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Early-Life Intranasal Colonization with Nontypeable *Haemophilus influenzae* Exacerbates Juvenile Airway Disease in Mice

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Accumulating evidence suggests a connection between asthma development and colonization with nontypeable *Haemophilus influenzae* (NTHi). Specifically, nasopharyngeal colonization of human infants with NTHi within 4 weeks of birth is associated with an increased risk of asthma development later in childhood. Monocytes derived from these infants have aberrant inflammatory responses to common upper respiratory bacterial antigens compared to those of cells derived from infants who were not colonized and do not go on to develop asthma symptoms in childhood. In this study, we hypothesized that early-life colonization with NTHi promotes immune system reprogramming and the development of atypical inflammatory responses. To address this hypothesis in a highly controlled model, we tested whether colonization of mice with NTHi on day of life 3 induced or exacerbated juvenile airway disease using an ovalbumin (OVA) allergy model of asthma. We found that animals that were colonized on day of life 3 and subjected to induction of allergy had exacerbated airway disease as juveniles, in which exacerbated airway disease was defined as increased cellular infiltration into the lung, increased amounts of inflammatory cytokines interleukin-5 (IL-5) and IL-13 in lung lavage fluid, decreased regulatory T cell-associated *FOXP3* gene expression, and increased mucus production. We also found that colonization with NTHi amplified airway resistance in response to increasing doses of a bronchoconstrictor following OVA immunization and challenge. Together, the murine model provides evidence for early-life immune programming that precedes the development of juvenile airway disease and corroborates observations that have been made in human children.

Asthma has nearly doubled in incidence in developed nations since 1980 and now affects almost 10% of all children and adults in the United States (1). One reason for this increase in incidence may be that the biological response to early-life antigen exposure is altered by emerging factors in the environment associated with industrialized societies (2, 3).

Asthma has traditionally been classified as a type 1 hypersensitivity disease that is characterized by a T_H2 response and the production of interleukin-4 (IL-4), IL-5, and IL-13. These cytokines play pivotal roles in mediating IgE production, mucus secretion, and airway hyperreactivity (AHR). Inflammation also promotes infiltration of regulatory T cells (Treg), which act to dampen IL-5 and IL-13 secretion by $CD4^+$ $CD25^-$ T_H2 cells in both human and mouse models of asthma (4, 5). Along with infiltration of eosinophils, these responses in the airways have been considered a defining feature of asthma. However, recent studies indicate that eosinophils are prominent in only 50% of asthmatic patients (2, 3, 6).

A subtype of noneosinophilic asthma called neutrophilic asthma is now recognized to afflict a significant number of patients and is associated with severe and persistent symptoms that are refractory to treatment with corticosteroids, a mainstay of moderate-severe asthma treatment (6, 7). Neutrophilic asthma is characterized by sputum with an increased number of neutrophils and increased levels of the proinflammatory cytokines IL-8 and IL-17 (6, 8, 9), known neutrophil chemoattractants (10). IL-17 also induces the secretion of several other proinflammatory cytokines and chemokines, including IL-1β, tumor necrosis factor alpha (TNF-α), and IL-8 (11). These cytokines are typically linked to a T_H1 inflammatory immune profile, which is also associated with infiltration of macrophages and effector T cells that coordinate cell-mediated immunity (12, 13). Since asthma treatment is directed to symptoms, it follows that different therapeutic approaches may be needed for the different inflammatory pathways that are invoked in neutrophilic asthma.

Nontypeable *Haemophilus influenzae* (NTHi) is a common cause of localized respiratory tract infections, including otitis media, sinusitis, pneumonia, and exacerbations of underlying lung disease, such as chronic obstructive pulmonary disease, cystic fibrosis, and asthma (14–18). The pathogenesis of NTHi disease begins with bacterial adherence to the nasopharyngeal epithelium and colonization of the nasopharynx. Bacterial adherence allows the organism to avoid clearance by the mucociliary escalator and other mechanical forces, such as coughing and sneezing. Studies using human adenoidal tissue or nasal turbinates in organ culture have demonstrated that NTHi preferentially adheres to nonciliated cells and to areas of damaged epithelium (14, 15, 19).

