorts which received LB1 by either regimen, or conversely, showed persistence of MEFs in cohorts administered only MPL (Fig. 3.4). The tympanic membranes of chinchillas administered only MPL demonstrated substantial inflammation (as evidenced by erythema along the junction between the external auditory canal and the tympanic membrane and across the tympanic membrane, in addition to capillary dilatation across the tympanic membrane), persistent and significant volume of MEF (evidenced by the presence of yellow/orange fluid behind the bulging tympanic membrane) and abnormal tympanograms throughout the 15-day study. In contrast, tympanic membranes of animals within the four cohorts that received LB1 by either regimen or dose exhibited almost complete return to a naive status, as evidenced by limited inflammation, absence of MEF and return to a normal tympanogram within the 15-day observation period. Collectively, these data demonstrated that the immune response induced by an exclusively or predominantly non-invasive immunization regimen effectively limited the duration of NTHI-induced experimental OM and ultimately resolved active disease, despite the rigorous challenge protocol used here.
Figure 3.4. Resolution of OM after transbullar challenge with NTHI.

Images depict representative tympanic membranes captured by video otoscopy. Insets display corresponding tympanometric plots. Receipt of 10 µg LB1 per dose IN/IN/IN, 10 µg or 30 µg per dose SQ/IN/IN induced resolution of OM, shown by reduction in inflammation along the tympanic membrane and absence of MEF behind the tympanic membrane. Note: top panel represents a healthy chinchilla middle ear.
Detection of immunogen-specific antibody in serum. To begin to explain the immunologic basis of the efficacy observed, we examined the relative quantity of LB1-specific antibody in the serum of chinchillas after receipt of each immunizing dose by both ELISA and biosensor-based assays. Increasing titers of immunogen-specific antibody was detected in the serum of chinchillas immunized with three sequential 10 µg or 30 µg LB1 per dose IN/IN/IN (Fig. 3.5A & B, see bars) which, as expected, was greater than titers observed for the cohort immunized with MPL alone (Fig. 3.5C). Similar trends were detected for each of these cohorts by biosensor assay (Fig. 3.5A-C, see line plots). Among cohorts immunized SQ/IN/IN with LB1, increasing titers of immunogen-specific antibody were similarly detected in serum (Fig. 3.5D & E). This result was not observed in the cohort administered MPL alone (Fig. 3.5F). Resonance units (RU) values obtained by biosensor assay correlated with ELISA-determined titers.

In terms of overall kinetics of the immune response elicited, both cohorts that received 10 µg LB1 per dose, by either an IN/IN/IN or SQ/IN/IN regimen, demonstrated a continuous increase in the relative quantity of LB1-specific antibody after receipt of each additional dose (Fig 3.5A & D), whereas the cohorts which received 30 µg LB1 per dose exhibited a reduction (Fig. 3.5B) or lag (Fig. 3.5E) in titer during the immunization schedule. Despite unique outcome of these two immunization regimens, these data demonstrated, by two complementary methods, that both induced a systemic immune response, as evidenced by the production of LB1-specific serum antibody. Based on clearance data (see Figs. 3.2 & 3.3), achieving a serum antibody GMT value ≥ 60 appeared to be associated with greatest ability to eradicate NTHI from the NP and middle ear.
Figure 3. 5. Relative quantity and affinity of LB1-specific antibody within pooled chinchilla serum collected prior to and after receipt of each immunizing dose.

Reciprocal titers were obtained by ELISA (black bars). Relative resonance units were obtained by biosensor assay, which measured the relative binding of immunogen-specific antibody, (dashed lines).
Use of commercially available reagents to detect isotype-specific antibody in fluids collected from the chinchilla. Whereas Protein A used in the ELISA assay to detect chinchilla antibodies favored IgG and IgM isotypes, the biosensor assay had the potential to detect antibody of all isotypes. Thereby, to fully characterize the immune response elicited by each immunization strategy, it was desirable to develop an assay that would allow us to discriminate among chinchilla antibody isotypes. However, there are no commercially available chinchilla-specific reagents. Of a panel of antibodies raised against molecules expressed by other species, including human, mouse and rat, greatest cross-reactivity with chinchilla IgG, IgA and IgM was observed when we used either rat- or human-directed reagents, a result with precedent for immunodetection in the chinchilla (133, 242, 284). To demonstrate the cross-reactivity of commercially available rat-directed reagents, we tested three chinchilla serum pools with known increasing LB1-specific serum titers as well as both naive serum and hyperimmune chinchilla serum directed at an unrelated immunogen in an ELISA assay in which LB1 served as the target antigen (Fig. 3.6). Both reciprocal titer- and dilution-specific differences in each serum pool were detected by use of goat anti-rat IgG. Further, the reactivity of the control serum pool (chinchilla anti-OMP26) or naive chinchilla serum was minimal at all dilutions tested. We were therefore confident in the utility of these commercially available reagents to characterize the immune response here.
Figure 3. 6. Detection of LB1-specific IgG in chinchilla serum by cross-reactive goat anti-rat IgG.

Chinchilla serum with an LB1-specific reciprocal titer of either 5000 (open triangle); 50,000 (closed square); or 100,000 (open diamond), (as determined previously via ELISA assay using Protein A), exhibited both reciprocal titer- and dilution-specific differences when detected with goat anti-rat IgG. Negative control antiserum directed against unrelated NTHI OMP26 (closed circle), demonstrated no reactivity above background, similar to results obtained when we assayed naive serum (open circle).
**Detection of immunogen-specific IgA and IgG in mucosal secretions.** To begin to explain the mechanism behind the immunization-induced clearance of NTHI from the NP and middle ear, we examined the relative quantity and isotype of antibody present within NP lavage fluids and MEF. In NP fluids collected from animals immunized with LB1 by either regimen, the overall quantity of LB1-specific IgA was comparable to animals that received MPL alone (Fig. 3.7A), except on day 15 when the cohorts immunized with either 30 µg LB1 IN/IN/IN or 10 µg LB1 SQ/IN/IN per dose demonstrated the greatest immunogen-specific IgA titer. With regard to immunogen-specific IgG in NP lavage fluids, receipt of either 10 or 30 µg LB1 per dose SQ/IN/IN or 10 µg LB1 IN/IN/IN induced at least two-fold greater LB1-specific IgG at all time points assessed, compared to the respective cohorts which received only MPL (Fig. 3.7B). Further, immunogen-specific IgG for these three cohorts peaked between 9 and 12 days after NTHI challenge. Based on the relative ability to eradicate NTHI from the NP among cohorts (see Fig. 3.2), achieving a GMT value of ≥ 20 for LB1-specific IgG in NP lavage fluids was associated with ability to clear this microbe from the nasal cavity.
Figure 3. 7. Detection of LB1-specific IgA or IgG within pooled NP lavage fluids.

Panel A, LB1-specific IgA; panel B, LB1-specific IgG. Within NP fluids, a greater relative quantity of LB1-specific IgG was detected in fluids retrieved from animals immunized with LB1, compared to LB1-specific IgA.
Both LB1-specific IgA and IgG were also detected in MEFs retrieved from chinchillas immunized with LB1 by either regimen (Fig. 3.8). Moreover, the relative quantity of LB1-specific IgG was consistently greater than that of LB1-specific IgA at the time points assessed. LB1-specific IgG and IgA titers peaked between days 6 and 9 after NTHI challenge among the cohorts from which it was possible to retrieve MEF. Rapid clearance of NTHI from the middle ears of animals immunized IN/IN/IN with 10 µg LB1 per dose eradicated our ability to recover MEF from this cohort beyond a time point of 3 days after direct challenge. Based on the relative ability to clear NTHI from the directly challenged middle ear space amongst cohorts (as shown in Fig. 3.3), induction of an LB1-specific GMT value of $\geq 60$ within a MEF was associated with greater protective efficacy in this model system.
Figure 3. Relative quantity of LB1-specific IgA and IgG within pooled MEF collected after NTHI challenge.

Immunogen-specific IgA (grey bars) and IgG (black bars) were detected in MEE, although a greater relative quantity of LB1-specific IgG was observed compared to IgA. *, no MEF present to recover.
Identification of the sites of immune induction after immunization. To begin to characterize the sites of immune induction after immunization via exclusively IN regimen, we harvested lymphoid tissues from immune chinchillas, isolated and labeled the lymphoid cells using the vital dye CFSE and examined the proliferation of CD3+ T-cells after \textit{in vitro} stimulation with LB1. Thus, only T-cells primed by immunization \textit{in vivo} were expected to proliferate after \textit{in vitro} stimulation with the same antigen.

Proliferation was detected as a reduction in CFSE-mediated fluorescence of CD3+ T-cells due to cellular division. As a positive control, concanavalin A served as a nonspecific stimulus of T-cell proliferation, and as shown in Fig. 3.9A, CD3+ T-cells isolated from the spleen and stimulated with concanavalin A exhibited a 40-fold reduction in CSFE-mediated fluorescence compared untreated cells (Fig. 3.9A), thus demonstrating the utility of this assay to measure T-cell proliferation in the chinchilla host.

