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Kinetic analysis and evaluation of the mechanisms involved in the resolution of experimental nontypeable *Haemophilus influenzae*-induced otitis media after transcutaneous immunization

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Abstract

Transcutaneous immunization (TCI) is a simple and needle-free method with which to induce protective immune responses. Using a chinchilla model of nontypeable *Haemophilus influenzae* (NTHI)-induced otitis media (OM), we examined the efficacy afforded by TCI with a novel chimeric immunogen called 'chimV4' which targets two critical adhesins expressed by NTHI, outer membrane protein P5 and the majority subunit of NTHI Type IV pilus, PilA. Experimental OM was first established in cohorts of animals, and then TCI performed via a therapeutic immunization regime by rubbing vaccine formulations on hydrated pinnae. The kinetics of resolution of established experimental disease was evaluated by clinically-relevant assessments of OM, bacterial culture of planktonic and adherent NTHI within the middle ear and gross examination of the relative amount of NTHI biomass within the middle ear space. Within seven days after primary TCI, a significant reduction in the signs of OM, significantly fewer NTHI adherent to the middle ear mucosa and significant resolution of mucosal biofilms was detected in animals that received chimV4 plus the adjuvant LT(R192G-L211A), compared to animals administered LT(R192G-L211A) alone or saline by TCI ($p < 0.05$) with eradication of NTHI within an additional seven days. The mechanism for rapid disease resolution involved efflux of activated dermal dendritic cells from the pinnae after TCI, secretion of factors chemotactic for CD4⁺ T-cells, induction of polyfunctional IFN γ - and IL-17-producing CD4⁺ T-cells and secretion of host defense peptide within the middle ear. These data support TCI as a therapeutic intervention against experimental NTHI-induced OM and begin to elucidate the host response to immunization by this noninvasive regimen.

Keywords

chimV4; OMP P5; Type IV pilus; chinchilla; mucosal biofilm; dendritic cell; dmLT

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Introduction

Otitis media (OM) is a common disease of childhood. It is estimated that 709 million cases of acute OM and 65–330 million episodes of chronic secretory OM occur each year worldwide, with the greatest burden of disease experienced by children <4 years of age [1, 2]. While mortality due to OM is not common in developed countries, the morbidity associated with OM is significant for all children. One complication of OM is hearing loss, an outcome that is associated with developmental delays in behavior, language, and education of this very young population [3–6]. Moreover, chronic OM is difficult to resolve, and may require prolonged treatment with an antimicrobial, which is of concern due to the emergence of multiple antibiotic-resistant bacteria in all three genera most predominant in OM [7, 8]. Thus, development of means to prevent or treat OM is of tremendous importance.

One of the primary bacterial agents that cause OM, and multiple diseases of the respiratory tract, is nontypeable *Haemophilus influenzae* (NTHI). This organism possesses numerous determinants that facilitate its persistence as a commensal inhabitant of the human nasopharynx, and under appropriate conditions these and other factors may be utilized to establish and exacerbate disease in other anatomical sites, such as the lung during episodes of bronchitis and chronic obstructive pulmonary disease, and the middle ear during OM. Our laboratory has focused many of its vaccine development efforts on two adhesins expressed by NTHI, outer membrane protein P5 (OMP P5) and the Type IV pilus (Tfp), proteins known to be critical for NTHI adherence to respiratory epithelial cells and for the establishment of biofilms [9–12]. We have designed two immunogens which target each protein individually [13, 14], and more recently, a single novel chimeric immunogen which targets both adhesins. This latter immunogen, called ‘chimV4’, is comprised of a truncated variant of mature PilA (the majority subunit of NTHI Tfp), which serves as immunogen and carrier for a 24-mer immunodominant and protective epitope derived from the N-terminal half of OMP P5 [13, 15–17]. Antibody directed against this 18 kDa recombinant chimeric protein demonstrates significant protective efficacy against NTHI-induced OM in a chinchilla model of viral-bacterial synergy [13]. Moreover, when administered via transcutaneous immunization (TCI), chimV4 admixed with the adjuvant LT(R192G-L211A), a double mutant of *E. coli* heat-labile enterotoxin (abbreviated ‘dmLT’) [18, 19], exhibits significant efficacy when utilized in preventative and therapeutic immunization regimes in experimental models of NTHI-induced OM [20].

TCI offers multiple advantages as an immunization strategy; it is noninvasive which may aid in patient acceptance and compliance; there are reduced costs associated with vaccine production and administration by this regimen as delivery devices may be simplified or eliminated, trained medical personnel are not required and the potential for dose-sparing could allow for wider vaccine distribution beyond developed countries [21–23]. TCI is known to induce both systemic and mucosal immune responses [24–26], an important feature as the mucosae represent a critical physical defensive barrier that can also respond immunologically to insult [27]. In both animals and humans, TCI with bacterial or viral proteins, toxoids, peptide antigens and nanoparticles is shown to induce the production of antigen-specific antibody and functional T-cell responses [28–31]. In animal models, protection is noted against subsequent bacterial or toxin challenge [32–35]. Clinical trials have also demonstrated the production of antigen-specific antibody and activated effector T-cells after administration of bacterial toxins, inactivated or live viruses; and although efficacy against subsequent challenge varies among these published reports, safety profiles indicate this route of immunization is well-tolerated [36–40]. Therefore, TCI exhibits potential as an efficacious and simple method to induce protective immune responses and therefore limit disease.

TCI engages the numerous antigen presenting cells resident within the dermis and epidermis of the skin, the dermal dendritic cells (DCs) and Langerhans cells, respectively [22, 25]. Whereas each cell type is capable of antigen uptake and presentation, it is proposed that Langerhans cells are primarily retained within the epidermis and are responsible for tolerogenic immunity to self-antigens and environmental stimuli, whereas dermal DCs exhibit greater migratory function and ability to stimulate T-cells to induce protective immunity [41–43]. Moreover, TCI can facilitate robust immune responses in close proximity to the site of administration, facilitated by homing of activated immune cells to nearby lymphoid tissues [44–46]. Previous work by our laboratory examined the migration of cutaneous DCs after application of NTHI OMP P5- and Tfp-targeted immunogens admixed with dmLT onto intact chinchilla pinnae (the outer, visible part of the ear). Within one hour, pinnae-derived dermal DCs that had taken up vaccine antigens were detected within the nasal-associated lymphoid tissue (NALT), a notable observation as this lymphoid aggregate is situated in close proximity to the middle ear [20, 47]. We further show that TCI with chimV4 plus dmLT promotes resolution of ongoing NTHI-induced OM within 14 days after primary immunization, as demonstrated by both a clinically-relevant tympanometric assessment and visualization of limited to no residual mucosal biofilms within the middle ear space [20]. As mucosal biofilms within the middle ear are a serious complicating factor which facilitates the chronicity and recalcitrance of OM [48, 49], the ability to administer NTHI adhesin-targeted immunogens in a regime that promotes local and robust immune responses has great potential to reduce the burden of this prevalent disease.

Herein, we begin to explore the mechanisms for the observed rapid resolution of established NTHI-induced OM after TCI with chimV4 plus dmLT in a chinchilla model. The kinetics of disease resolution was assessed over a period of 14 days via multiple parameters. Video otoscopy and tympanometry were performed as clinically-relevant evaluations of the incidence and severity of OM in addition to examination of mucosal biofilms within the middle ear to rank disease severity grossly. Culture of NTHI from middle ear fluids and mucosal biofilms provided an assessment of the bacterial load within both the planktonic and adherent populations, respectively, over time. The quantity of immunogen-specific antibody in middle ear fluids was determined to identify the point at which an adaptive immune response contributed to disease resolution. Moreover, as it became apparent that innate immune elements within the middle ear mucosa played a role early in this disease resolution process, the relative amount of the host defense molecule chinchilla β -defensin-1 (cBD-1, an orthologue of human β -defensin 3), was examined. Lastly, the functional activation of dermal DCs induced by TCI of chinchilla pinnae and the phenotype of the resultant CD4⁺ T-cell response was analyzed. Collectively, these data supported a proposed model wherein TCI with a chimeric immunogen directed against two critical NTHI adhesins and delivered with a potent adjuvant stimulated the activation and migration of dermal DCs to the NALT, the expansion and differentiation of CD4⁺ T-cells to a polyfunctional phenotype and subsequent production of specific antibody to facilitate the eradication of NTHI from the middle ear and resolution of both established mucosal biofilms resident within the middle ear and active disease.