There is mounting evidence that NTHi has a role in the development and exacerbation of asthma. In recent work, Bisgaard et al. found that children colonized with NTHi within the first 4 weeks of life were 2 to 4 times more likely to develop symptoms of asthma or to be hospitalized for wheezing by age 5 years than children without detectable NTHi in the nasopharynx (20). No-

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tably, colonization at later than 4 weeks of life was not associated with a greater risk of asthma symptoms, indicating that early exposure is required for development of asthma. This information suggests that exposure during a vulnerable window may result in reprogramming of the immune system. In related work, Hilty and coworkers observed that *Haemophilus* was significantly more likely to be present in the lower airways of adult patients with asthma than in patients with normal lung function (21). Finally, in studies examining induced sputum from symptomatic or asymptomatic asthmatics, *Haemophilus* was among the most common organisms isolated and was associated with the presence of increased neutrophils in the airway (22, 23). Taken together, these data suggest that the presence of *Haemophilus* may modify or exacerbate the immune response typically seen during an inflammatory airway event, such as an acute asthma attack.

Although mouse models have provided key insights into asthma pathogenesis (24), they have not been applied to understanding the role of NTHi exposure during infancy in the development of asthma-like disease, particularly neutrophilic asthma. Preexisting murine asthma models commonly use adult inbred BALB/c or C57BL/6 mice sensitized with any of several different allergic or nonallergic antigens by repeated exposure to the antigen either through the peritoneal cavity or directly through the airway. The sensitized animals are then challenged, typically via aerosol, some days later with the same specific antigen. AHR and immune profiles are then assessed between 24 and 72 h after challenge. Similar to human disease, the murine immune and airway responses vary by antigen type and route of exposure (25). However, in general, antigen challenge typically results in increased levels of serum IgE and infiltration of eosinophils and T_H2-family cytokines into the airways between 24 and 72 h after the final challenge, thus failing to model the increasingly important form of neutrophilic asthma.

We hypothesized that colonization with NTHi in early life may result in immunological reprogramming that leads to the induction, modification, or exacerbation of airway disease in later life. To test our hypotheses, we examined whether early-life colonization with NTHi either modified or induced asthma-like disease in a mouse model of allergic airway hypersensitivity. We found that early-life colonization with NTHi in 3-day-old mice led to more severe airway disease in juveniles. More severe airway disease postcolonization was accompanied by increased infiltration of immune effector cells (neutrophils and eosinophils) and inflammatory cytokines into the airways, coupled with decreased expression of Treg-associated FOXP3. We also observed that previously NTHi-colonized, allergen-sensitized mice secreted increased amounts of mucus into the airways and had heightened sensitivity to aerosolized bronchoconstrictor medication.

MATERIALS AND METHODS

Bacterial growth and preparation for inoculation. Nontypeable *Haemophilus influenzae* (NTHi) strain TN106.P2 was used for all the described studies. This strain is a spontaneous streptomycin-resistant variant of a strain originally isolated from a patient with pneumonia (26). The strain expresses the HMW1/HMW2 and Hap adhesins. Bacteria were streaked from freezer stocks onto chocolate agar (BD), grown overnight at 37°C in 5% CO_2, and inoculated into brain heart infusion (BHI) broth supplemented with hemin and NAD to an optical density at 600 nm (OD_{600}) of 0.2 as described previously (27). Cultures were grown with shaking to an OD_{600} of 0.8, at which point the bacteria were pelleted, washed in phosphate-buffered saline (PBS), and resuspended in PBS to a final concentration of 2×10^8 CFU/ml. Aliquots of this suspension were diluted and plated to determine the actual inoculating dose for each experiment.