Of the tissues examined from chinchillas primed parenterally, T-cell proliferation was detected only in those cells isolated from the spleen of animals immunized with LB1 plus MPL (Fig. 3.9B), and not chinchillas that received MPL alone. This immunization regimen did not appear to prime T-cells within the NALT, as no change in CFSE-mediated fluorescence was detected when cells recovered from that tissue were assayed (Fig. 3.9C). Also, after exclusively IN immunization with LB1 plus MPL or MPL alone, no proliferation of T-cells was noted by cells isolated from the spleen (Fig. 3.9D). Only cells isolated from the NALT of animals immunized IN/IN/IN with LB1 plus MPL, but not MPL alone, demonstrated a reduction in CFSE-mediated fluorescence after \textit{in vitro} simulation with LB1 (Fig. 3.9E). No proliferation was observed by T-cells isolated from lymphoid tissues collected from animals immunized with the control peptide by either
regimen (data not shown). These data therefore indicated that, relative to the time point
tested, immunization with LB1 via SQ/IN/IN regimen primarily primed T-cells within the
spleen whereas an exclusive IN immunization regime resulted in priming of primarily T-
cells within the NALT.
Figure 3. 9. *In vitro* proliferation of CD3$^+$ T-cells.

Cells were isolated from the NALT and spleens of chinchillas, labeled with CFSE and stimulated with LB1 *in vitro*. CD3$^+$ CFSE$^+$ cells were detected by flow cytometry. CD3$^+$ T-cells isolated from the spleen and stimulated *in vitro* with concanavalin A were induced to proliferate, as shown by a reduction in CFSE-mediated fluorescence (panel A, blue histogram), compared to unstimulated cells (panel A, black histogram). CD3$^+$ T-cells isolated from the spleens of chinchillas immunized SQ/IN/IN with LB1 plus MPL were induced to proliferate after *in vitro* stimulation with LB1 (panel B, red histogram), but no proliferation was detected from cells isolated from the NALT (panel C, red histogram) of the same animals. No proliferation was detected by cells isolated from the spleen or NALT of animals parenterally primed with MPL alone (panels B and C, green histograms). CD3$^+$ T-cells isolated from the spleens of chinchillas after exclusively IN immunization with LB1 plus MPL did not proliferate (panel D, red histogram). However, CD3$^+$ T-cells from the NALT did proliferate (panel E, red histogram). No proliferation was detected by cells isolated from the spleen or NALT of animals immunized IN with MPL alone (panels D & E, green histograms).
Discussion

OM is a highly prevalent pediatric disease the burden of which is felt both in the associated morbidity and due to the relative socioeconomic impact of diagnosis and treatment (7, 158). Whereas antibiotics and/or insertion of tympanostomy tubes is relied upon to manage OM medically and surgically, these approaches have inherent risks, including emergence of antibiotic resistance among the bacteria associated with OM as well as the invasive nature and potential risks of surgery under general anesthesia (47, 342). Development of vaccines to prevent OM is therefore a desirable and important goal. We have demonstrated pre-clinically, that parenteral immunization with a NTHI OMP P5-derived immunogen, LB1, affords significant protection against experimental NTHI-induced OM (14, 18, 162, 176). In the current study, we wanted to examine alternative immunization regimens which had the potential for comparable efficacy as we hypothesized that LB1 had the potential to be efficacious when administered mucosally.

Intranasal immunization has many benefits, including the non-invasive nature of delivery and ability to induce both systemic and mucosal immune responses. The induction of mucosal immunity is an important consideration as OM is a disease of the uppermost respiratory tract mucosa. Moreover, there is now greater appreciation for the existence of compartmentalization within the mucosal immune system itself, with evidence for immune cells within the gastrointestinal and respiratory tracts preferentially migrating to local lymph nodes after engagement with antigen (35, 347). Immunization via an IN route distributes antigen within the nasal cavity where the bacteria which induce OM normally reside thereby likely also promoting correct (and potentially site-specific) processing events needed to elicit a clinically efficacious response. Moreover,
prior to the development of OM, the relative bacterial load within the pediatric NP increases (75, 77). As such, targeted delivery of immunogen to immune cells within lymphoid tissues proximal to the nasal cavity or within the upper respiratory tract mucosa via an IN immunization route could thus potentially limit or inhibit the development of disease.

We observed that delivery of LB1 by pediatric microaerosol sprayer distributed the immunogen throughout the chinchilla nasal cavity, which likely facilitated uptake by nasal mucosa DCs, migration to local lymphoid tissues and initiation of an immune response. This premise is supported by prior work in which a single dose of LB1 administered IN to chinchillas was sufficient to induce the migration of nasal cavity DCs to the NALT (242), a lymphoid aggregate important as an immune inductive site for the upper respiratory tract (173), and particularly after IN immunization (9, 347). Moreover, the current work demonstrated that IN immunization primed CD3+ T-cells within the chinchilla NALT. As the NALT is in close proximity to both the NP and middle ear, we speculate that the initiation of an immune response within this lymphoid aggregate may explain the rapidity with which NTHI were cleared from the NP and middle ears of immune animals.

Within mucosal fluids collected from immune animals, both immunogen-specific IgA and IgG were detected and the presence of both antibody isotypes was found to correlate with clearance of NTHI and resolution of OM. Moreover, the detection of a greater relative quantity of immunogen-specific IgG within each mucosal secretion was a beneficial outcome as nearly all NTHI contain the iga gene that encodes an IgA1 protease, and up to a third have an additional gene (igaB) which encodes a second IgA protease (80, 191). It was therefore likely that immunogen-specific IgG, either locally
produced or serum-derived, was required (in addition to LB1-specific IgA) in order to resolve experimental NTHI-induced OM.

The two immunization regimens employed in this study were designed to investigate whether an exclusively mucosal regimen was sufficient to induce a protective immune response, or whether parenteral priming prior to mucosal boosts was required to enhance the elicited response. In general, immunization by either regimen resulted in both clearance of NTHI from the NP and significantly earlier resolution of OM after direct challenge when compared to cohorts that received only adjuvant by the same regime. Furthermore, the rapidity of resolution of OM was faster than we have observed using traditional parenteral immunization in this model (18). These data demonstrated that a mucosally-based immunization regimen whereby LB1 served as the immunogen, could induce protection against experimental NTHI-induced OM in a chinchilla model. Moreover, the systemic and mucosal immune responses elicited were comparable between the two regimens assessed.

The greatest difference observed here, among cohorts, was related to the response observed relative to the total dose of immunogen delivered IN. Interestingly, receipt of 30 µg LB1 IN/IN/IN did not augment clearance of NTHI from the nasopharynx or middle ear compared to delivery of 10 µg per dose by the same route, the latter of which was protective. Cutter et. al. observed a similar trend after IN delivery of 15 µg versus 5 µg isolated NTHI HapS protein to mice, despite an approximately 3-fold greater serum titer induced by receipt of 15 µg per dose (56). As comparable serum titers were detected among all immune cohorts here, one can speculate that IN delivery of 30 µg LB1 plus MPL per dose perhaps induced counter-productive inflammation within the nasal mucosa. The OMP P5 epitope that is incorporated into LB1 has been demonstrated to
be a pathogen-associated molecular pattern (i.e. PAMP) (242), thus, whereas IN delivery of 10 µg per dose was extremely effective to induce mucosal immunity, delivery of 3-times as much immunogen in combination with the adjuvant MPL, also an immunostimulant, may have ultimately been excessive, and thereby dampened the resulting immune response. This was evidenced by a lesser quantity of specific IgG within NP lavage fluids or of immunogen-specific IgA and IgG within MEE, compared to the other immune cohorts.

Collectively, these data provided evidence of the protective efficacy that could be afforded by mucosal delivery of a low dose of LB1 to prevent experimental OM due to NTHI. We continue to refine our immunization strategies to incorporate additional NTHI adhesin-based immunogens as well as additional alternative and noninvasive delivery methods into our research program to design and test vaccine candidates for the prevention of this highly prevalent pediatric disease.
Chapter 4

Transcutaneous immunization as preventative and therapeutic regimens to protect against experimental otitis media due to nontypeable Haemophilus influenzae

Introduction

Nontypeable Haemophilus influenzae (NTHI) is a predominant bacterial agent of the prevalent pediatric disease otitis media (OM) and is also responsible for multiple diseases of the upper and lower respiratory tracts of both children and adults (89). The economic burden of NTHI-induced diseases, including OM, is significant due to treatment and surgical management costs (158). Complications of OM, for example hearing loss, are associated with behavioral, educational and language development delays of this very young population (23). With the goal to prevent NTHI-induced OM, many research efforts have focused on the development of vaccines that target outer membrane proteins (OMPs), other surface proteins and lipooligosaccharide expressed by this bacterium (216).

Our lab has concentrated on two of the multiple adhesins expressed by NTHI, OMP P5 and the Type IV pilus (Tfp). Specifically, we have designed three vaccine candidates: a 40-mer synthetic chimeric peptide immunogen called ‘LB1’ which incorporates a 19-mer B-cell epitope from OMP P5 that has been co-linearly synthesized
with a T-cell promiscuous epitope from measles virus fusion protein (14); a recombinant protein called ‘rsPilA’ which represents a mature, N-terminally truncated and soluble PilA subunit protein of the Tfp (236) and a chimeric immunogen called ‘chimV4’ in which modified rsPilA serves as both immunogen and carrier molecule for a 24-mer epitope of OMP P5 that is positioned at its N-terminus (236). Antibody induced by parenteral immunization with any of these immunogens confers significant protection against NTHI-induced OM in a chinchilla model of viral-bacterial superinfection (162, 236). We now wanted to expand our vaccine delivery strategies to develop a non-invasive, but potentially equally efficacious method, transcutaneous immunization (TCI).