2. Materials and Methods

2.1 Animals

Sixty-one adult chinchillas (*Chinchilla lanigera*; Rauscher's Chinchilla Ranch, LaRue, Ohio; mean mass 550 ± 12 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 3 cohorts of 20–21 chinchillas each. Animal care and all procedures were performed in concordance with institutional and federal guidelines, and were conducted under an approved protocol.

2.2 Immunogen and adjuvant

ChimV4 is a novel, chimeric immunogen wherein a modified and N-terminally truncated form of PilA, the majority subunit of NTHI Tfp, serves as an immunogenic carrier for 24 amino acids of a B-cell epitope from surface-exposed region 3 of NTHI OMP P5 [13]. 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. The amino acid substitutions render dmLT nontoxic while maintaining adjuvant properties [18, 19, 50].

2.3 Bacterial 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, a rat model of pulmonary clearance and a murine model of OM [51–58].

2.4 NTHI challenge and transcutaneous immunization regimen

Chinchillas were challenged with NTHI exclusively transbullarily with 1000 CFU NTHI strain 86-028NP delivered in 0.3 ml sterile, pyrogen-free 0.9% sodium chloride (Hospira, Inc., Lake Forest, IL) per bulla and the challenge dose was confirmed by plate count. This challenge model is shown to result in the formation of a mucosal biofilm in >83% of middle ears within four days [20, 59]. Four days after NTHI challenge (day 0), all animals were immunized by TCI. The pinnae of alert animals was first hydrated by placement of gauze soaked in sterile, pyrogen-free 0.9% sodium chloride on the inner surface for 5 min. Pinnae were then blotted with dry gauze and 50 µl of each vaccine formulation was applied to the center of the inner face of each pinna using a pipet. The pinnae were then folded in half and opposing surfaces gently rubbed together [20, 59]. Formulations consisted of 10 µg chimV4 admixed with 10 µg dmLT, 10 µg dmLT alone or 50 µl pyrogen-free 0.9% sodium chloride and were delivered twice at a weekly interval (day 0 and day 7).

2.5. Otoscopy and tympanometry

Video otoscopy using a 0°, 3-in. probe connected to a digital camera system (MedRx, Largo, FL) was utilized to monitor signs of tympanic membrane inflammation and/or presence of fluid within the middle ear space. Middle ear pressure, tympanic membrane compliance and tympanic width were monitored via tympanometry using a MADSEN OTOflex 100 (GN Otometrics, Schaumburg, IL). Overall signs of OM were blindly rated on a scale of 0 to 4+ as previously described [20, 51–53]. Middle ears with a score of ≥ 2.0 were always considered positive for otitis media as middle ear fluid (MEF) was visible behind the tympanic membrane. Each middle ear was considered independent (n=4 middle ears per cohort on day 0, n= 6 middle ears per cohort for days 3 through 14), and for each cohort, the percentage of middle ears with OM was calculated.

2.6 Collection of samples

On days 0, 3, 5, 7, 9, 11 and 14 after primary TCI, three chinchillas from each cohort were sacrificed. MEF was collected by epitympanic tap, serially diluted and plated on to chocolate agar to semi-quantitate CFU planktonic NTHI. Fluid from each middle ear was considered independent (n=4 middle ears per cohort on day 0, n= 6 middle ears per cohort for days 3 through 14), and for each cohort, the mean CFU NTHI/ml middle ear fluid ± SEM was presented. Middle ear mucosal biofilm, if present, was collected after evaluation as described below, homogenized and plated on to chocolate agar to semi-quantitate the CFU NTHI adherent to the middle ear mucosa. Each middle ear was considered independent

(n=4 middle ears per cohort on day 0, n= 6 middle ears per cohort for days 3 through 14) and for the cohort, the mean CFU NTHI/mg tissue $\pm$ SEM was presented.

2.7 Evaluation of mucosal biofilms within the middle ear

Upon sacrifice, the inferior bullae from each animal were dissected, opened to reveal the middle ear space and washed with 1.0 ml sterile, pyrogen-free 0.9% sodium chloride to remove residual MEF and loosely adherent bacterial biofilm. The bullae and remaining adherent mucosal biofilm were then imaged with a digital camera. Each image was blindly ranked on the relative amount of residual mucosal biofilm using a 0 to 4+ scale, wherein 0= no mucosal biofilm; 1= mucosal biofilm fills <25% of middle ear space; 2= mucosal biofilm fills 25–50% of middle ear space; 3= mucosal biofilm fills 50–75% of middle ear space and 4= mucosal biofilm fills 75–100% of middle ear space [20, 59]. The mean mucosal biofilm score $\pm$ SEM for the cohort (n=4 middle ears per cohort on day 0, n= 6 middle ears per cohort for days 3 through 14) was reported.

2.8 Enzyme-linked immunosorbent assays

To determine the relative quantity of immunogen-specific IgG and IgA in clarified, pooled MEFs, endpoint ELISA was performed. Samples were incubated in chimV4-coated wells (0.2 μ g protein/well) for 3 hr at 25°C and bound 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). Endpoint 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) with 95% confidence intervals.

To quantitate the host defense peptide cBD-1 within mucosal homogenates collected on day 3, a sandwich ELISA was performed. Clarified homogenates of middle ear mucosa and mucosal biofilm were incubated in wells of a Microfluor 2 black U-bottom microtiter plate (Thermo Scientific, Rochester, NY) coated with Protein G-purified rabbit anti-recombinant cBD-1 (0.5 μ g antibody/well) [60] overnight at 4°C. Host defense peptide was detected by addition of FITC-conjugated purified rabbit anti-recombinant cBD-1. The concentration of cBD-1 in each mucosal sample was calculated by comparison of the mean fluorescence of each well versus a standard curve generated with purified recombinant cBD-1. The mean concentration of cBD-1 $\pm$ SEM for each cohort from three independent assays is reported.

2.9 Functional responses of dermal DCs after TCI

To evaluate functional responses of cutaneous DCs induced by TCI, we examined the efflux of dermal DCs from the chinchilla pinnae and secretion of immune effectors [61]. Three hr after TCI with chimV4 admixed with 10 μ g dmLT, 10 μ g dmLT alone, or sterile, pyrogen-free 0.9% sodium chloride, three chinchillas per formulation were sacrificed and both pinnae removed, placed in 70% v/v ethanol for 5 min and transferred to sterile petri dishes to dry. The pinnae were trimmed to 2.5 cm², the dorsal and ventral faces peeled apart, cartilage removed and the pinnae placed on to 8 μ m pore size Transwell membranes. Two milliliters of RPMI 1640 (Corning cellgro, Manassas, VA) plus 0.5% w/v bovine serum albumin (Sigma Aldrich) were added to the basolateral chamber and incubated for 20 hrs at 37°C, 5% CO₂, in a humidified atmosphere. Cells that had migrated into the basolateral chamber were separated based on expression of CD11c⁺ using MACS magnetic microbeads (Miltenyi Biotech, Cambridge, MA) then incubated with antibody directed against CD11b (eBiosciences, Inc., San Diego, CA) and DC-SIGN (R&D Systems, Minneapolis, MN) for discrimination of dermal DCs. The number of dermal DC per ml culture medium was determined as the number of events detected within a 25 μ l volume using a calibrated

Accuri C6 flow cytometer (BD Biosciences, Sparks, MD). Each pinna was considered independent and the mean $\pm$ SEM for each cohort presented (N=6 pinnae per cohort).

The clarified supernatants from each of the six cultured pinnae per cohort described above were applied to Proteome Profiler Human cytokine array kit (R&D Systems) and assayed following the manufacturer's instructions. Relative pixel intensity was determined with a BioRad GS800 densitometer and analyzed with Quantity One software (BioRad, Hercules, CA). The fold increase in pixel intensity between pinnae collected from chinchillas administered dmLT alone or chimV4+dmLT was compared to pinnae administered saline.

2.10 Determination of T-cell phenotype

We have previously shown that dendritic cells migrate from the pinnae to the chinchilla NALT after TCI of the chinchilla pinnae [20], indicating that this lymphoid aggregate may serve as an immune inductive site. To examine the phenotype of the immune response induced following TCI, we examined cytokine production by CD4⁺ T-cells within the NALT. One week after receipt of the second immunizing dose as described above, the NALT was collected from three chinchillas per cohort; homogenized individually using GentleMACs dissociator (Miltenyi Biotech) and CD3⁺ cells isolated using MACS magnetic microbeads. A total of 1×10^6 cells/0.5 ml RPMI 1640 plus 0.5% w/v bovine serum albumin was incubated with Leukocyte activation cocktail plus GolgiPlug (BD Biosciences) for 5 hr at 37°C, 5% CO₂ in a humidified atmosphere prior to stain with human Th1/Th2/Th17 phenotyping kit according to manufacturer's instructions (BD Biosciences). 20,000 CD4⁺ lymphocytes were acquired using an Accuri C6 flow cytometer and data analyzed with FloJo software (Tree Star, Inc., Ashland OR). One of three representative assays is presented.