Animal infections. All animal procedures were performed according to protocols approved by the Duke Institutional Animal Care and Use Committee (IACUC). BALB/c mice were bred in the Duke Division of Laboratory Animal Resources breeding facility. Females confirmed to be pregnant were transferred from the breeding facility into biosafety level 2 caged housing. Three-day-old BALB/c mice (weight, ~1.5 g) were held supine with gentle hand restraint and inoculated intranasally with 5×10^5 to 1×10^6 CFU of streptomycin-resistant NTHi strain TN106.P2 in 5 μl of PBS. Control animals received an intranasal dose of 5 μl of PBS alone. Subgroups of animals inoculated with NTHi were euthanized on days 1, 2, 3, 5, 14, 20, 49, and 53 postinoculation. Lung, intestine, liver, spleen, and nasopharyngeal tissues were harvested, homogenized in sterile PBS, and plated on supplemented BHI agar containing 200 μg/ml streptomycin to measure the bacterial burden in each organ or site. Data were analyzed using the GraphPad Prism software package.

Induction of allergic airway disease. We used an ovalbumin (OVA) allergy model to induce airway disease in mice essentially as described previously (8, 28, 29). OVA (catalog number A5378; lot number SLBB770V; Sigma) was absorbed to alum over an hour-long incubation at room temperature with mixing. The absorbed protein-alum conjugate was pelleted and resuspended in PBS. One dose absorbed to a final concentration of 1 mg alum was injected intraperitoneally at either a subsensitizing dose (16 μg, or a low OVA dose) (30) or a sensitizing dose (50 μg, or a high OVA dose) at 1 to 2 weeks after weaning or at 4 to 5 weeks of age. Control animals received alum alone. Seven days later, sensitized animals were placed in a Plexiglas pie chamber (Braintree Scientific) and subjected to aerosolized OVA, which was nebulized by use of a Shuco model S5000 air compressor, at a low dose (0.1% in PBS) or a high dose (1% in PBS) for 15 min each day for 3 days. Control animals were exposed to aerosolized PBS only. On the fourth day, one group of animals was sacrificed for collection of lavage fluid and tissue samples, and another group of animals was subjected to dynamic compliance and total respiratory system resistance measurements using a FlexiVent apparatus (SciReq, Montreal, Quebec, Canada). Briefly, animals subjected to FlexiVent measurement were anesthetized with ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg), injected intraperitoneally. A 19-gauge tracheal cannula was placed by cut down just below the cricothyroid membrane and secured with 5-0 silk. Throughout the procedure, the animals were monitored by electrocardiography and received thermal support with a water blanket. Methacholine was delivered directly into the trachea at the doses indicated below via a nebulizer. At approximately 1 min following the delivery of each aerosol dose, pulmonary function testing was performed by the forced oscillometry technique (31, 32). Following the final measurement, the anesthetized animals were euthanized by intraperitoneal injection of a euthanasia solution (pentobarbital sodium and phenytoin sodium [Euthasol]). The lungs were lavaged, removed, sampled, and fixed as described below.

Sample collection and preparation. Following induction of allergic airway disease, the lungs were inflated at 30 cm of pressure with 10% formalin for 20 min. The cardiac lobe was then submerged in 10% formalin for 24 h. The organs were then moved to 70% ethanol, embedded in paraffin, and sectioned for staining. Duplicate 5-μm sections were mounted onto charged slides. Alternatively, prior to inflation with fixative, the left lobe of the lung was tied off with 5-0 silk at the bronchial branch point, removed, minced, and immediately placed in 1 ml TRIzol reagent (Invitrogen). In other experiments, groups of animals were lavaged with 3 serial 1-ml volumes of ice-cold PBS via a 20-gauge cannula inserted into the trachea. Volumes from each lung were pooled, and infiltrating cells were harvested by centrifugation. Cells were then counted using a hemacytometer, deposited in equal total numbers on charged slides by the cytospin method, and stained using Wright's differential stain. At least 200 cells from each sample were then counted by an analyst