TCI, the application of a vaccine onto intact skin, induces an immune response by engaging antigen-presenting cells present within the epidermis and dermis, the Langerhan’s cells and dermal dendritic cells, respectively (334). There are multiple benefits to this immunization route, which include the simplicity and noninvasive nature of delivery, reduced cost as syringes, needles and trained medical professionals are not required to deliver the vaccine and the prospect for greater vaccine distribution beyond developed countries due to typically cheaper production costs (105). Previous studies show that TCI with bacterial or viral proteins and other peptide antigens induces an immune response in both animals and humans (29, 190, 332). Furthermore, via use of animal models, there is evidence of protection against subsequent bacterial, viral or toxin challenge (96, 270, 297, 311). Whereas parenteral immunization elicits primarily a systemic immune response, TCI induces both systemic and mucosal immunity (201). OM is a disease of the uppermost respiratory tract, therefore the ability to induce immunity at the mucosae of this anatomical region has the potential to reduce, or preferably prevent, the onset of disease in the middle ear.
NTHI-induced diseases of the respiratory tract, including OM, can be chronic and/or recurrent in nature, a consequence of biofilms present on the respiratory mucosae (219). Specific to OM, NTHI biofilms are shown on the middle ear mucosa of children (116) and upon gross examination of the middle ear in animal models (72). These bacterial communities are recalcitrant to antibiotic treatment and resist immune-mediated clearance. Among other NTHI proteins, OMP P5 and Tfp are identified as components of the biofilm matrix (91, 154). We therefore hypothesized that immunization with NTHI OMP P5- and Tfp-directed immunogens could serve to target each element \textit{in vivo}, and thus facilitate resolution of OM. Herein, we utilized a chinchilla model of experimental NTHI-induced OM to examine the immune response elicited by TCI with NTHI OMP P5- and Tfp-directed immunogens when delivered with a potent adjuvant, a double mutant of \textit{E. coli} heat-labile enterotoxin (dmLT) (311). Moreover, we examined the efficacy of TCI when utilized in both preventative and therapeutic immunization strategies to determine the potential of this non-invasive approach to protect against as well as resolve experimental NTHI-induced OM.
Materials and Methods

**Animals.** To examine the efficacy of TCI utilized as a preventative immunization strategy, 70 adult chinchillas (*Chinchilla lanigera*; Rauscher’s Chinchilla Ranch, LaRue, Ohio; mean mass $662 \pm 15$ g) with no evidence of middle ear disease as determined by video otoscopy (MedRx, Largo, FL) and tympanometry (EarScan, Murphy, N.C.) were enrolled and divided into 7 cohorts of 10 animals each. To test TCI as a therapeutic immunization strategy, 21 adult chinchillas ($625 \pm 23$ g) were enrolled and divided into 7 cohorts of 3 animals each. Animal care and all procedures were performed in concordance with institutional and federal guidelines, and were conducted under an approved protocol.

**Immunogens and adjuvant.** NTHI OMP P5 was observed expressed by 100% of a panel of middle ear and nasopharyngeal NTHI isolates collected from children with chronic OM (20). Multiple algorithmic analyses of the deduced amino acid sequence of OMP P5 predict four surface-exposed regions within the N-terminus of this adhesin (68, 335) and within the third surface exposed region is a 19-mer B-cell epitope that is incorporated into LB1 (19). Among NTHI strains tested, there is limited amino acid sequence diversity within this moiety which allows for segregation of isolates into three groups, 76% of which cluster into one majority group. The immunogen LB1 is a 40-mer synthetic chimeric peptide comprised of the aforementioned 19-mer B-cell epitope from OMP P5 that is co-linearly synthesized with a T-cell promiscuous epitope from measles virus fusion protein, incorporated to be broadly permissive in binding to MHC class II molecules.
PilA, the majority subunit of NTHI Tfp, has deduced minimal amino acid sequence diversity. Among a panel of 23 isolates tested, the product of the pilA gene exhibits ≥ 81% sequence identity to that of NTHI strain 86-028NP (16). Recombinant, soluble PilA (rsPilA) is a 122 amino acid recombinant protein which represents a mature, N-terminally truncated PilA subunit of the NTHI Tfp (16, 154, 236). ChimV4 is a novel, chimeric immunogen wherein modified rsPilA serves as an immunogenic carrier for a slightly larger (24 amino acids) variant of the OMP P5 B-cell epitope described within the immunogen LB1 (236). A double mutant form of E. coli heat-labile enterotoxin, called LT(R192G-L211A) and abbreviated dmLT, wherein glycine is substituted for arginine at position 192 and alanine is substituted for lysine at position 211, served as the adjuvant (311). The amino acid substitutions render dmLT nontoxic while maintaining its adjuvant properties.

**NTHI strain.** NTHI strain 86-028NP was isolated from the nasopharynx of a child undergoing tympanostomy and tube insertion for chronic OM at Nationwide Children’s Hospital, Columbus, OH. This strain has been characterized and extensively used in chinchilla models of OM and a rat model of pulmonary clearance (18, 162, 176, 239).

**Immunization via a preventative regimen and NTHI challenge.** TCI via a preventative immunization regimen was performed as follows: both pinnae of each alert animal were hydrated for 5 min. by placement of gauze soaked in sterile, pyrogen-free 0.9% sodium chloride (Hospira, Inc., Lake Forest, IL) on the inner surface prior to vaccination. The inner surface of each pinna was then blotted with dry gauze and 50 µl
of each vaccine formulation was applied using a pipet. The pinnae were then folded in half and opposing surfaces gently rubbed together. Formulations consisted of 10 µg LB1, rsPilA or chimV4 delivered alone or admixed with 10 µg LT(R192G-L211A), also called ‘dmLT’, or 10 µg dmLT alone. Two doses were delivered at weekly intervals.

NTHI challenge was performed as previously described (18). Briefly, one week after receipt of the second immunizing dose, all chinchillas were challenged IN with 10⁸ CFU NTHI strain 86-028NP delivered in 0.2 ml pyrogen-free saline divided equally between the nares, as well as transbullarly with 1000 CFU NTHI delivered in 0.3 ml sterile pyrogen-free saline per bulla. Challenge doses were confirmed by plate count.

**NTHI challenge and immunization via a therapeutic strategy.** TCI following a therapeutic regimen involved challenge of all chinchillas exclusively transbullarly with 1000 CFU NTHI strain 86-028NP prior to immunization. Four days after NTHI challenge, animals were immunized by TCI as described above. Formulations consisted of 10 µg LB1, rsPilA or chimV4 delivered alone or admixed with 10 µg dmLT or 10 µg dmLT alone and were delivered twice a weekly intervals. One week after receipt of the second immunizing dose, all animals were sacrificed.

**Histology of chinchilla pinnae.** Pinnae were collected and trimmed to the central 2.5 mm² portion. The pinna was then cut into 4 strips of equal size and fixed in 2% w/v paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 24 hrs. Strips were processed for routine histology and embedded in paraffin. Five micron serial sections were cut and tissue stained with hematoxylin and eosin.
**Collection of blood and mucosal secretions.** Blood for serum was collected via cardiac puncture prior to immunization (pre) and one week after receipt of the second immunizing dose (immune) for all animals immunized via a preventative regimen. Animals immunized via a therapeutic regimen were bled prior to NTHI challenge and at study end.

NP lavages were performed on animals immunized via a preventative regimen prior to immunization, one week after receipt of the final dose and on days 3, 7, 10, and 14 after bacterial challenge by passive inhalation of 500 µl sterile pyrogen-free saline as previously described (18).

Video otoscopy (using a 0°, 3-in. probe connected to a digital camera system) to monitor signs of tympanic membrane inflammation and/or presence of fluid within the middle ear space was performed and overall signs of OM were rated on a scale of 0 to 4+ as previously described (18, 162, 239). Middle ears with a score of ≥ 2.0 were always considered positive for OM as MEF is visible behind the tympanic membrane. Each middle ear was considered independent, and for each cohort, the percentage of middle ears with OM was calculated.

Epitympanic taps to retrieve middle ear effusions were performed on any chinchilla whose tympanic membrane was rated as ≥ 2.5 on a scale of 0 to +4.0. Epitympanic taps were not performed on ears ranked 2.0, due potential for perforation of the tympanic membrane to retrieve the low volume of MEF. NP lavage and middle ear fluids were serially diluted and plated onto chocolate agar supplemented with 15 µg ampicillin/ ml medium or chocolate agar, respectively, to semi-quantitate CFU NTHI per ml fluid type. The mean CFU NTHI/ ml fluid was reported for each cohort.
**Evaluation of middle ear biomass.** Upon sacrifice of animals immunized after NTHI challenge, the inferior bullae from each animal were dissected, opened to reveal the middle ear space and washed with 1 ml sterile, pyrogen-free saline to remove residual MEF and loosely adherent biomass. The bullae and remaining adherent biomass were then imaged with a digital camera. The images from the left and right bulla were scrambled and scored by nine blinded observers who ranked the relative residual biomass on a 0 to 4+ scale, wherein 0= no biomass; 1= biomass fills <25% of middle ear space; 2= biomass fills 25-50% of middle ear space; 3= biomass fills 50-75% of middle ear space and 4= biomass fill 75-100% of middle ear space. The mean biomass score for each bulla was reported.