2.11 Statistical analyses

Data analyses were performed using GraphPad Prism v. 5.01 (La Jolla, CA). Statistical differences among cohorts in bacterial concentration within MEFs and mucosal biofilms, antibody titers and dendritic cell migration were determined using Kruskal-Wallis one-way analysis of variance on ranks and Dunn's method for multiple comparisons. A *p*-value of 0.05 was considered significant. Significant differences in the percentage of middle ears with OM and differences among mucosal biofilm scores were analyzed by repeated measures analysis of variance and Bonferroni's multiple comparison test. A *p*-value of 0.05 was considered significant.

3. Results

3.1 Resolution of established experimental otitis media

As a clinically-relevant assessment for the resolution of established experimental OM, each tympanic membrane was examined to document signs of disease including erythema, the presence of middle ear fluid behind the tympanic membrane and changes in tympanometric read outs. As expected, on day 0, 100% of middle ears (122/122) were positive for the presence of MEF behind the tympanic membrane as each middle ear had been directly inoculated with NTHI four days prior (Fig. 1). Within three days after receipt of the first immunizing dose, a 33% (2/6 middle ears) reduction in OM was observed after receipt of the adjuvant dmLT alone, however, within seven days after administration of the primary dose, the percentage of animals with OM increased to 100% (6/6 middle ears). The reduction in the incidence of OM after receipt of dmLT suggested that an initial, non-specific immune response was induced following administration of this potent adjuvant, a trend observed again after receipt of the second immunizing dose on day 7. Delivery of chimV4 admixed with dmLT resulted in a greater reduction in the percentage of middle ears

with OM to 50% (3/6 middle ears) three and five days after receipt of the first dose, statistically significant results compared to receipt of saline ($p < 0.05$). Moreover, seven days after primary TCI with chimV4 admixed with dmLT only 17% (1/6 middle ears) demonstrated signs on OM and although this percentage increased to 33% (2/6 middle ears) on day nine, receipt of the second immunizing dose subsequently induced rapid and complete resolution of experimental disease. On each day after receipt of the second immunizing dose, significantly fewer middle ears in the cohort that received chimV4+dmLT exhibited signs of experimental OM, compared to either cohort that received saline or dmLT alone ($p < 0.05$). These results indicated that incorporation of chimV4 with dmLT facilitated an enhanced and NTHI-targeted immune response. Moreover, these data demonstrated that delivery of chimV4+dmLT induced an immune response in which clinically-relevant signs of OM were abrogated by 50% within three days after administration and experimental disease had resolved within 14 days.

3.2 Eradication of NTHI from middle ear fluids

To examine clearance of planktonic NTHI after TCI, MEFs were collected when their presence was indicated by video otoscopy and cultured to determine the relative concentration of bacteria within these fluids. On day 0, four days after direct challenge of middle ears with 1000 CFU NTHI, and prior to administration of any vaccine formulation, the inoculum had multiplied to 9.7×10^6 to 1.1×10^7 CFU/ml in all animals (Fig. 2), as is expected in this challenge model [20, 51]. No significant difference in bacterial concentration was observed among the three cohorts until one week after receipt of the primary immunizing dose. Whereas beginning on day 3 we detected a significant 50% reduction in middle ears with signs of OM in the cohort that received chimV4+dmLT by video otoscopy and tympanometry (see Fig. 1), the bacterial concentration within the MEFs of chinchillas observed to have OM ranged from 2.4×10^7 to 5.0×10^7 CFU/ml and did not allow for statistical discrimination from the cohorts administered saline or dmLT only (1.0×10^7 to 5.3×10^7 CFU/ml). However, as shown in Fig. 2, beginning seven days after primary TCI, and specifically upon receipt of the second immunizing dose, a rapid and significant 6-log reduction in NTHI concentration within middle ear fluids of animals administered chimV4+dmLT was observed, compared to receipt of saline or dmLT alone ($p < 0.05$), with complete eradication of NTHI from the middle ears of animals in this cohort by day 14. These data demonstrated that TCI with chimV4+dmLT induced an immune response that effectively contributed to the elimination of planktonic NTHI within middle ear fluids, a response that was most effective after administration of the boosting dose.

3.3 Resolution of established mucosal biofilms from the middle ear after direct challenge

To evaluate the kinetics of mucosal biofilm resolution afforded by TCI, the middle ears of all chinchillas were inoculated with NTHI and a robust biofilm allowed to form. In this model, >83% of middle ears develop a mucosal biofilm that fills 75–100% of the middle ear space within four days [20, 59]. Following immunization, any residual NTHI biofilm was evaluated and ranked on a 0 to 4+ scale wherein a score of 0 indicated that no mucosal biofilm was present and 4+ designated that 75–100% of the middle ear space contained an NTHI mucosal biofilm. As shown in Fig. 3, on day 0 (four days after direct challenge of the middle ear), the mean mucosal biofilm scores for each of the three cohorts ranged from 3.7 to 4.0, which indicated that 75–100% of the middle ear space of all animals contained a mucosal biofilm, as expected for this model. No change in rank was observed in the cohort that received saline, as a mean score of 3.0 was maintained for the duration of the study. Receipt of dmLT alone, however, afforded a 25% reduction in mucosal biofilm with scores of 2.3 to 2.8 detected after receipt of the second dose. These data provided additional evidence to support the induction of a nonspecific immune response by this powerful adjuvant.

Notably, administration of chimV4 with dmLT resulted in a statistically significant reduction in mucosal biofilm from the middle ear five days after primary TCI, compared to receipt of saline ($p < 0.01$). At this time point, the mean biofilm score equaled 2.3 ± 0.2 and indicated that approximately 50% of the middle ear space contained a mucosal biofilm. Over time, biofilm scores for this cohort consistently decreased; seven days after primary TCI, a rank of 1.7 ± 0.2 was achieved (25–50% of the middle ear space had a mucosal biofilm) and by day 14, 75% of the biofilm was eliminated ($p < 0.01$ compared to saline and dmLT cohorts). These data demonstrated that TCI with chimV4+dmLT was effective to stimulate the resolution of an established mucosal biofilm from the middle ear.

3.4 Eradication of NTHI from established mucosal biofilms in the middle ear

To examine the relative concentration of NTHI resident within a biofilm adherent to the mucosa in the middle ear, compared to the planktonic population available for culture from MEFs as described in Fig. 2, the middle ear mucosa and accompanying NTHI biofilm was collected, homogenized and cultured. Four days after direct inoculation of the middle ear, the mean bacterial concentration of NTHI was equivalent among the three cohorts as expected and ranged from 5.3×10^3 to 1.8×10^4 CFU NTHI/mg mucosa (Fig. 4). Within three days after primary TCI, significantly fewer NTHI were detected in mucosal homogenates from animals administered either dmLT alone or chimV4+dmLT (8.5×10^2 and 1.5×10^3 CFU/mg mucosa, respectively), compared to saline (3.5×10^4 CFU; $p < 0.05$). Moreover, the latter cohorts exhibited a 10-fold reduction in CFU NTHI relative to three days prior, influenced by administration of dmLT, as the two cohorts that received this adjuvant were distinct from the cohort administered saline, yet not significant from each other. Furthermore, this result could not likely be attributed to development of adaptive immune response, as only three days had passed since primary immunization and no immunogen-specific antibody was yet detectable within MEFs, as described below. Over the following four days, the cohort that received dmLT alone maintained a bacterial concentration of $1.5\text{--}1.8 \times 10^3$ CFU NTHI/mg mucosa, however middle ears from chinchillas immunized with chimV4+dmLT exhibited a sequential 11- and 24-fold reduction in CFU NTHI on days 5 and 7 after primary TCI, respectively ($p < 0.05$ compared to animals administered saline on days 5 and 7; $p < 0.05$ compared to animals administered dmLT alone on day 7 only). Moreover, within the latter 7 days of the study, only 10 to 30 CFU NTHI/mg tissue were detected ($p < 0.05$ compared to receipt of saline; $p < 0.05$ compared to receipt of dmLT alone on day 14 only). Thus, while TCI with dmLT alone appeared to stimulate an initial reduction in CFU NTHI within mucosal biofilms in the middle ear, the combined influence of chimV4+dmLT yielded consistently fewer NTHI at each subsequent time point.