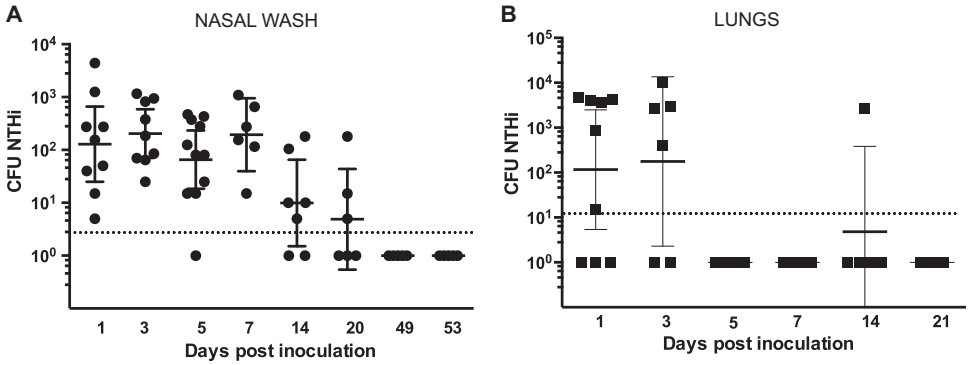

FIG 1 NTHi CFU counts from nasal washes (A) and homogenized lungs (B) on the indicated day postinoculation. The majority of neonatal animals remained colonized with *H. influenzae* until at least 14 days postinfection. Groups of 3-day-old mice were intranasally inoculated with 10^6 CFU of NTHi. Horizontal solid lines, geometric means; error bars, standard deviations of the means; dashed lines, limit of detection. Each symbol represents the total number of CFU in a sample from an individual.

blind to the conditions and categorized on the basis of the staining pattern. The remaining lavage fluid was stored at −80°C.

Immunohistochemistry. Paraffin-embedded lung tissue sections were stained for FOXP3 expression essentially as described previously (33). Briefly, 5-μm sections were mounted onto charged slides, cleared in Histo-Clear clearing agent (National Diagnostics), and rehydrated. Sections were then heated to just below boiling in 10 mM citrate buffer with 0.05% Tween 20 for 10 min, washed in water, and quenched in 0.3% hydrogen peroxide for 10 min. After blocking in 0.2% Roche blocking reagent (Sigma) in PBS–0.05% Tween 20 for 1 h at room temperature, the slides were incubated with purified anti-FOXP3 antibody (eBioscience) or an isotype control antibody (Pierce) at 1:200 in blocking buffer overnight at 4°C. The slides were then washed repeatedly in PBS–0.05% Tween and incubated in 1:200 biotinylated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) for 30 min at room temperature in blocking buffer and then 1:200 streptavidin-linked horseradish peroxidase (Bio-Rad), also in blocking buffer, for 30 min. The signal was detected with 3,3-diaminobenzidine (DAB) substrate (Sigma), and sections were counterstained with Gill's hematoxylin (Polysciences, Inc.). The slides were then dehydrated, cleared, and mounted with Permount mounting medium (Thermo Fisher). Positively stained cells in at least 5 fields from at least 5 samples per experimental group were counted by an analyst blind to the conditions under ×40 magnification.

Cytokine ELISA. Cytokine levels in bronchoalveolar lavage fluid were measured using multi- and single-analyte ELISArray plates (Qiagen) or eBioscience enzyme-linked immunosorbent assay (ELISA) kits for IL-5 and IL-13, following the manufacturer's protocol. In brief, 50 or 100 μl of undiluted bronchoalveolar lavage fluid was added to precoated capture ELISA plates and tested according to the manufacturer's instructions.