**Enzyme-linked immunosorbent assay.** ELISA was performed on serum to detect immunogen-specific IgG and IgA in immune serum and MEFs. Samples were incubated in LB1-, rsPilA- or chimV4-coated wells (0.2 µg protein/ well) for 3 hr at 25° C and antibody was detected with HRP-conjugated goat anti-rat IgG or IgA (Bethyl Laboratories, Montgomery, TX). Color was developed with 3,3′,5,5′-tetramethylbenzidine (TMB; Pierce Biotechnology, Rockford, IL). Reciprocal titers were defined as the dilution that yielded an OD_{450 nm} value of 0.1 above control wells that were incubated without sample fluids. Assays were performed a minimum of three times and reciprocal titers reported as the geometric mean (GMT).

**Flow cytometry to detect recognition of native proteins on NTHI.** To detect the recognition of native OMP P5 and Tfp as expressed on the surface of NTHI by antibodies in NP lavage fluids, flow cytometric analysis was performed. NTHI strain 86-
028NP was cultured overnight on chocolate agar, then suspended in brain heart infusion broth supplemented with 2 µg each β-NAD and heme (sBHI; Sigma Aldrich, St. Louis, MO). The optical density was adjusted to 0.6 at 490 nm, further diluted 1:6 in fresh sBHI and the culture incubated static for 3 hr at 37° C, previously shown to be time of maximal pil promoter activity (16). NTHI were then incubated with a 1:10 dilution of pooled clarified NP lavage fluids for 1 hr at 37° C on a rotational rocker. Chinchilla immunoglobulins were detected with Protein A conjugated to FITC (Zymed, South San Francisco, CA). A total of 20,000 events were collected per sample using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and data analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). Assays were performed a minimum of three times.

**In vivo migration of dendritic cells from the pinnae.** To track the migration of chinchilla dendritic cells from the pinnae to lymphoid tissues, we applied the amine-reactive vital dye, carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA) with LB1+dmLT, rsPilA+dmLT, chimV4+dmLT or dmLT alone to passively label cells which sampled the vaccine formulations after delivery (182, 242). To confirm a minimal contribution of nonspecific activation of DCs by the dye itself, CFSE alone was also applied. One hr after TCI with vaccine formulations and CFSE, animals were sacrificed and the cervical, brachial, axillary and mediastinal lymph nodes were removed in addition to the spleen. The nasal-associated lymphoid tissue (NALT) was also removed. Tissues were crushed into Dulbecco’s PBS without calcium or magnesium (Mediatech, Inc., Manassas, VA) and cells passed through a 40 µm cell strainer. The spleen was also crushed, strained and the cells centrifuged with Ficoll-Paque. DCs were labeled
with mouse anti-rat CD11c-Alexa fluor 647 (AbD Serotec, Raleigh, NC) and CD11c+ CFSE+ cells were detected by flow cytometry. A total of 10,000 events were collected.

To discriminate between Langerhan’s cells and dermal dendritic cells, 10⁶ cells from each lymphoid tissue cell suspension were incubated with mouse anti-human DC-SIGN- APC, then permeabilized and incubated with mouse anti-human CD207- PE (R&D Systems, Minneapolis, MN) or appropriate isotype control antibody and assayed via flow cytometry. A total of 10,000 events were collected. Assays were performed a minimum of three times and representative scatter plot shown.

To confirm that the CFSE+ signal detected within the NALT was specifically due to the migration of DCs from the pinnae and not due to diffusion of the dye within the lymphatics, we investigated whether in vitro-activated, bone marrow-derived DCs would similarly follow homing signals to the NALT when applied to the pinnae. Thus, chinchilla bone marrow cells were harvested and cultured in the presence of 40 ng each of human recombinant GM-CSF and IL-4 (R&D Systems, Minneapolis, MN)/ ml to induce differentiation of precursor cells as previously described (242). After six days, the immature DCs were labeled with 10 µM CFSE prior to incubation for 30 min. with either 10 µg chimV4 plus 10 µg dmLT or 10 µg dmLT alone. The treated DCs were washed to remove unbound immunogen, adjusted to 10⁶ cells/ 100 µl, and injected intradermally to alert chinchillas in a volume of 50 µl per pinna. The animals were sacrificed 1 h later, and the lymphoid tissues were processed as described above. CFSE+ cells were detected by flow cytometry. A total of 10,000 viable events were collected for each of three independent assays and representative plots are shown.
**Statistical analysis.** Statistical differences between antibody titers, bacterial counts in NP lavage fluids and MEF in were determined using a Kruskal-Wallis one way analysis of variance on ranks and Dunn’s method for multiple comparisons. A $p$-value $\leq 0.05$ was considered significant. Significant differences in relative biomass among cohorts and percentage of middle ears with OM were assessed by a repeated measures analysis of variance and Bonferroni’s multiple comparison test. A $p$-value $\leq 0.05$ was considered significant.
Results

*Histologic analysis of chinchilla pinna.* Pinnae from naive chinchillas were collected to examine the histological organization of this tissue. A cross section of a pinna is shown in Fig. 4.1, wherein the epidermis (Ep) and dermis (D), supported by elastic connective tissue (Ct), were discernible on each side of hyaline cartilage (Hc) which runs central through the pinna. Similar to human thin skin, the pinna contained hair follicles (Hf) and sebaceous glands (Sg). An abundant stratum corneum (Sc) was visible as the outermost layer of the epidermis. Therefore, hydration of this keratinized epithelial layer was deemed necessary to enhance the permeability of the skin and thus facilitate both entry of topically applied molecules and sampling by cutaneous antigen-presenting cells, as has been reported (319).
Figure 4. 1. Cross section through a naive chinchilla pinna stained with hematoxylin and eosin.

Sc, stratum corneum; Ep, epidermis; D, dermis; Ct, connective tissue, Hc, hyaline cartilage; Hf, hair follicle and associated sebaceous gland; Sg, sebaceous glands. Chinchilla pinna exhibited characteristics of thin skin and thus was amenable to TCI upon hydration of the stratum corneum. Image captured at 10X magnification.
**TCI prior to NTHI challenge- resolution of nasopharyngeal colonization.** To examine the ability of TCI with NTHI OMP P5-directed candidate ‘LB1’, the Tfp-directed candidate ‘rsPilA’ and the chimeric OMP P5- plus Tfp-directed immunogen ‘chimV4’, to induce clearance of NTHI from the nasopharynges (NP) of challenged chinchillas, periodic NP lavages were performed and the recovered fluids cultured to semi-quantitate the relative bacterial load at this anatomical site. Cohorts administered any of the three immunogens without adjuvant and the cohort immunized with dmLT alone had $10^4$ - $5 \times 10^6$ CFU NTHI in NP lavage fluids beginning 3 days after challenge, and maintained this bacterial load for the remainder of the study period (Fig. 4.2A). In contrast, cohorts that received rsPilA, LB1 or chimV4 admixed with dmLT demonstrated 2- to 4-log fewer NTHI within NP lavage fluids on each of days 7, 10 and 14 after bacterial challenge, a statistically significant result at each time point, compared to cohorts that received respective immunogen only or dmLT only ($p < 0.05$). Moreover, only the three cohorts that received immunogen plus dmLT eliminated NTHI from the NP within 10 or 14 days after challenge.

As an additional assessment of efficacy, the relative percentage of animals with culture-positive NP lavage fluids was calculated for each cohort. Three days after NTHI challenge, 100% of animals administered immunogen alone or dmLT alone had culture-positive NP lavage fluids and at least 5/10 (50%) animals in each of these four cohorts remained culture-positive for the entire 14-day study period (Fig. 4.2B). In contrast, a 20- 40% reduction in culture- positive NP lavage fluids was observed three days after NTHI challenge in cohorts that received any of the three tested immunogens plus dmLT. Further, 7 days after NTHI challenge, only 1/10 (10%) animals in the cohorts that received LB1+dmLT or rsPilA+dmLT and 4/10 (40%) animals within the cohort
administered chimV4+dmLT had culture-positive NP lavage fluids, and by 14 days after bacterial challenge all of these latter cohorts had eliminated NTHI from the NP. These data therefore demonstrated that TCI with OMP P5- and Tfp-directed candidates, when delivered in combination with dmLT, resulted in an effective immune response which reduced, then completely eliminated, NTHI from the NP.
Figure 4. 2. TCI via a preventative regimen induced clearance of NTHI from the nasopharynges of chinchillas.