3.5 Analysis of immunogen-specific antibody and cBD-1 in MEFs

To begin to examine the mechanisms for the protective efficacy observed, we examined the relative quantity of immunogen-specific IgG and IgA within MEFs by ELISA. ChimV4-specific IgG within MEFs collected from animals immunized with chimV4+dmLT was detected at levels above background beginning 5 days after primary TCI (GMT= 80; Table 1). Whereas a slight, but non-significant decrease in titer was observed on day 7, receipt of the second immunizing dose induced a 2.5-fold increase in specific IgG to a GMT of 160, which was maintained for an additional two days ($p < 0.05$ compared to day 0). By day 14, seven days after receipt of the second immunizing dose, the GMT of chimV4-specific IgG in the middle ear equaled 190 ($p < 0.05$ compared to day 0). Whereas chimV4-specific IgA was also detected within MEFs beginning five days after primary TCI, the relative quantity of this antibody isotype was 2.0- to 4.6-fold less than IgG at each time point tested and no significant increase in quantity of IgA was achieved relative to pre-immunization values (Table 1). Previously, we showed that the ability to eradicate NTHI from the middle ear was associated with a GMT value of at least 160 for IgG [20]. Review of the kinetics of NTHI

clearance from the MEF of chinchillas immunized with chimV4+dmLT (Fig. 2) further supports this observation as on days 9 through 14, time points at which the GMT of chimV4-specific IgG 160, significantly fewer NTHI were present within MEFs and a >5-log decrease in CFU NTHI was achieved.

As reduction of established NTHI mucosal biofilms began within three days after primary TCI (Fig. 4) and prior to detection of antibody in MEFs (Table 1), we hypothesized that production of innate host defense molecules by the middle ear epithelium contributed to this observation. Toward that end, we focused on one of many such effectors, cBD-1, the orthologue to human β -defensin-3, known to be expressed in the chinchilla uppermost respiratory tract that exhibits bactericidal activity against NTHI *in vitro* and shown to have a role in the control of NTHI during experimental colonization of the chinchilla nasopharynx [60, 62, 63]. Three days after primary immunization (seven days after NTHI challenge), 50.3 ± 16.7 ng cBD-1/ml was detected within homogenates of middle ear mucosa collected from animals that received saline, compared to 643.4 ± 57.3 ng/ml and 849.8 ± 49.0 ng/ml in mucosae from animals administered dmLT only or chimV4+dmLT, respectively. These data suggested that the reduced bacterial concentration detected within mucosal biofilms adherent to the middle ear mucosa of animals immunized with dmLT alone and chimV4+dmLT may, in part, be attributed to significantly more cBD-1 present within this milieu, compared to the cohort that received saline ($p < 0.05$).

3.6 Activation of dermal DCs

As a means to examine the functional activation of dermal DCs after TCI, pinnae were collected 3 hr after immunization and cultured *ex vivo*. At this arbitrarily selected time point, 1.5×10^3 CD11c⁺CD11b⁺DC-SIGN⁺ dermal DC/ml had emigrated from the pinnae into the culture medium, a value which can be considered as steady-state migration in this assay system. TCI with dmLT alone resulted in 12-times more dermal DCs (1.9×10^4 cells/ml), although not a significant result. The greatest and significant efflux of dermal DCs was observed from pinnae on which chimV4+dmLT was applied; a total of 6.8×10^4 cells/ml was observed, 44- and 4-fold more cells compared to application of saline or dmLT alone, respectively ($p < 0.01$).

Activated DCs can secrete cytokines and chemokines as a means of functional modulation via autocrine or paracrine manner. The supernatants from the cultured pinnae were assayed for the presence of inflammatory mediators via membrane array. Of a panel of 36 effectors, seven were expressed at least 2-fold greater after TCI of pinnae with dmLT or chimV4+dmLT compared to administration of saline (Fig. 5). Of note is the 10- and 5-fold increase in CCL4 induced by TCI with chimV4+dmLT, compared to saline or dmLT alone, respectively. While many of the listed mediators are pluripotent and are involved in the induction of inflammatory responses, a majority of these factors share a common role as chemotactic agents for specific cell types. IL-23, IL-16, CCL5 and CCL4 in particular, are noted to induce the migration and differentiation of CD4⁺ T-cells, critical first steps toward the initiation of an immune response [64–66].

3.7 Induction of polyfunctional CD4⁺ T-cells

Lastly, we examined the phenotype of the resultant immune response, specifically, the pattern of cytokine production by CD4⁺ T-cells within the NALT after TCI with any of the three vaccine formulations. The NALT was examined as the primary lymphoid aggregate to which dermal DCs trafficked after TCI in this model [20]. Compared to the NALT isolated from chinchillas to which saline was applied, TCI with dmLT alone yielded 5.1% of CD4⁺ T-cells that produced both IFN- γ and IL-17 (Fig 6), further support for the induction of a modest response by administration of this potent adjuvant alone. ChimV4 admixed with

dmLT resulted in a 5.7-fold increase in the percentage of T-cells that produced both IFN- γ and IL-17 to 29.3%, relative to receipt of dmLT alone. Moreover, within the positive population highlighted, two subtypes were observed; one of lesser dual fluorescence and one of greater dual fluorescence. While not clearly defined at this time, each population may represent CD4⁺ T-cells of greater and lesser activation states.

The greater percentage of CD4⁺ T-cells observed within the NALT of animals immunized by TCI with chimV4+dmLT, compared to receipt of dmLT or saline alone, indicated that this immunization regime induced the greatest expansion of CD4⁺ T-cells resident within this lymphoid aggregate of the three formulations. Moreover, increase in the number of cells that expressed both IFN- γ and IL-17, represented by the greater overall fluorescent signal within this population, demonstrated robust differentiation to cells that appeared functionally polarized toward a dual Th1-Th17 phenotype. These polyfunctional T-cells have the potential to further facilitate the development of a broadly protective and multifactorial immune response that could be highly efficacious against NTHI-induced experimental OM, an outcome which was observed herein.

4. Discussion

OM is a common disease of childhood and while the associated mortality is very limited at least in developed countries, the morbidity associated with this disease in terms of developmental delays, quality of life and economic impact is substantial. The chronic nature of OM may be attributed to the ability of the bacterial causative agents, including NTHI, to form mucosal biofilms within the middle ear space [48, 49, 67]. These highly structured communities are recalcitrant to antibiotic therapies and resist immune-mediated clearance [68–70]. Among many other NTHI proteins, and an abundance of host- and bacterial-derived extracellular DNA, NTHI OMP P5 and Tfp are identified as components of the biofilm matrix [11, 71]. Prior work established that TCI with chimV4, a chimeric immunogen designed to target both of these critical NTHI adhesins (outer membrane protein P5 and Tfp), delivered with dmLT by simply rubbing the formulations on to the pinnae of chinchillas was significantly efficacious to both prevent NTHI-induced OM and resolve active OM [20]. The rapidity with which biofilms that had been established within the middle ear resolved was striking. We therefore proposed that TCI with NTHI-adhesin directed immunogens plus dmLT induced the activation and subsequent migration of cutaneous DCs to the NALT, which is located in close proximity to the site of experimental disease, and thereby facilitated the efficacy observed.

Our current work sought to elucidate the mechanisms that afforded the observed efficacy and examine the kinetics of disease resolution. We utilized an established chinchilla model wherein cohorts of animals were first challenged by direct inoculation of the middle ear with NTHI to induce OM [20, 59]. The chinchilla serves as a relevant model for this pediatric disease as it is permissive to infection with the viral and bacterial agents that cause OM in humans and by appropriate viral-bacterial co-infection, one can experimentally mimic the natural disease course of OM observed in the child [48, 72–75]. It has shown reproducibility in numerous preclinical trials wherein the efficacy of NTHI-targeted immunogens to prevent or resolve NTHI-induced OM was assessed [20, 51, 52, 54, 59] and is shown to be predictive of a clinical trial outcome in examined in children [53, 76]. Moreover, the chinchilla also serves as a robust host that both we and others employ to study characteristics of bacterial biofilms associated with OM [11, 12, 77–85].