Alcian blue-stained mucus quantification. Lungs from mice that had undergone OVA aerosol challenge were inflated, fixed, embedded, sectioned, and stained for acidic mucins with alcian blue and counterstained with nuclear fast red (Sigma) (34). Two independent images from a ×10 magnification for each animal were coded, imaged by an analyst blind to the conditions, and set against a 9-square grid. Sections were given 1 point for each square on the grid that contained alcian blue-stained mucus. The number of points per image was graphed and used to quantify total visible mucus secretion.

qPCR. The left lobe from each lung was homogenized in 1 ml TRIzol reagent (Invitrogen), and RNA was extracted according to the manufacturer's instructions. To measure IL-5 and IL-13 gene transcript levels, 3 μg of total RNA from lung tissue in a 40-μl total volume was transcribed to cDNA using an iScript reverse transcriptase kit (Bio-Rad). The resulting cDNA was diluted 1:1 in nuclease-free water, and 2 μl of this dilution was used in each quantitative PCR (qPCR). Transcript levels of the gene for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were used as internal controls. Primer sets validated to be specific for mouse FOXP3, GAPDH, IL-5, and IL-13 genes were obtained from Integrated DNA Technologies (Coralville, IA). To measure *MUC5AC* and *FOXP3* transcript levels, 2 μg of total RNA was transcribed to cDNA using an oligo(dT)$_{20}$ primer and SuperScript III reverse transcriptase according to the manufacturer's instructions (Invitrogen). Two microliters of cDNA was then used directly in each qPCR. The following cycling conditions were used for all qPCR assays: 1 cycle of 1 min at 95°C and 40 cycles of 15 s at 95°C and 60 s at 60°C. Actual transcript levels were measured by determination of the amount of EvaGreen dye (Biotium Inc., Hayward, CA) incorporated into amplified DNA in a StepOnePlus thermocycler (Thermo Fisher). Negative controls for the qPCRs included samples with no template and no reverse transcriptase (straight RNA). The *MUC5AC*-specific primers used have been described by Ehre et al. (35). The *MUC5AC* transcript was directly quantified against a standard curve. To generate template DNA for the *MUC5AC* standard curve, an intron-spanning portion of *MUC5AC* was directly synthesized (Genewiz, South Plainfield, NJ) and ligated into pUC57-amp.

Statistical analysis. All data were analyzed in GraphPad Prism (version 6.0) software. Statistical significance was determined using Sidak-Bonferroni, Mann-Whitney nonparametric, and Student's *t* tests where appropriate.

RESULTS

Previous studies have demonstrated that respiratory tract colonization with NTHi in immunocompetent adult mice lasts approximately 72 h and is then cleared (36, 37). In contrast, neonatal colonization with NTHi has not been tested. Accordingly, we first sought to determine whether NTHi could establish sustained colonization of neonatal mice. As shown in Fig. 1A, inoculation of 1×10^6 CFU of NTHi directly into the nares of 3-day-old mice resulted in sustained nasal colonization in all mice for 7 days postinoculation and in the majority of mice for 14 days postinoculation. Conversely, this dose and route of inoculation produced only transient colonization of the lungs, with clearance occurring by day 5 in almost all animals (Fig. 1B). NTHi was never recovered by culture from the blood, spleens, intestines, or livers at any time point postcolonization (data not shown), indicating that the bacteria did not spread beyond the respiratory tract. Furthermore, we found that colonization with NTHi resulted in no detectable differences between the growth of the colonized animals and that of the uncolonized controls (weights at 5 days postinfection, 6.5 ± 0.4 g for control mice and 5.9 ± 0.7 g for NTHi-infected mice;