Panel A, colonization kinetics which demonstrated that receipt of rsPilA+dmLT, LB1+dmLT or chimV4 +dmLT resulted in rapid clearance of NTHI from the NP relative to the cohorts administered rsPilA, LB1, chimV4 or dmLT alone. *, statistically significant compared to receipt of respective immunogen-only cohort and dmLT alone cohort (p<0.05). Panel B, percentage of colonized NP per cohort showing a reduction in colonization after receipt of LB1+dmLT, rsPilA+dmLT or chimV4+dmLT compared to the cohorts administered rsPilA, LB1, chimV4 or dmLT alone.
**TCI prior to NTHI challenge- resolution of experimental OM.** As a more rigorous assessment of the protection afforded by TCI, the same chinchillas as challenged intranasally above were also challenged by direct inoculation of the middle ears with 1000 CFU NTHI to initiate active OM. Middle ear fluids (MEF), when present as observed by video otoscopy and confirmed by tympanometry, were collected and cultured to determine the relative concentration of NTHI within these fluids. Within 3 days after challenge, the inoculum of 1000 CFU NTHI had multiplied to 1.1x10^7 CFU NTHI in MEF retrieved from animals that received dmLT alone (Fig. 4.3A). As 10^8 CFU NTHI is typical for sham-immunized chinchillas (alum or monophosphoryl lipid A) (18), the observed reduction after receipt of dmLT suggested that this adjuvant induced a nonspecific protective effect. Animals administered LB1, rsPilA or chimV4 alone also exhibited an increase in bacterial concentration to 8.4 x10^5- 2.1x10^6 CFU NTHI at this time point. Compared to receipt of adjuvant alone, fewer NTHI were detected, which demonstrated that delivery of any of the three immunogens which target NTHI OMP P5 or Tfp induced an enhanced immune response. In contrast, three days after direct challenge, an increase from 1000 CFU to approximately 8 x10^3- 8 x10^4 CFU NTHI was observed in MEF collected from animals immunized with any of the three immunogens delivered with dmLT. Moreover, whereas approximately 10^6 to 10^7 CFU NTHI was detected in MEF collected from animals immunized with only one of three immunogens or with dmLT alone between 7 and 14 days after challenge, significantly fewer NTHI (approximately 2- to 7-logs less) were observed in MEF collected from animals immunized with LB1, rsPilA or chimV4 that had been admixed with dmLT (p< 0.05). Notably, animals immunized with chimV4+dmLT resolved OM within 10 days after challenge.
In terms of the percentage of animals with culture-positive MEF, similar to that observed in NP lavage fluids, receipt of rsPilA, LB1, chimV4 or dmLT alone resulted in a minimum of 10/20 ears (50%) with culture-positive MEF for the entire 14-day study period in all four cohorts (Fig. 4.3B). However, three days after direct challenge of the middle ear, between 80-90% of middle ears from animals immunized with any of the three immunogens plus dmLT had resolved OM. Ten days after challenge, only 1/20 (5%) middle ears in each cohort administered LB1 or rsPilA plus dmLT remained culture-positive for NTHI, and those that received chimV4+dmLT had eliminated the bacterium from the middle ear. Therefore, these data demonstrated that TCI with the NTHI OMP P5- and Tfp-directed candidates delivered with dmLT was efficacious and induced elimination of NTHI from the middle ear fluids of challenged animals as well as rapid resolution of experimental OM.
Figure 4.3. Resolution of OM in directly challenged middle ears after immunization via a preventative regimen.

Panel A, immunization with rsPilA+dmLT, LB1+dmLT or chimV4 +dmLT induced clearance of NTHI from middle ears of chinchillas compared to the cohorts that received rsPilA, LB1, or chimV4 or the cohort that was immunized with dmLT alone. *, statistically significant compared to receipt of respective immunogen-only cohort or dmLT alone ($p < 0.05$). Panel B, reduction in the percentage of culture-positive middle ears per cohort after receipt of rsPilA+dmLT, LB1+dmLT or chimV4 +dmLT relative to the cohorts that received immunogen or dmLT alone.
**TCI prior to NTHI challenge- analysis of antibody in serum.** To begin to identify the mechanism(s) for the protection observed, we first examined the relative quantity of immunogen-specific antibody produced systemically. TCI with any of the three immunogens alone induced a 4-fold increase in the serum antibody geometric mean titer (GMT) of immunogen-specific IgG and a 2- to 4-fold increase in specific IgA (Table 4.1) after receipt of all immunizing doses, compared to respective pre-immune serum. Inclusion of dmLT in each formulation significantly enhanced the resultant GMT ($p< 0.05$), as a 16- to 64-fold increase in immunogen-specific IgG and 8- to 16-fold increase in specific IgA was detected, relative to pre-immune serum. Based on the ability to eradicate NTHI from the NP or middle ear, achieving and maintaining a GMT value of at least 160 for immunogen-specific IgG was associated with most rapid clearance observed here.
Table 4.1. Geometric mean titers of immunogen-specific IgG and IgA in serum after TCI in a preventative regimen.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Immunogen-specific antibody in serum</th>
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<td></td>
<td></td>
<td>IgG</td>
<td>IgA</td>
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<tr>
<td></td>
<td>Cohort</td>
<td>pre</td>
<td>immune</td>
<td>pre</td>
<td>Immune</td>
</tr>
<tr>
<td>LB1</td>
<td>10</td>
<td>40</td>
<td>10</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>LB1+dmLT</td>
<td>10</td>
<td>320*</td>
<td>10</td>
<td>160*</td>
<td></td>
</tr>
<tr>
<td>rsPilA</td>
<td>10</td>
<td>40</td>
<td>10</td>
<td>40</td>
<td></td>
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<tr>
<td>rsPilA+dmLT</td>
<td>10</td>
<td>160*</td>
<td>10</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>chimV4</td>
<td>10</td>
<td>40</td>
<td>10</td>
<td>20</td>
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<tr>
<td>chimV4+dmLT</td>
<td>10</td>
<td>640*</td>
<td>10</td>
<td>160*</td>
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*p< 0.05 compared to respective immunogen-only titer.
Recognition of native proteins expressed by NTHI by antibody present within NP lavage fluids induced by a preventative immunization regimen. Whereas TCI with any of the three candidates induced the production of antibody in serum which recognized the immunogen delivered when assayed by ELISA, we wanted to determine if antibody produced mucosally, at the site of NTHI colonization, would bind to native structures expressed by viable NTHI, as these structural proteins would be the target for the antibodies during disease. By flow cytometry, 3.5%, 3.7% or 1.0% more live, unfixed NTHI were positively labeled by antibody in NP lavage fluids collected after TCI with LB1, rsPilA or chimV4 alone, respectively, relative to receipt of dmLT alone (Table 4.2). Incubation of NTHI with immune NP lavage fluids collected from animals immunized with LB1, rsPilA or chimV4 plus dmLT, however, demonstrated in an increase of 12.6%, 11.7% or 10.0% in positive labeling of NTHI to respectively, compared to dmLT alone. Relative to fluids collected from animals that received only immunogen, 3.2- to 10-fold greater labeling of NTHI was achieved when NP lavage fluids from immune animals were assayed which suggested a greater relative antibody titer and/or greater affinity of the antibody for its target was present within these mucosal secretions. Thus, TCI with the synthetic peptide or recombinant protein immunogens plus dmLT induced the production of mucosal antibody that recognized native structures expressed by NTHI which likely contributed to the rapid bacterial clearance observed.
Table 4. 2. Recognition of native proteins expressed by NTHI by antibody in NP lavage fluids as assessed by flow cytometry.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Percentage of labeled NTHI relative to labeling by serum from animals that received dmLT</th>
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<tbody>
<tr>
<td>LB1</td>
<td>3.5%</td>
</tr>
<tr>
<td>LB1+dmLT</td>
<td>12.6%</td>
</tr>
<tr>
<td>rsPilA</td>
<td>3.7%</td>
</tr>
<tr>
<td>rsPilA+dmLT</td>
<td>11.7%</td>
</tr>
<tr>
<td>chimV4</td>
<td>1.0%</td>
</tr>
<tr>
<td>chimV4+dmLT</td>
<td>10.0%</td>
</tr>
</tbody>
</table>
Resolution of established NTHI biomass in the middle ear via use of a therapeutic immunization regimen. To examine the therapeutic potential of TCI with the NTHI OMP P5- and Tfp-directed immunogens, we now first challenged chinchillas by direct inoculation of the middle ear with NTHI strain 86-028NP and allowed a robust biofilm to form in the middle ear space. Prior work has established that when using this protocol within 4 days after direct challenge, 83-100% of all middle ears develop a biomass that occupies approximately 75-100% of the middle ear space. Thus, after these biomasses were established, animals were immunized by TCI to determine if the resulting antibodies could resolve these structures. One week after receipt of the second dose, each middle ear was blindly ranked based on a 0 to 4+ scale of relative biomass, wherein a score of 0 indicated no biomass was detected within the middle ear space and 4.0+ designated that 75-100% of the middle ear space was filled with biomass. The average biomass score for the cohort that received dmLT alone was 2.8, which indicated approximately 50-75% of the middle ear space was filled with biomass (Fig. 4.4). In contrast, use of the therapeutic immunization regimen with LB1, rsPilA or chimV4, alone or delivered with dmLT, resulted in a significantly reduced biomass ($p < 0.05$). The average biomass score after receipt of LB1 or rsPilA, alone or with dmLT was between 0.9 and 1.2, thus 75% of the biomass had resolved after TCI. Receipt of chimV4 alone or with dmLT resulted in greater reduction in the relative biomass scores to 0.6 and 0.3, respectively. Based on relative biomass score alone, the addition of dmLT did not appear to significantly influence the outcome achieved in immunized animals. Nonetheless, overall, therapeutic immunization with the OMP P5- and Tfp-targeted immunogens elicited an immune response which resulted in a significant reduction in biomass within the middle ear.
Figure 4. Mean NTHI biomass scores for each bulla after TCI following a therapeutic regimen.