In the rigorous challenge model described herein, after a period of four days to permit NTHI to establish robust biofilms within the middle ear, animals were immunized via TCI. The administration of saline served as a negative control and provided the ability to examine the

immune response elicited by application of dmLT alone. This adjuvant is a form of *E. coli* heat-labile enterotoxin wherein two amino acid substitutions inactivate a trypsin cleavage site and modifies a potential pepsin cleavage site, thus rendering the molecule nontoxic, while adjuvant properties are retained [19, 25, 86]. It is proposed that dmLT promotes activation of various cell types, including DCs, with a resultant mixed Th1/Th2-type immune response [19].

Compared to delivery of saline, three days after primary TCI with dmLT alone, an initial reduction in clinically-relevant signs of OM, in addition to a significant decrease in CFU NTHI within middle ear biomasses was achieved. However, this was a transient outcome, as two days later no differences were observed between the two cohorts. This early response likely reflected nonspecific activation and function of dermal DCs. However, without a sustained antigenic stimulus, this response appeared temporary. In contrast, the formulation in which chimV4 was administered with dmLT induced a similar, yet, prolonged and effective outcome. Significant differences in clinically-relevant signs of OM, significant reduction in the amount of biomass within the middle ear space and significantly fewer NTHI were present within the biomasses beginning three days after receipt of the first immunizing dose. As immunogen specific IgG and IgA was not detected within the middle ear until 5 days after primary TCI, these early effects likely represent activation of innate immune elements, *i.e.* dendritic cell activation, production of host defense peptides, cytokine/chemokine signaling. The observed greater efflux of dDCs from the pinnae and detection of a greater concentration of cBD-1 support this hypothesis. Thus, our data suggested that dmLT played an early, critical role in immune activation that was further enhanced and focused against NTHI with the addition of chimV4 in the vaccine formulation.

Host defense peptides are multifunctional proteins that possess both antimicrobial and immunomodulatory characteristics. The β -defensins are one class of host defense peptide and are shown to be produced by epithelial cells, constitutively or immediately in response to infection [87, 88]. We tested middle ear mucosal homogenates for the presence of cBD-1, the orthologue to human β -defensin 3, as a potential innate immune factor that contributed to the early reduction in signs of OM and concentration of NTHI within mucosal biofilms. A correlation between a greater concentration of cBD-1 and fewer NTHI was detected, particularly within the middle ears of animals that received dmLT alone or chimV4+dmLT. It is also reported that exposure of neutrophils to human β -defensin-3 suppresses apoptosis of these critical immune cells; moreover this molecule is chemotactic for neutrophils and macrophages [89, 90]. While these functions are not yet described for cBD-1, it is intriguing to speculate that this molecule may possess similar functional characteristics, the result of which would be the elimination of NTHI from the middle ear, as observed.

Specific to the kinetics of disease resolution, the first seven days after primary TCI yielded the most dramatic resolution of disease, shown by multiple assessments. Via video otoscopy and tympanometry, delivery of chimV4+dmLT resulted in a 90% reduction in the percentage of middle ears with OM within this cohort. Grossly, by day 7 the mean mucosal biofilm score for this cohort equaled 1.7, a 50–75% reduction relative to day 0. Moreover, a >5-log reduction in CFU NTHI was detected within mucosal biofilms collected from the middle ear. The only assessment that did not follow this trend was the examination of CFU NTHI within MEFs. No differences were detected among the cohorts until beginning on day 7, at which time the planktonic NTHI were rapidly eradicated. Interestingly, the time point for resolution of NTHI from MEFs correlated with the detection of chimV4-specific IgG in MEFs at a GMT of 160. As previously mentioned, a GMT of 160 is associated with resolution of experimental OM in this model.

Collectively, our data demonstrated two phases of disease resolution in this experimental model: an early phase from days 0 to 7 after primary TCI in which dmLT-induced activation of dermal DCs with subsequent migration to the NALT, secretion of cytokines and chemokines by activated DCs to stimulate expansion and polarization of CD4⁺ T-cells and production of at least one of many host defense peptides known to have activity against NTHI, cBD-1. Inclusion of chimV4 within the formulation prolonged this response. Phase two began 7 days after primary TCI, upon administration of the second immunizing dose as immunogen-specific antibody that targets determinants expressed by NTHI was now detected. While the timeframe proposed by this model incorporates the basic tenets for the initiation of innate and adaptive immune responses, to our knowledge, this is the first report of a kinetic analysis of disease resolution after TCI that also incorporates challenge with a biological agent. These data will be utilized as we further refine this effective immunization method, with the goal to enhance the immune response elicited, while maintaining the simplicity of the regimen.

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References

1. Monasta L, Ronfani L, Marchetti F, Montico M, Vecchi Brumatti L, Bavcar A, et al. Burden of disease caused by otitis media: systematic review and global estimates. *PLoS One*. 2012; 7(4):e36226. [PubMed: 22558393]
2. Diagnosis and management of acute otitis media. *Pediatrics*. 2004 May; 113(5):1451–65. [PubMed: 15121972]
3. Baldwin RL. Effects of otitis media on child development. *Am J Otol*. 1993 Nov; 14(6):601–4. [PubMed: 7507647]
4. Johnson DL, McCormick DP, Baldwin CD. Early middle ear effusion and language at age seven. *J Commun Disord*. 2008 Jan-Feb; 41(1):20–32. [PubMed: 17418231]
5. McCormick DP, Johnson DL, Baldwin CD. Early middle ear effusion and school achievement at age seven years. *Ambul Pediatr*. 2006 Sep-Oct; 6(5):280–7. [PubMed: 17000418]
6. Hunter LL, Margolis RH, Giebink GS. Identification of hearing loss in children with otitis media. *Ann Otol Rhinol Laryngol Suppl*. 1994 May; 163:59–61. [PubMed: 8179273]
7. Song JH, Dagan R, Klugman KP, Fritzell B. The relationship between pneumococcal serotypes and antibiotic resistance. *Vaccine*. 2012 Apr 5; 30(17):2728–37. [PubMed: 22330126]
8. Leibovitz E, Broides A, Greenberg D, Newman N. Current management of pediatric acute otitis media. *Expert Rev Anti Infect Ther*. 2010 Feb; 8(2):151–61. [PubMed: 20109045]
9. Bakaletz LO, Baker BD, Jurcisek JA, Harrison A, Novotny LA, Bookwalter JE, et al. Demonstration of Type IV pilus expression and a twitching phenotype by *Haemophilus influenzae*. *Infect Immun*. 2005 Mar; 73(3):1635–43. [PubMed: 15731063]
10. Sirakova T, Kolattukudy PE, Murwin D, Billy J, Leake E, Lim D, et al. Role of fimbriae expressed by nontypeable *Haemophilus influenzae* in pathogenesis of and protection against otitis media and relatedness of the fimbrial subunit to outer membrane protein A. *Infect Immun*. 1994 May; 62(5): 2002–20. [PubMed: 7909539]
11. Jurcisek JA, Bakaletz LO. Biofilms formed by nontypeable *Haemophilus influenzae* in vivo contain both double-stranded DNA and type IV pilin protein. *J Bacteriol*. 2007 May; 189(10): 3868–75. [PubMed: 17322318]
12. Jurcisek JA, Bookwalter JE, Baker BD, Fernandez S, Novotny LA, Munson RS Jr, et al. The PilA protein of non-typeable *Haemophilus influenzae* plays a role in biofilm formation, adherence to epithelial cells and colonization of the mammalian upper respiratory tract. *Mol Microbiol*. 2007 Sep; 65(5):1288–99. [PubMed: 17645732]