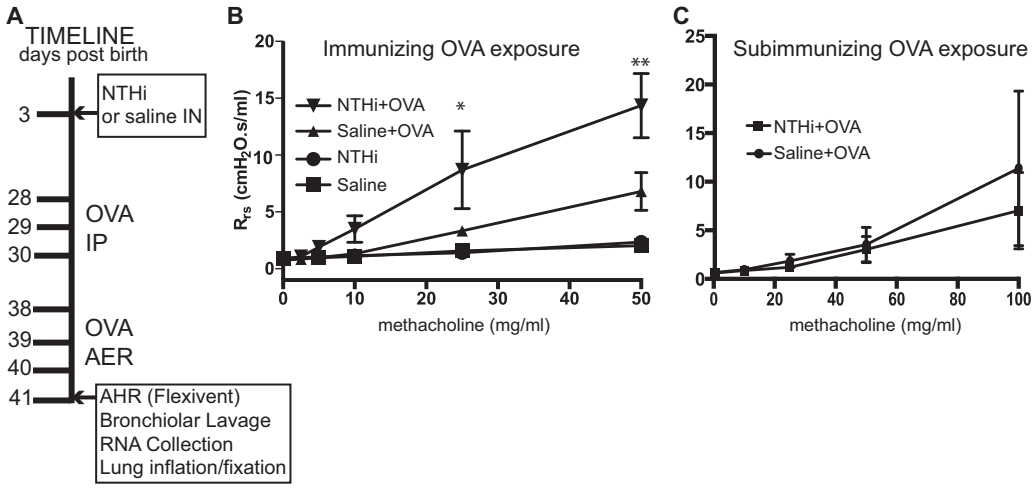

FIG 2 Airway hypersensitivity increased in animals colonized with *H. influenzae* early in life. (A) Sensitization timeline. Neonatal mice were colonized on day of life 3, sensitized, and then challenged with aerosolized OVA for three consecutive days starting 1 week following sensitization. Airway hyperreactivity and immune factors were then measured as described in the text. (B) Animals were given sensitizing doses of OVA by intraperitoneal injection and then challenged with aerosolized OVA. The data shown are representative of those from triplicate experiments. Statistical significance was determined using the Sidak-Bonferroni method. *, $P = 0.004$; **, $P = 0.0004$. (C) Animals were given subsensitizing doses of OVA by intraperitoneal injection and then challenged by aerosolized OVA a week later. The results shown are representative of those from duplicate experiments. Abbreviations: OVA, ovalbumin; IP, intraperitoneal; IN, intranasal; AER, aerosol; AHR, airway hyperreactivity; R_{rs}, respiratory system resistance; NTHi+OVA, NTHi-colonized, OVA-sensitized mice; Saline+OVA, saline-treated, OVA-sensitized mice; Saline, saline-treated mice without OVA sensitization; NTHi, mice colonized with NTHi without OVA sensitization.

weights at 46 days postinfection, 21.5 ± 2.1 g for control mice and 19.31 ± 1.2 g for NTHi-infected mice; $n \geq 10$ mice per group).

We then inoculated neonatal mice with ~10^6 CFU of NTHi or vehicle alone at 3 days of life. This inoculum was chosen to allow the reproducible introduction of bacteria into the small-caliber nasal passage of the neonatal mouse. NTHi-colonized and noncolonized control animals were sensitized according to the timeline in Fig. 2A. Mice were injected with either a sensitizing dose or a subsensitizing dose (as described in the Materials and Methods section) of ovalbumin (OVA) by intraperitoneal injection at 4 weeks of age. A subset of the NTHi-colonized animals and a subset of the noncolonized animals received alum only as an additional control. One week later, these mice were challenged with aerosolized OVA at 0.1% (low dose) or 1% (high dose) on three consecutive days. By using either a low dose or a high dose of OVA, we could test whether NTHi colonization might act as an adjuvant to subsensitizing doses (low OVA dose) of antigen or as an accelerant to sensitizing doses (high OVA dose) of antigen. At approximately 24 h after the last aerosol challenge, the respiratory system resistance of each animal was measured following administration of increasing doses of the bronchoconstrictor methacholine.

As shown in Fig. 2B, when airway disease was induced using sensitizing doses of OVA, animals with early-life exposure to NTHi had increased airway resistance starting at 10 mg/ml methacholine and further increased airway resistance at 25 mg/ml compared with that of animals not colonized in early life (Fig. 2B). There was no significant difference between NTHi-colonized and noncolonized animals when low doses of OVA were administered (Fig. 2C). These data suggest that NTHi exposure in early life primed for more severe airway disease in the juvenile period but did not act as an adjuvant for what would otherwise be subsensitizing antigen exposure.