Scores were based on blinded evaluation and ranked on a 0 to 4+ scale of relative residual biomass. *, statistically significant compared to receipt of dmLT alone ($p< 0.05$). Receipt of LB1, rsPiiA or chimV4, admixed with dmLT resulted in significantly enhanced resolution of NTHI biofilms established within the middle ear space.
**Induction of immunogen-specific IgG and IgA in serum and middle ear fluids after immunization by a therapeutic regimen.** After TCI as a therapeutic regimen, predominantly immunogen-specific IgG was detected in serum from animals that received LB1, rsPilA or chimV4 admixed with dmLT, GMT which was 4- to 16-times greater compared to that of animals administered respective immunogen alone (Table 4.3). Within MEF, again, IgG was the predominant antibody isotype observed, and inclusion of dmLT in each vaccine formulation resulted in a 4- to 8-fold increase in specific antibody, compared to cohorts administered immunogen alone. Similar to that observed after TCI via a preventative immunization regimen (shown in Table 4.1), a GMT of ≥160 was associated with enhanced resolution of biomasses within the middle ears. No consistent trend was observed with regard to biomass resolution for immunogen-specific IgA in either serum or MEF recovered from immune animals. Thus, TCI via a therapeutic immunization regimen induced the production of both systemic and mucosal antibody (primarily IgG), which likely contributed to the observed resolution of NTHI-induced biomass from the middle ear.
Table 4. Geometric mean titers of immunogen-specific IgG and IgA in serum and MEF after TCI in a therapeutic immunization regimen.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Immunogen-specific antibody in serum</th>
<th>Immunogen-specific antibody in MEF</th>
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<tr>
<td></td>
<td>IgG</td>
<td>IgA</td>
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<tr>
<td>LB1</td>
<td>40</td>
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<tr>
<td>LB1+dmLT</td>
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<td>rsPilA</td>
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<td>rsPilA+dmLT</td>
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<td>chimV4</td>
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<tr>
<td>chimV4+dmLT</td>
<td>640*</td>
<td>160*</td>
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</table>

*p< 0.05 compared to respective immunogen-only titer.
Resolution of middle ear fluid after TCI via a therapeutic immunization regimen. As an additional and clinically-relevant assessment for the resolution of established NTHI-induced OM, prior to sacrifice each tympanic membrane was observed to document signs of OM. Specifically, video otoscopy and tympanometry were performed and relative signs of inflammation and presence of MEF behind the tympanic membrane were scored 14 days after NTHI challenge. In the cohort that received dmLT alone, 100% (6/6) of ears were positive for the presence of MEF (Fig. 4.5). Receipt of LB1 alone or when delivered with dmLT induced a 17-33% reduction in the number of ears with signs of OM, respectively. Moreover, a 33-50% reduction in middle ears with signs of OM was achieved after immunization with rsPilA or with chimV4 alone, respectively. Of note, immunization via a therapeutic regimen with rsPilA+dmLT resulted in 66% fewer ears with signs of OM, whereas no MEFs were observed behind the tympanic membranes of animals first challenged with NTHI, then immunized with chimV4+dmLT, each a statistically significant result compared to that obtained following immunization with dmLT alone (p<0.05). Unlike our observations for biomass reduction alone, wherein we were unable to detect the influence of dmLT, here our data showed a greater reduction in signs of OM after TCI with any of the three immunogens admixed with dmLT, compared to immunogen alone. Therefore, TCI after direct challenge of the middle ear resulted in resolution of signs of ongoing NTHI-induced OM in addition to mediating a marked reduction in resident biomass within the middle ear.
Figure 4.5. Percentage of middle ears with OM in each cohort after immunization via a therapeutic regimen, based on video otoscopy and tympanometry.

*, statistically significant compared to receipt of dmLT alone ($p<0.05$). Resolution of clinical signs of OM was enhanced after receipt of LB1, rsPiiA, chimV4 or LB1+dmLT compared to receipt of dmLT alone. Further, resolution was significantly greater after receipt of rsPiiA+dmLT or of chimV4+dmLT, compared to administration of dmLT alone.
Migration of dendritic cells from the pinnae. Lastly, we examined the migration of dendritic cells (DCs) from the pinna after TCI, to identify potential sites of immune induction after immunization by this route. To do so, the amine-reactive dye CFSE was applied to the pinnae along with each vaccine formulation to allow for discrimination among cells resident within lymphoid tissues and DCs that had migrated to these sites from the pinnae (182, 242). We observed that within one hour after TCI only 13.6% of cells isolated from the NALT of animals immunized with adjuvant alone were CFSE⁺CD11c⁺ (Fig. 4.6A). In contrast, TCI with either rsPilA, LB1 or chimV4 each delivered with dmLT resulted in a 2.5- to 5.4-fold relative increase (37.2%, 34.4% and 72.9%, respectively) in the percentage of CFSE⁺ DCs detected within this lymphoid aggregate (Fig. 4.6B-D). CFSE⁺CD11c⁺ DCs were also detected in the axillary and brachial lymph nodes, although only 1.1- to 2.0-fold more CFSE⁺ DCs were observed in lymph nodes after TCI with any immunogen versus the adjuvant alone (data not shown). No difference in the percentage of CFSE⁺CD11c⁺ DCs within the cervical or mediastinal lymph nodes or spleen of animals that received an immunogen and those immunized with dmLT alone was found at this time point. These data suggested that DCs within the pinnae sampled the NTHI adhesin-derived immunogens that had been applied to the surface of the pinnae and were induced to mature. DC maturation resulted in the preferential migration of these cells to primarily the NALT, and also the axillary and brachial lymph nodes.

Further analysis was performed to distinguish between the dendritic cells types which migrated to the lymphoid tissues. We specifically focused on discrimination between Langerhan’s cells versus dermal DCs, the primary antigen presenting cells found within the skin. A total of approximately 69% of CFSE⁺ cells within the NALT were
positively labeled with antibody directed against DC-SIGN, specific for dermal DCs (94), as opposed to 0.3% of cells which labeled positively for CD207, which is selectively expressed by Langerhan’s cells (324) (Fig. 4.6E). Therefore, these data indicated that the primary antigen-presenting cell involved in induction of the immune responses observed herein were DCs.

To further confirm that the positive signal described above was due to the migration of DCs to lymphoid tissues and not to diffusion of the dye alone through the lymphatics to that site, we labeled bone marrow-derived DCs with CFSE, activated them with chimV4+dmLT or dmLT alone in vitro, then injected the DCs intradermally into the pinnae. One hour later, a population of CFSE+ cells was detected within the NALT after receipt of DCs activated with chimV4+dmLT (Fig. 4.6F), but not when activated with dmLT alone (Fig 6F, inset). Moreover, 4.3% of the cells isolated from the NALT were CFSE+, whereas ≤0.6% was observed within the cervical, axillary and brachial lymph nodes and spleen (data not shown). Thus, the migration of DCs resident within the pinnae after TCI and those instilled into the pinnae after in vitro activation were similar, a result which suggested preferential homing to this local lymphoid aggregate as mediated via local cytokine/chemokine signals. Collectively, our tracking data demonstrated that immunogen-activated DCs within the pinnae migrated primarily to a proximal lymphoid aggregate, the NALT. Moreover, the cell type activated after TCI was the dermal DC, which likely played a critical role in the initiation of the protective immune response observed herein.
Figure 4.6. Migration of DCs from the pinnae to the NALT after TCI and discrimination of cutaneous DC type.

Presence of CD11c+CFSE+ cells in the NALT after TCI with (A) dmLT alone; (B) rsPilA+dmLT; (C) LB1+dmLT or (D) chimV4+dmLT was detected by flow cytometry. Numbers in each box represent the percentage of CFSE+ cells within each sample. (E) CFSE+ cells were identified to be DC-SIGN+ dermal DCs. (F) Bone marrow-derived DCs activated with chimV4+dmLT ex vivo then injected subdermally into pinnae migrated to the NALT, whereas dmLT-activated DCs did not (inset).
Discussion

OM is a prevalent disease of children worldwide and while commonly managed by prescription of antibiotics and surgical intervention where available, the incidence and cost attributed to this disease is substantial (158). Moreover, the emergence of multiple antibiotic resistant bacteria, including strains of NTHI, is cause for concern (342). Thus, immunization against OM has the potential to alleviate this socioeconomic burden by prevention or resolution of disease. At present, a licensed 10-valent pneumococcal capsular conjugate vaccine wherein NTHI Protein D serves as a carrier molecule (Synflorix™), has demonstrated 35.3% protective efficacy against NTHI-induced OM after parenteral immunization of children in a clinical trial in Slovakia and the Czech Republic (262). Although shown as a secondary outcome measure, this trial nonetheless demonstrated, for the first time, that parenteral delivery of a NTHI-derived antigen could provide protection against OM due to NTHI. To increase the modest coverage observed against NTHI-induced OM, examination of additional NTHI-specific targets is needed.

We have focused our vaccine development efforts on two of multiple adhesins expressed by NTHI, OMP P5 and Tfp, and recently utilized an established chinchilla model of viral-bacterial superinfection shown to be predictive of the aforementioned clinical trial outcome (162, 239). Collectively, these pre-clinical data demonstrated that antibody induced by parenteral immunization with NTHI OMP P5- and Tfp-derived vaccine candidates (called LB1, rsPilA and chimV4) provides significant protection against experimental NTHI-induced OM (236). We now sought to expand our vaccine delivery routes to include noninvasive administration regimens. Currently, children in the U.S. receive up to 25 vaccines by injection during the first two years of life (170). This
can be cause for concern by parents who may ultimately delay or refuse immunization of their child. Also, while not of considerable concern in developed countries, the reuse of needles in developing countries poses serious risk for transmission of blood-borne diseases (149, 244). Thus, while vaccination by injection is proven to be extremely effective as a preventative intervention, development of alternative delivery strategies for current or future formulations could potentially serve as equally effective means to induce protective immunity while simultaneously addressing bottlenecks associated with injectable vaccines.