13. Novotny LA, Adams LD, Kang DR, Wiet GJ, Cai X, Sethi S, et al. Epitope mapping immunodominant regions of the PilA protein of nontypeable *Haemophilus influenzae* (NTHI) to facilitate the design of two novel chimeric vaccine candidates. *Vaccine*. 2009 Dec 10; 28(1):279–89. [PubMed: 19699813]
14. Bakaletz LO, Leake ER, Billy JM, Kaumaya PT. Relative immunogenicity and efficacy of two synthetic chimeric peptides of fimbrin as vaccinogens against nasopharyngeal colonization by nontypeable *Haemophilus influenzae* in the chinchilla. *Vaccine*. 1997 Jun; 15(9):955–61. [PubMed: 9261941]
15. Novotny LA, Jurcisek JA, Pichichero ME, Bakaletz LO. Epitope mapping of the outer membrane protein P5-homologous fimbrin adhesin of nontypeable *Haemophilus influenzae*. *Infect Immun*. 2000 Apr; 68(4):2119–28. [PubMed: 10722609]
16. Novotny LA, Pichichero ME, Denoel PA, Neyt C, Vanderschrick S, Dequesne G, et al. Detection and characterization of pediatric serum antibody to the OMP P5-homologous adhesin of nontypeable *Haemophilus influenzae* during acute otitis media. *Vaccine*. 2002 Oct 4; 20(29–30): 3590–7. [PubMed: 12297406]
17. Novotny LA, Bakaletz LO. The fourth surface-exposed region of the outer membrane protein P5-homologous adhesin of nontypeable *Haemophilus influenzae* is an immunodominant but nonprotective decoying epitope. *J Immunol*. 2003 Aug 15; 171(4):1978–83. [PubMed: 12902501]
18. Summerton NA, Welch RW, Bondoc L, Yang HH, Pleune B, Ramachandran N, et al. Toward the development of a stable, freeze-dried formulation of *Helicobacter pylori* killed whole cell vaccine adjuvanted with a novel mutant of *Escherichia coli* heat-labile toxin. *Vaccine*. 2010 Feb 3; 28(5): 1404–11. [PubMed: 19897067]
19. Norton EB, Lawson LB, Freytag LC, Clements JD. Characterization of a mutant *Escherichia coli* heat-labile toxin, LT(R192G/L211A), as a safe and effective oral adjuvant. *Clin Vaccine Immunol*. 2011 Apr; 18(4):546–51. [PubMed: 21288994]
20. Novotny LA, Clements JD, Bakaletz LO. Transcutaneous immunization as preventative and therapeutic regimens to protect against experimental otitis media due to nontypeable *Haemophilus influenzae*. *Mucosal Immunol*. 2011 Jul; 4(4):456–67. [PubMed: 21326197]
21. Giudice EL, Campbell JD. Needle-free vaccine delivery. *Adv Drug Deliv Rev*. 2006 Apr 20; 58(1): 68–89. [PubMed: 16564111]
22. Warger T, Schild H, Rechtsteiner G. Initiation of adaptive immune responses by transcutaneous immunization. *Immunol Lett*. 2007 Mar 15; 109(1):13–20. [PubMed: 17320194]
23. Kenney RT, Frech SA, Muenz LR, Villar CP, Glenn GM. Dose sparing with intradermal injection of influenza vaccine. *N Engl J Med*. 2004 Nov 25; 351(22):2295–301. [PubMed: 15525714]
24. Mishra D, Mishra PK, Dubey V, Nahar M, Dabaghao S, Jain NK. Systemic and mucosal immune response induced by transcutaneous immunization using Hepatitis B surface antigen-loaded modified liposomes. *Eur J Pharm Sci*. 2008 Apr 23; 33(4–5):424–33. [PubMed: 18359615]
25. Lawson LB, Clements JD, Freytag LC. Mucosal Immune Responses Induced by Transcutaneous Vaccines. *Curr Top Microbiol Immunol*. 2010 Nov 9.
26. Glenn GM, Scharton-Kersten T, Vassell R, Matyas GR, Alving CR. Transcutaneous immunization with bacterial ADP-ribosylating exotoxins as antigens and adjuvants. *Infect Immun*. 1999 Mar; 67(3):1100–6. [PubMed: 10024549]
27. Russell MW, Ogra PL. Mucosal decisions: tolerance and responsiveness at mucosal surfaces. *Immunol Invest*. 2010; 39(4–5):297–302. [PubMed: 20450281]
28. Maeba S, Otake S, Namikoshi J, Shibata Y, Hayakawa M, Abiko Y, et al. Transcutaneous immunization with a 40-kDa outer membrane protein of *Porphyromonas gingivalis* induces specific antibodies which inhibit coaggregation by *P. gingivalis*. *Vaccine*. 2005 Mar 31; 23(19): 2513–21. [PubMed: 15752838]
29. Belyakov IM, Hammond SA, Ahlers JD, Glenn GM, Berzofsky JA. Transcutaneous immunization induces mucosal CTLs and protective immunity by migration of primed skin dendritic cells. *J Clin Invest*. 2004 Apr; 113(7):998–1007. [PubMed: 15057306]
30. Vogt A, Mahe B, Costagliola D, Bonduelle O, Hadam S, Schaefer G, et al. Transcutaneous anti-influenza vaccination promotes both CD4 and CD8 T cell immune responses in humans. *J Immunol*. 2008 Feb 1; 180(3):1482–9. [PubMed: 18209043]

31. Hansen S, Lehr CM. Nanoparticles for transcutaneous vaccination. *Microb Biotechnol*. 2012 Mar; 5(2):156–67. [PubMed: 21854553]
32. Skountzou I, Quan FS, Jacob J, Compans RW, Kang SM. Transcutaneous immunization with inactivated influenza virus induces protective immune responses. *Vaccine*. 2006 Aug 28; 24(35–36):6110–9. [PubMed: 16766095]
33. Mawas F, Peyre M, Beignon AS, Frost L, Del Giudice G, Rappuoli R, et al. Successful induction of protective antibody responses against *Haemophilus influenzae* type b and diphtheria after transcutaneous immunization with the glycoconjugate polyribosyl ribitol phosphate-cross-reacting material 197 vaccine. *J Infect Dis*. 2004 Sep 15; 190(6):1177–82. [PubMed: 15319869]
34. Hickey DK, Aldwell FE, Beagley KW. Transcutaneous immunization with a novel lipid-based adjuvant protects against Chlamydia genital and respiratory infections. *Vaccine*. 2009 Oct 19; 27(44):6217–25. [PubMed: 19698810]
35. Naito S, Maeyama J, Mizukami T, Takahashi M, Hamaguchi I, Yamaguchi K. Transcutaneous immunization by merely prolonging the duration of antigen presence on the skin of mice induces a potent antigen-specific antibody response even in the absence of an adjuvant. *Vaccine*. 2007 Dec 17; 25(52):8762–70. [PubMed: 18023509]
36. Glenn GM, Flyer DC, Ellingsworth LR, Frech SA, Frerichs DM, Seid RC, et al. Transcutaneous immunization with heat-labile enterotoxin: development of a needle-free vaccine patch. *Expert Rev Vaccines*. 2007 Oct; 6(5):809–19. [PubMed: 17931160]
37. McKenzie R, Bourgeois AL, Frech SA, Flyer DC, Bloom A, Kazempour K, et al. Transcutaneous immunization with the heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC): protective efficacy in a double-blind, placebo-controlled challenge study. *Vaccine*. 2007 May 4; 25(18):3684–91. [PubMed: 17313998]
38. Etchart N, Hennino A, Friede M, Dahel K, Dupouy M, Goujon-Henry C, et al. Safety and efficacy of transcutaneous vaccination using a patch with the live-attenuated measles vaccine in humans. *Vaccine*. 2007 Sep 28; 25(39–40):6891–9. [PubMed: 17764789]
39. Hirobe S, Matsuo K, Quan YS, Kamiyama F, Morito H, Asada H, et al. Clinical study of transcutaneous vaccination using a hydrogel patch for tetanus and diphtheria. *Vaccine*. 2012 Feb 27; 30(10):1847–54. [PubMed: 22230592]
40. Frech SA, Dupont HL, Bourgeois AL, McKenzie R, Belkind-Gerson J, Figueroa JF, et al. Use of a patch containing heat-labile toxin from *Escherichia coli* against travellers' diarrhoea: a phase II, randomised, double-blind, placebo-controlled field trial. *Lancet*. 2008 Jun 14; 371(9629):2019–25. [PubMed: 18554712]
41. Santegoets SJ, Gibbs S, Kroeze K, van de Ven R, Scheper RJ, Borrebaeck CA, et al. Transcriptional profiling of human skin-resident Langerhans cells and CD1a+ dermal dendritic cells: differential activation states suggest distinct functions. *J Leukoc Biol*. 2008 Jul; 84(1):143–51. [PubMed: 18436579]
42. Mathers AR, Larregina AT. Professional antigen-presenting cells of the skin. *Immunol Res*. 2006; 36(1–3):127–36. [PubMed: 17337773]
43. Kaplan DH. In vivo function of Langerhans cells and dermal dendritic cells. *Trends Immunol*. 2010 Dec; 31(12):446–51. [PubMed: 21035396]
44. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. *Nat Med*. 2005 Apr; 11(4 Suppl):S45–53. [PubMed: 15812489]
45. Czerkinsky C, Holmgren J. Mucosal Delivery Routes for Optimal Immunization: Targeting Immunity to the Right Tissues. *Curr Top Microbiol Immunol*. 2010 Nov 5.
46. Wu HY, Russell MW. Nasal lymphoid tissue, intranasal immunization, and compartmentalization of the common mucosal immune system. *Immunol Res*. 1997; 16(2):187–201. [PubMed: 9212364]
47. Jurcisek JA, Durbin JE, Kusewitt DF, Bakaletz LO. Anatomy of the nasal cavity in the chinchilla. *Cells Tissues Organs*. 2003; 174(3):136–52. [PubMed: 12835577]
48. Murphy TF, Bakaletz LO, Smeesters PR. Microbial interactions in the respiratory tract. *Pediatr Infect Dis J*. 2009 Oct; 28(10 Suppl):S121–6. [PubMed: 19918134]
49. Hall-Stoodley L, Hu FZ, Gieseke A, Nistico L, Nguyen D, Hayes J, et al. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA*. 2006 Jul 12; 296(2):202–11. [PubMed: 16835426]