We next assessed the pulmonary inflammatory status in each of the groups. Twenty-four hours after the final OVA aerosol challenge, the animals were sacrificed and the lavaged contents of their lungs were analyzed for immune cell infiltration. We found that significantly more infiltrating eosinophils and neutrophils were recovered from the lung lavage fluid of NTHi-colonized, OVA-sensitized animals than from the lung lavage fluid of control, allergic animals (Fig. 3). There was no significant difference between the numbers of infiltrating macrophages or total lymphocytes in NTHi-colonized animals and the numbers in noncolonized animals following the induction of airway disease (data not shown). These data suggest that the early-life presence of NTHi influenced later-life immune signaling to dictate increased cellular infiltration during an acute allergic event.

Cellular infiltration into the lung is driven by cytokine chemoattractants. With this information in mind, we performed an initial multianalyte ELISA with samples from a limited number of NTHi-colonized allergic animals and sham-colonized allergic animals to screen for the upregulation of several different cytokines involved in T_H1, T_H2, and T_H17 immune responses (data not shown). The T_H1 cytokines screened for were IL-2, IL-10, IL-12, gamma interferon (IFN-γ), and TNF-α, while the T_H2-related cytokines screened for were IL-2, IL-4, IL-5, IL-10, and IL-13. Cytokines IL-6, IL17A, IL-23, TNF-α, and transforming growth factor β1 (TGF-β1) are associated with T_H17 cells. In this initial assay, we observed measurable differences only in the levels of IL-5 and IL-13, cytokines that are involved in recruiting eosinophils to the lung (38). Thus, we tested the bronchoalveolar lavage fluid for the presence of these cytokines by quantitative, single-analyte sandwich ELISA. We found that the lung lavage fluid from NTHi-colonized, OVA-sensitized animals had significantly higher levels of both IL-13 and IL-5 (Fig. 4A and B) than the lung lavage fluid from the control OVA-sensitized animals. These data were reflected in the IL-5 and IL-13 gene expression data, in which, following induction of airway disease, animals colonized at day of life 3 subsequently had higher but not significantly higher IL-5 and

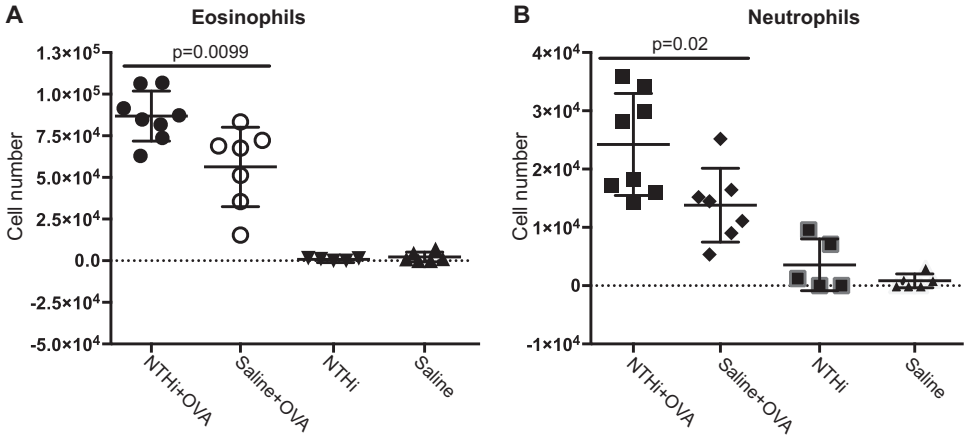

FIG 3 The lungs of mice colonized by NTHi as neonates prior to induction of airway disease had greater eosinophil and neutrophil infiltration than the lungs of mice treated with saline. Cells were isolated from bronchoalveolar lavage fluid and categorized on the basis of the differential staining pattern. Abbreviations: NTHi+OVA, NTHi-colonized, OVA-sensitized mice; Saline+OVA, saline-treated, OVA-sensitized mice; Saline, saline-treated mice without OVA sensitization; NTHi, mice colonized with NTHi without OVA sensitization. The statistical significance of the differences between the NTHi-colonized, OVA-sensitized and saline-treated, OVA-sensitized groups was determined using the Mann-Whitney nonparametric test.