TCI is a simple and noninvasive method for vaccine administration. In addition to the ease of delivery, both systemic and mucosal immunity is induced by this regimen. OM is a disease of the uppermost respiratory tract mucosa, thus the potential to initiate mucosal immunity, particularly in the nasopharynx where NTHI normally reside, holds promise to limit or prevent its onset. We focused on the chinchilla pinnae as an easily accessible site for administration that did not require manipulation, such as shaving, prior to delivery. Moreover, as there is greater appreciation for compartmentalization within the mucosal immune system due to homing of immune cells that result in the most powerful response induced at the site proximal to delivery (57, 131), we wanted to favor development of an immune response in proximity to the NP and middle ear. This premise is supported by work in a murine host, where after TCI, the greatest cytotoxic lymphocyte activity was detected by cells isolated from lymphoid tissues in close proximity to site of administration whereas less activity was observed by cells from distal tissues (29).

A proof-of-concept study demonstrated that TCI induced an immunogen-specific immune response in the chinchilla host, and that bilateral (both pinnae) administration
induced more robust immunity compared to unilateral delivery (one pinna; data not shown). Further, upon challenge with NTHI, we observed clearance of NTHI from the NP and middle ear. Herein, we expanded upon that preliminary study to examine the protection afforded by TCI with the OMP P5-derived candidate LB1, the Tfp-directed candidate rsPilA, and the chimeric OMP P5- plus Tfp-directed immunogen, chimV4 when delivered with a double mutant of *E. coli* heat-labile enterotoxin, a potent adjuvant.

Our current data demonstrated that TCI prior to NTHI challenge with any of the three immunogens induced the production of both immunogen-specific IgA and IgG and while clearance of NTHI was associated with the production of both antibody isotypes, a greater relative quantity of IgG was consistently observed. NTHI possess the *iga* gene which encodes an IgA1 protease, and up to a third of strains have an additional gene (*igaB*) which encodes a second IgA protease (80, 191). Whereas IgA is the conventional antibody isotype detected at mucosal surfaces, mucosal IgG, either produced locally or present via transudation from serum is also known to contribute to protection against mucosal infection (131). We observed a correlation between antibody GMT of ≥160 and enhanced resolution of disease after TCI via use of a preventative or therapeutic regimen. Protection against NTHI-induced OM is primarily antibody mediated, although the contribution of T-cell mediated mechanism cannot be ruled out, as it is required for clearance of NTHI from the rat lung (174). The data presented herein demonstrated that TCI induced the production of antibodies which targeted adhesins expressed by NTHI and as such, was likely the primary mechanism for eradication of the bacterium from the nasopharynx and middle ear.

TCI prior to bacterial challenge with rsPilA, LB1 or chimV4 delivered with dmLT resulted in a rapid reduction in the bacterial load of NTHI within the NP of immune
chinchillas, in addition to a reduction in the percentage of culture-positive animals per cohort, compared to receipt of immunogen or adjuvant alone. Within middle ears, whereas an increase in bacterial concentration is typically observed after direct challenge due to initial bacterial replication (18), only a modest initial increase in CFU NTHI was observed in cohorts that received any of the three immunogens plus dmLT and was followed by a rapid elimination of bacteria from the middle ear. These data clearly demonstrated that TCI with OMP P5 or Tfp-targeted immunogens with dmLT induced an efficacious immune response. Among these three cohorts, differences were observed specific to the time to clear NTHI from the NP and middle ear after challenge. It is known that OMP P5 is constitutively expressed and is important for enabling NTHI to establish colonization in the NP (296). NTHI Tfp are temporally expressed and necessary for NTHI to maintain long-term colonization at this site (154). Moreover, these adhesins are utilized for both adherence and biofilm formation within the middle ear. Thus, the presence of antibody specific to each adhesin, or both adhesins, likely served to prevent NTHI adherence, which facilitated elimination of NTHI from the NP and middle ear upon challenge.

OM can be a chronic and/or recurrent disease, a consequence of biofilms established by NTHI within the middle ear. Based on the efficacy afforded by immunization with the OMP P5- and Tfp-targeted immunogens prior to bacterial challenge, we wondered whether TCI after NTHI challenge might also effectively resolve OM by eradicating an already established NTHI biofilm from the middle ear. Immunization via a therapeutic regimen resulted in a reduction in the signs of OM based on a clinically-relevant scoring system, in addition to overall resolution of an established NTHI biomass in the middle ear as determined by blinded gross examination of the
middle ear space. Whereas a significant reduction in biomass was grossly observed for all immune animals compared to those that received dmLT alone, only cohorts that received rsPilA or chimV4 admixed with dmLT demonstrated a significant reduction in signs of disease as determined by video otoscopy and tympanometry. The two techniques, one of gross examination and the other of observation for signs of disease, do not discredit each other. Rather, they indicated that while TCI via a therapeutic route resulted in reduction of middle ear biomass, MEF had yet to drain through the Eustachian tube or be absorbed by the middle ear mucosa in all animals, although overall signs of OM were reduced. Therapeutic vaccines for control of other viral or bacterial infections have been examined in experimental and clinical trials with promising results (113, 245, 327) and currently in use is a therapeutic canine melanoma vaccine to combat oral melanoma (3). Thus, the strategy to resolve active disease by therapeutic vaccination is promising.

An additional observation from each study presented herein was the enhanced immune response and resultant bacterial clearance by formulation of each immunogen with dmLT, compared to administration of immunogen alone. Derivatives of cholera toxin and *E. coli* heat-labile enterotoxin have shown utility as adjuvants for mucosal and cutaneous immunization regimens, wherein the toxicity associated with mucosal application of each holotoxin is not observed (34). Specific to dmLT, the engineered amino acid substitutions at position 192 inactivates a trypsin cleavage site and at position 211 modifies a potential pepsin cleavage site (311). The resultant molecule is insensitive to proteolysis by either enzyme, as by design. Further, no toxicity is observed in a patent mouse enterotoxicity model (311). While the exact mechanism is
undefined, it is believed that adjuvant activity is an overall outcome of the activation of various cell types, including DCs (90, 179).

We also examined the role of cutaneous DCs in the observed rapid and protective immune response. Our data demonstrated that TCI with the OMP P5- and Tfp- targeted immunogens delivered with dmLT induced the maturation and subsequent migration of DCs resident within the pinnae, a response not observed after application of dmLT alone. As a complimentary approach, DCs activated \textit{ex vivo}, then injected subdermally into the pinnae exhibited similar maturation and migration characteristics to \textit{in vivo}-activated DCs. By each approach, immunogen-activated DCs migrated primarily to the NALT, an immune inductive site (352) likely due to local cytokine/chemokine signaling. Migratory cells within the NALT were identified to be predominantly dermal DCs, not Langerhan’s cells, based on the expression of DC-SIGN \textit{versus} CD207. While not completely defined, it is proposed that Langerhan’s cells are responsible for tolerogenic immunity, \textit{i.e.} to self antigens, whereas dermal DCs are involved in development of protective immunity (195). Moreover, compared to Langerhan’s cells, dermal DCs exhibit faster migration and populate lymph nodes in areas closer to B-cell follicles (166), facilitating antigen presentation to B-cells and initiation of immune responses. Therefore, TCI served to engage dermal DCs within the chinchilla pinnae which facilitated the development of a local and systemic immune response. We continue to examine the contribution of additional innate immune effects to the rapid resolution observed herein.

As we continue to develop TCI as a delivery method, it is important to consider practical application of this method to humans, particularly to very young children. To promote development of a mucosal immune response in the uppermost airway of a
child, it is rational to propose vaccine application to skin behind the ear or back of the
neck. There is evidence that the Waldeyer's tonsillar ring in the neck and pharynx
functions similar to the rodent NALT, thus the potential exists to induce an immune
response within lymphoid tissues proximal to the human nasopharynx and middle ear,
as desired. Further, to facilitate widespread application, the TCI method must be simple
and inexpensive, preferably deviceless, yet able to efficiently deliver the formulation to
the dermal dendritic cells through the stratum corneum. Whereas there are multiple
strategies in development to breach this protective barrier (22), we suggest that direct
application of a gel or cream on to the skin also has the potential to hydrate the stratum
corneum, restrict diffusion of the antigen and promote long-term sampling by DCs.

In summary, TCI served to engage dermal DCs within the chinchilla pinnae
which resulted in the development of a local mucosal, in addition to systemic, immune
response. Administration of the NTHI OMP P5- and Tfp-directed immunogens admixed
with dmLT was efficacious to both prevent experimental NTHI-induced OM and to
resolve active OM. These data therefore demonstrated that TCI is an effective way to
immunize against experimental NTHI-induced OM and has potential application to
prevent other diseases of the respiratory tract due to NTHI. Moreover, the reduced
costs associated with non-invasive routes of immunization such as TCI hold great
promise in terms of expanding the use of vaccines to prevent OM beyond the
boundaries of developed countries.
Chapter 5

Summary

OM is the most common disease of childhood, affecting 60-330 million children worldwide (109). With costs exceeding $5 billion annually in just the United States alone, this disease exerts a substantial burden on the child, caretaker and health care system (7, 93, 158, 231). While morbidity and quality of life is an issue worldwide, mortality is typically only experienced in developing countries (36, 295, 340).

NTHI is often referred to as an opportunistic pathogen, it exists within the human nasopharynx as a commensal microorganism, however under specific conditions, is able to cause multiple diseases of the upper and lower respiratory tracts, such as COPD, cystic fibrosis, chronic bronchitis, sinusitis, conjunctivitis and OM (25, 74, 219, 287). NTHI has an impressive armament at its disposal for adaptation to its environment: multiple mechanisms to acquire iron in the nutrient limited conditions of its host, numerous adhesins to facilitate attachment and colonization of the respiratory epithelium, mechanisms to evade host clearance such as IgA and matrix proteases, ability to decorate LOS with host molecules, possession of multifunctional transporters to degrade host defense peptides and the ability to form highly resilient biofilms, to name a few (88, 217, 255, 303).