50. Martinez-Becerra FJ, Kissmann JM, Diaz-McNair J, Choudhari SP, Quick AM, Mellado-Sanchez G, et al. Broadly protective *Shigella* vaccine based on type III secretion apparatus proteins. *Infect Immun*. 2012 Mar; 80(3):1222–31. [PubMed: 22202122]
51. Bakaletz LO, Kennedy BJ, Novotny LA, Duquesne G, Cohen J, Lobet Y. Protection against development of otitis media induced by nontypeable *Haemophilus influenzae* by both active and passive immunization in a chinchilla model of virus-bacterium superinfection. *Infect Immun*. 1999 Jun; 67(6):2746–62. [PubMed: 10338477]
52. Kennedy BJ, Novotny LA, Jursisek JA, Lobet Y, Bakaletz LO. Passive transfer of antiserum specific for immunogens derived from a nontypeable *Haemophilus influenzae* adhesin and lipoprotein D prevents otitis media after heterologous challenge. *Infect Immun*. 2000 May; 68(5): 2756–65. [PubMed: 10768970]
53. Novotny LA, Jursisek JA, Godfroid F, Poolman JT, Denoel PA, Bakaletz LO. Passive immunization with human anti-protein D antibodies induced by polysaccharide protein D conjugates protects chinchillas against otitis media after intranasal challenge with *Haemophilus influenzae*. *Vaccine*. 2006 May 29; 24(22):4804–11. [PubMed: 16616975]
54. Kyd JM, Cripps AW, Novotny LA, Bakaletz LO. Efficacy of the 26-kilodalton outer membrane protein and two P5 fimbria-derived immunogens to induce clearance of nontypeable *Haemophilus influenzae* from the rat middle ear and lungs as well as from the chinchilla middle ear and nasopharynx. *Infect Immun*. 2003 Aug; 71(8):4691–9. [PubMed: 12874350]
55. Mason KM, Munson RS Jr, Bakaletz LO. A mutation in the *sap* operon attenuates survival of nontypeable *Haemophilus influenzae* in a chinchilla model of otitis media. *Infect Immun*. 2005 Jan; 73(1):599–608. [PubMed: 15618200]
56. Johnson RW, McGillivray G, Denoel P, Poolman J, Bakaletz LO. Abrogation of nontypeable *Haemophilus influenzae* Protein D function reduces phosphorylcholine decoration, adherence to airway epithelial cells, and fitness in a chinchilla model of otitis media. *Vaccine*. 2010 Dec 15.
57. Krishnamurthy A, McGrath J, Cripps AW, Kyd JM. The incidence of *Streptococcus pneumoniae* otitis media is affected by the polymicrobial environment particularly *Moraxella catarrhalis* in a mouse nasal colonisation model. *Microbes Infect*. 2009 Apr; 11(5):545–53. [PubMed: 19306940]
58. Hong W, Mason K, Jursisek J, Novotny L, Bakaletz LO, Swords WE. Phosphorylcholine decreases early inflammation and promotes the establishment of stable biofilm communities of nontypeable *Haemophilus influenzae* strain 86-028NP in a chinchilla model of otitis media. *Infect Immun*. 2007 Feb; 75(2):958–65. [PubMed: 17130253]
59. Goodman SD, Obergefell KP, Jursisek JA, Novotny LA, Downey JS, Ayala EA, et al. Biofilms can be dispersed by focusing the immune system on a common family of bacterial nucleoid-associated proteins. *Mucosal Immunol*. 2011 Nov; 4(6):625–37. [PubMed: 21716265]
60. McGillivray G, Mason KM, Jursisek JA, Peebles ME, Bakaletz LO. Respiratory syncytial virus-induced dysregulation of expression of a mucosal beta-defensin augments colonization of the upper airway by non-typeable *Haemophilus influenzae*. *Cell Microbiol*. 2009 Sep; 11(9):1399–408. [PubMed: 19500108]
61. Stoitzner P, Zanella M, Ortner U, Lukas M, Tagwerker A, Janke K, et al. Migration of langerhans cells and dermal dendritic cells in skin organ cultures: augmentation by TNF-alpha and IL-1beta. *J Leukoc Biol*. 1999 Sep; 66(3):462–70. [PubMed: 10496317]
62. Harris RH, Wilk D, Bevins CL, Munson RS Jr, Bakaletz LO. Identification and characterization of a mucosal antimicrobial peptide expressed by the chinchilla (*Chinchilla lanigera*) airway. *J Biol Chem*. 2004 May 7; 279(19):20250–6. [PubMed: 14996845]
63. Lim DJ, Chun YM, Lee HY, Moon SK, Chang KH, Li JD, et al. Cell biology of tubotympanum in relation to pathogenesis of otitis media - a review. *Vaccine*. 2000 Dec 8; 19(Suppl 1):S17–25. [PubMed: 11163458]
64. Taub DD, Conlon K, Lloyd AR, Oppenheim JJ, Kelvin DJ. Preferential migration of activated CD4+ and CD8+ T cells in response to MIP-1 alpha and MIP-1 beta. *Science*. 1993 Apr 16; 260(5106):355–8. [PubMed: 7682337]
65. Schall TJ, Bacon K, Camp RD, Kaspari JW, Goeddel DV. Human macrophage inflammatory protein alpha (MIP-1 alpha) and MIP-1 beta chemokines attract distinct populations of lymphocytes. *J Exp Med*. 1993 Jun 1; 177(6):1821–6. [PubMed: 7684437]