While NTHI is one of the predominant causative agents of OM, its presence within the middle ear does not ultimately benefit the organism. The healthy middle ear is a sterile site and likely due to the proximity to the inner ear and brain, the presence of an
endotoxin-expressing organism in this space elicits a robust immune response against the offending organism (217). As the ET serves as the conduit from the NP to the middle ear, this organ also has mechanisms to protect the middle ear. Mucus and serous secretions trap particulate matter, which is pushed by ciliary action to the proximal or nasopharyngeal orifice of the ET for subsequent clearance by swallowing (183, 185). Moreover, the ET epithelium expresses receptors such as TLR 2 and 4 to detect the presence of bacterial or viral agents in proximity to the privileged site (300). An example for spatial distribution is shown such that relative to the middle ear, TLR4 expression is greatest at the distal half of the ET, whereas host defense peptide expression is greatest within the proximal half, closest to the site of NTHI colonization (13).

Healthy individuals can maintain a ‘colonizing load’ of NTHI within the NP (204); however several factors can tip the balance in favor of the bacterium. Environmental, behavioral and genetic risk factors can preclude to development of OM (40, 115, 276). Moreover, dysregulation of host defenses, for example due to viral URTI can similarly favor unrestricted growth of NTHI within the NP, ascension through the ET, and entry into the middle ear (13, 122, 277). Those most at risk for development of OM are children <7 years of age due to both immunological and craniofacial immaturity (30, 280, 291). Although a period of ‘watchful waiting’ is now recommended by the American Academy of Pediatrics for otherwise healthy children with OM, prior overuse of antimicrobials has resulted in the emergence of multiple antibiotic-resistant organisms (1, 2). The most effective way to manage OM is to prevent its onset.

Many labs have focused vaccine development efforts on adhesins expressed by NTHI with the goal to limit or prevent adherence of the organism to the respiratory
mucosae. While there are multiple outer membrane protein targets on the organism, there are several tenets to rational vaccine design. The adhesin must be conserved, expressed by all strains, be surface-exposed and be the target of functional antibody (11). Toward that end, the most promising vaccine candidates to date target NTHI Tfp, OMP P6, OMP P5, OMP26 and Protein D (11, 51, 260). Currently, Protein D is most advanced in terms of licensure as a protein carrier in the pediatric pneumococcal conjugate vaccine, Synflorix™ (263). The remaining candidates are still in development.

To overcome technical difficulties with endotoxin contamination, protein purification or yield, or to focus the resultant immune response on to a protective epitope, each of the aforementioned NTHI OMPs has been adapted from their native form. OMP P6 and OMP26 are currently tested as recombinant proteins or presented in expression systems such as bacterial ghosts (73, 136, 176, 269). Epitope mapping studies revealed immunodominant epitopes within Tfp PilA and OMP P5, such that candidates derived from each protein are subsets of the total molecule and elicit protective immune responses in vivo (236, 237, 240). Moreover, the mapping studies aided in the design of a novel chimeric immunogen wherein protective epitopes of OMP P5 and PilA are optimally presented, therefore two NTHI adhesins are targeted with one molecule (236). The latter three immunogens are the focus of work presented herein.

In a chinchilla passive transfer superinfection model the OMP P5 and Tfp-directed candidates [called LB1 and rsPilA, respectively] and a LB1-rsPilA chimeric immunogen [called chimV4], have demonstrated significant protective efficacy against the development of experimental NTHI-induced OM (18, 162, 236). As we continue development of each candidate with the goal for clinical application, we also wanted to examine alternate delivery strategies. In the first two years of life, children can receive
up to 25 vaccines by injection, and multiple vaccines are administered at each visit (170). This can be cause for concern by parents, and may result in non-compliance with the recommended pediatric vaccine schedule. Moreover, while highly effective for prevention of multiple diseases, there is concern regarding possible adverse effects or interference as multiple antigens and carrier molecules are administered to this young population (59).

We therefore expanded our vaccine design strategies to include intranasal and transcutaneous immunization regimens. There are multiple benefits to noninvasive immunization routes, which include the simplicity and noninvasive nature of delivery, reduced cost as syringes, needles and trained medical professionals are not required to deliver the vaccine and the prospect for greater vaccine distribution beyond developed countries due to typically cheaper production costs (105). Moreover, each route induces both systemic and mucosal immune responses, locally and at sites distal to administration (61, 64, 131, 252). The development of a mucosal immune response is an important consideration as NTHI causes multiple diseases of the respiratory mucosa, including OM.

In chinchilla models of experimental OM, we examined the efficacy afforded by each noninvasive immunization route. Compared to parenteral immunization, immunogen-specific antibody titers were several thousand-fold less after IN or TCI with LB1, rsPilA or chimV4 (18). The immunization regimens employed here are shown to yield lower antibody titers, compared to parenteral immunization (57, 58). However, as the immune responses elicited by each route was protective, these data suggested and demonstrated that the antibody induced effectively bound its respective target on NTHI. Importantly, each noninvasive regimen elicited the production of specific systemic and
mucosal antibody, as by design. An additional observation was the predominance of antibody of an IgG isotype. All NTHI strains possess at least one IgA protease, and up to a third of strains have a gene that encodes a second, thus the greater relative quantity of IgG compared to IgA was not unexpected (80, 191).

IN immunization, regardless of regimen [parenteral prime, followed by IN boost with LB1+MPL or exclusively IN immunization with LB1+MPL], resulted in fewer animals with signs of OM in each cohort, compared to the respective adjuvant only cohort. Moreover, within six days after challenge no middle ear fluids were visible by video otoscopy and tympanometry in animals immunized exclusively IN with LB1+MPL, a significant response compared to animals that received only adjuvant. Therefore IN immunization with LB1 induced an effective immune response to facilitate resolution of signs of experimental OM.

TCI with LB1, rsPilA or chimV4 plus dmLT induced NTHI middle ear clearance kinetics previously not observed with traditional parenteral immunization strategies. Animals that received any of the three immunogens admixed with dmLT exhibited a modest increase in CFU NTHI per ml MEF three days after direct challenge of the middle ear, relative to the inoculum. Plus, the bacterial counts obtained were representative of MEF present in only 1 or 2 middle ears within a total of 20 ears in each cohort. Therefore TCI with any of the three immunogens admixed with dmLT induced rapid elimination of NTHI and resolution of experimental OM.

To begin to understand the mechanism for the efficacy observed, we focused on the potential role of DCs, either present within the nasal mucosa for IN immunization or within the pinnae dermis for TCI. One of the first aims for this research was to confirm the presence of chinchilla-specific DCs. While it was intuitive that this rodent possessed
DCs, there were no reports in the literature for culture of the specialized antigen-presenting cells. Using rat- and human-specific reagents, we induced the differentiation of precursor cells from chinchilla bone marrow to DCs and confirmed by phenotype and functional assays that the resultant cells exhibited characteristics comparable to DCs from other species. The resultant cells were utilized in vivo for tracking assays to identify potential immune inductive sites after application of ex vivo activated DCs into the nasal cavity or pinnae. Additionally, DCs resident within each site were tracked to examine the migration of exogenous DCs after immunization of the chinchilla host.

Both noninvasive immunization regimens induced the migration of DCs, activated ex vivo or endogenous to the site of immunization, to primarily the NALT. Moreover, the exogenous DCs detected within the NALT after TCI were shown to express DC-SIGN, which indicated that the primary antigen-presenting cell involved in the induction of the immune response was the dermal DC and not the Langerhan’s cell. Langerhan’s cells play a role in tolerogenic immune responses, whereas dermal DCs are involved in protective immunity, therefore our observations are supported by known functions for each cell type (195).

Tracking DCs to the NALT after immunization indicated that this lymphoid aggregate may serve as the inductive site for immunization regimens that target the head, as shown here. As the rodent NALT is reported to have functional similarities to the human Waldeyer’s lymphoid ring of the neck and pharynx, the ability to target lymphoid tissues proximal to the site of colonization in the NP or infection within the middle ear is promising for the prevention of NTHI-induced OM (39, 61, 173, 302, 347). Moreover, the finding that one may eradicate an established NTHI biofilm from the chinchilla middle ear after TCI with adhesin-targeted immunogens and the reduction in
clinically-relevant signs of OM holds promise for continued development of therapeutic immunization strategies against NTHI-induced OM.

Future work includes understanding the signals which guide DCs to the NALT and the interaction with T-cells within this tissue after TCI. Moreover identify the mechanisms for the rapid biomass resolution achieved only seven days after the second weekly immunization with a focus on the involvement of innate immune effectors. Additionally, continue to refine TCI in both preventative and therapeutic immunization approaches to improve upon the protection observed herein.

In summary, the adaptation of established cellular immunology techniques to work performed in chinchilla models offers to enhance the overall understanding of the mechanisms for the observed protection against experimental OM. These data demonstrated that IN immunization and TCI with OMP P5- and Tfp-targeted immunogens were efficacious against experimental NTHI-induced OM. Moreover, the development of noninvasive immunization strategies and socially responsible application of this technology has the potential to expand the use of vaccine to prevent OM, and perhaps other diseases of the respiratory tract due to NTHI, beyond the boundaries of developed countries.
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