66. Morelli AE, Rubin JP, Erdos G, Tkacheva OA, Mathers AR, Zahorchak AF, et al. CD4+ T cell responses elicited by different subsets of human skin migratory dendritic cells. *J Immunol.* 2005 Dec 15; 175(12):7905–15. [PubMed: 16339526]
67. Murphy TF, Kirkham C. Biofilm formation by nontypeable *Haemophilus influenzae*: strain variability, outer membrane antigen expression and role of pili. *BMC Microbiol.* 2002 Apr 15;2:7. [PubMed: 11960553]
68. Flemming HC, Wingender J. The biofilm matrix. *Nat Rev Microbiol.* 2010 Sep; 8(9):623–33. [PubMed: 20676145]
69. Starner TD, Shrout JD, Parsek MR, Appelbaum PC, Kim G. Subinhibitory concentrations of azithromycin decrease nontypeable *Haemophilus influenzae* biofilm formation and diminish established biofilms. *Antimicrob Agents Chemother.* 2008 Jan; 52(1):137–45. [PubMed: 17954687]
70. Slinger R, Chan F, Ferris W, Yeung SW, St Denis M, Gaboury I, et al. Multiple combination antibiotic susceptibility testing of nontypeable *Haemophilus influenzae* biofilms. *Diagn Microbiol Infect Dis.* 2006 Nov; 56(3):247–53. [PubMed: 16769194]
71. Gallaher TK, Wu S, Webster P, Aguilera R. Identification of biofilm proteins in non-typeable *Haemophilus influenzae*. *BMC Microbiol.* 2006; 6:65. [PubMed: 16854240]
72. Vesa S, Kleemola M, Blomqvist S, Takala A, Kilpi T, Hovi T. Epidemiology of documented viral respiratory infections and acute otitis media in a cohort of children followed from two to twenty-four months of age. *Pediatr Infect Dis J.* 2001 Jun; 20(6):574–81. [PubMed: 11419498]
73. Suzuki K, Bakaletz LO. Synergistic effect of adenovirus type 1 and nontypeable *Haemophilus influenzae* in a chinchilla model of experimental otitis media. *Infect Immun.* 1994 May; 62(5):1710–8. [PubMed: 8168932]
74. Brockson ME, Novotny LA, Jurcisek JA, McGillivray G, Bowers MR, Bakaletz LO. Respiratory Syncytial Virus Promotes *Moraxella catarrhalis*-Induced Ascending Experimental Otitis Media. *PLoS One.* 2012; 7(6):e40088. [PubMed: 22768228]
75. Giebink GS, Berzins IK, Marker SC, Schiffman G. Experimental otitis media after nasal inoculation of *Streptococcus pneumoniae* and influenza A virus in chinchillas. *Infect Immun.* 1980 Nov; 30(2):445–50. [PubMed: 7439990]
76. Prymula R, Peeters P, Chrobok V, Kriz P, Novakova E, Kaliskova E, et al. Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae*: a randomised double-blind efficacy study. *Lancet.* 2006 Mar 4; 367(9512):740–8. [PubMed: 16517274]
77. Ehrlich GD, Veeh R, Wang X, Costerton JW, Hayes JD, Hu FZ, et al. Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *JAMA.* 2002 Apr 3; 287(13):1710–5. [PubMed: 11926896]
78. Pang B, Hong W, Kock ND, Swords WE. Dps promotes survival of nontypeable *Haemophilus influenzae* in biofilm communities in vitro and resistance to clearance in vivo. *Front Cell Infect Microbiol.* 2012; 2:58. [PubMed: 22919649]
79. Bouchet V, Hood DW, Li J, Brisson JR, Randle GA, Martin A, et al. Host-derived sialic acid is incorporated into *Haemophilus influenzae* lipopolysaccharide and is a major virulence factor in experimental otitis media. *Proc Natl Acad Sci U S A.* 2003 Jul 22; 100(15):8898–903. [PubMed: 12855765]
80. Leroy M, Cabral H, Figueira M, Bouchet V, Huot H, Ram S, et al. Multiple consecutive lavage samplings reveal greater burden of disease and provide direct access to the nontypeable *Haemophilus influenzae* biofilm in experimental otitis media. *Infect Immun.* 2007 Aug; 75(8):4158–72. [PubMed: 17517860]
81. Daines DA, Bothwell M, Furrer J, Unrath W, Nelson K, Jarisch J, et al. *Haemophilus influenzae luxS* mutants form a biofilm and have increased virulence. *Microb Pathog.* 2005 Sep; 39(3):87–96. [PubMed: 16099134]
82. Greiner LL, Watanabe H, Phillips NJ, Shao J, Morgan A, Zaleski A, et al. Nontypeable *Haemophilus influenzae* strain 2019 produces a biofilm containing N-acetylneuraminic acid that may mimic sialylated O-linked glycans. *Infect Immun.* 2004 Jul; 72(7):4249–60. [PubMed: 15213170]

83. Jurcisek J, Greiner L, Watanabe H, Zaleski A, Apicella MA, Bakaletz LO. Role of sialic acid and complex carbohydrate biosynthesis in biofilm formation by nontypeable *Haemophilus influenzae* in the chinchilla middle ear. *Infect Immun*. 2005 Jun; 73(6):3210–8. [PubMed: 15908345]
84. Weimer KE, Armbruster CE, Juneau RA, Hong W, Pang B, Swords WE. Coinfection with *Haemophilus influenzae* promotes pneumococcal biofilm formation during experimental otitis media and impedes the progression of pneumococcal disease. *J Infect Dis*. 2010 Oct 1; 202(7): 1068–75. [PubMed: 20715928]
85. Hong W, Pang B, West-Barnette S, Swords WE. Phosphorylcholine expression by nontypeable *Haemophilus influenzae* correlates with maturation of biofilm communities in vitro and in vivo. *J Bacteriol*. 2007 Nov; 189(22):8300–7. [PubMed: 17573475]
86. Freytag LC, Clements JD. Mucosal adjuvants. *Vaccine*. 2005; 23(15):1804–13. [PubMed: 15734046]
87. Pazgier M, Hoover DM, Yang D, Lu W, Lubkowski J. Human beta-defensins. *Cell Mol Life Sci*. 2006 Jun; 63(11):1294–313. [PubMed: 16710608]
88. Dhople V, Krukemeyer A, Ramamoorthy A. The human beta-defensin-3, an antibacterial peptide with multiple biological functions. *Biochim Biophys Acta*. 2006 Sep; 1758(9):1499–512. [PubMed: 16978580]
89. Rohrl J, Yang D, Oppenheim JJ, Hehlhans T. Human beta-defensin 2 and 3 and their mouse orthologs induce chemotaxis through interaction with CCR2. *J Immunol*. 2010 Jun 15; 184(12): 6688–94. [PubMed: 20483750]
90. Nagaoka I, Niyonsaba F, Tsutsumi-Ishii Y, Tamura H, Hirata M. Evaluation of the effect of human beta-defensins on neutrophil apoptosis. *Int Immunol*. 2008 Apr; 20(4):543–53. [PubMed: 18308714]

Highlights

Transcutaneous immunization with chimV4+dmLT resolved ongoing NTHI-induced experimental OM.

Significant reduction in signs of OM, NTHI concentration and biomass in the middle ear was achieved.

Activated DCs, polyfunctional T-cells and host defense peptide contributed to rapid resolution.

Immunogen-specific IgG in middle ear fluids also contributed to eradication of NTHI.

Transcutaneous immunization is a simple strategy to induce efficacious immune responses.

Figure 1.

Resolution of clinically-relevant signs of experimental OM as determined by video otoscopy and tympanometry. Boxes around days 0 and 7 on x-axis indicate days of vaccination. *, $p < 0.05$ compared to receipt of saline; +, $p < 0.05$ compared to receipt of dmLT alone. N=4 middle ears per cohort on day 0; N=6 middle ears per cohort on days 3 through 14.

Figure 2.

Eradication of NTHI from middle ear fluids. NTHI was inoculated into the middle ears of all chinchillas four days prior to primary immunization (inoc). Mean CFU NTHI per middle ear fluid $\pm$ SEM for each cohort is presented. Boxes around days 0 and 7 on x-axis indicate days of vaccination. *, $p < 0.05$ compared to receipt of saline; +, $p < 0.05$ compared to receipt of dmLT alone. N=4 middle ears per cohort on day 0; N=6 middle ears per cohort on days 3 through 14.

Figure 3.

Resolution of an established NTHI mucosal biofilms from within the middle ear. Mean NTHI mucosal biofilm scores $\pm$ SEM for each cohort were based on a 0 to 4+ scale of relative residual mucosal biofilm. Boxes around days 0 and 7 on x-axis indicate days of vaccination. *, $p < 0.01$ compared to receipt of saline; +, $p < 0.01$ compared to receipt of dmLT alone. N=4 middle ears per cohort on day 0; N=6 middle ears per cohort on days 3 through 14.

Figure 4.

Relative concentration of NTHI adherent to middle ear mucosa. Mean CFU NTHI $\pm$ SEM within biofilm that remained adherent to the middle ear mucosa over time is presented. Boxes around days 0 and 7 on x-axis indicate days of vaccination. *, $p < 0.05$ compared to receipt of saline; +, $p < 0.05$ compared to receipt of dmLT alone. N=4 middle ears per cohort on day 0; N=6 middle ears per cohort on days 3 through 14.

Figure 5.

Secretion of cytokines and chemokines by dermal DCs activated by TCI. Supernatants collected from cultured chinchilla pinnae from which dermal DCs had emigrated were assessed for the production of inflammatory mediators by membrane array. The fold change in pixel intensity as determined by densitometry relative to the saline-treated pinnae is shown. N=6 pinnae for each cohort.

Figure 6.

Induction of polyfunctional CD4⁺ T-cells within the chinchilla NALT after TCI. One week after receipt of the second immunizing dose, the NALT from each chinchilla was collected and processed to examine intracellular cytokine production by CD4⁺ T-cells in order to discern Th phenotype. The shaded region in each density plot indicates the positive staining for IFN-γ- and IL-17-producing CD4⁺ T-cells, relative to isotype antibody controls. One of three representative plots is presented for each cohort.

Table 1

Geometric mean of the reciprocal titer for chimV4-specific IgG and IgA within MEFs collected from chinchillas immunized by TCI with chimV4+dmLT. 95% confidence intervals of the geometric mean are indicated in brackets. N=4 middle ears for day 0, N=6 middle ears for days 3 through 14.

Days	chimV4-specific IgG	chimV4-specific IgA
0 (primary TCI)	10	10
3	10	10
5	80	28 [15–53]
7 (boost)	65 [24–171]	32 [12–86]
9	160 * [56–459]	35 [17–72]
11	160 * [65–394]	50 [28–91]
14	190 * [66–546]	56 [30–107]

*, $p < 0.05$ compared to day 0