IL-13 transcript levels (Fig. 4C) than animals that were not colonized as neonates.

Regulatory T cells (Treg) can dampen inflammation in the lung in mouse models of allergic airway disease, restricting IL-5 and IL-13 production, which is reflected in lower measured levels in lavage fluid (4). We hypothesized that lung tissue from NTHi-colonized, OVA-sensitized mice may have fewer functional Treg and measured expression of *FOXP3*, a marker of Treg abundance and activity (39). We found that NTHi-colonized, OVA-sensitized mice had significantly less *FOXP3* expression in lung tissue than all other experimental groups (Fig. 5A). To determine if we could observe fewer Treg in the lung tissue of NTHi-colonized, OVA-sensitized mice than in the lung tissue of the control groups, we immunostained paraffin-embedded, sectioned lung tissue for the FOXP3 protein. We found that saline-treated, OVA-sensitized mice had more detectable FOXP3-expressing cells than the NTHi-colonized, OVA-sensitized groups (Fig. 5B), suggesting that a greater number of Treg had infiltrated the airways of the saline-treated, OVA-sensitized mice.

As another approach to quantify the level of airway obstruction during allergic airway disease, we measured the amount of mucus secreted into the airway and the upregulation of *MUC5AC*, the major driver of mucus production in mice (35). As show in Fig. 6A, we found that *MUC5AC* transcript levels trended higher but not significantly higher in juvenile animals that were colonized as neonates and then sensitized to OVA (Fig. 6A). Consistent with the higher *MUC5AC* transcript levels, mice that were colonized with NTHi as neonates had more visible mucus in the major airways than mice that were not previously colonized by NTHi (Fig. 6B and C).

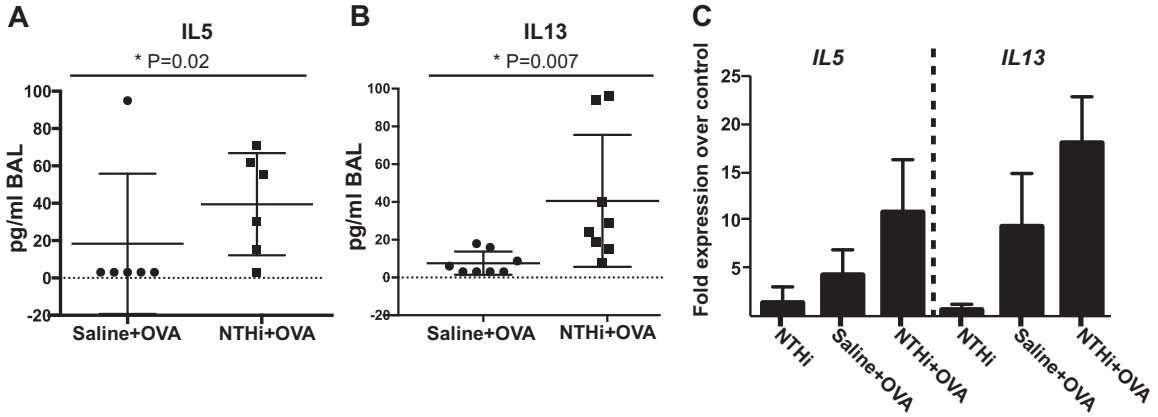

FIG 4 Animals colonized on day of life 3 had higher levels of IL-5 and IL-13 cytokine infiltration into the lung lavage fluid than noncolonized animals after induction of airway disease. Bronchoalveolar lavage fluid was collected from each animal approximately 24 h after airway induction and tested by sandwich ELISA for the presence of the cytokine IL-5 (A) or IL-13 (B). (C) IL-5 and IL-13 gene transcript levels following induction of airway disease were internally normalized to the level of GAPDH expression and expressed as the fold increase over the levels in mice treated with saline only. The data shown are representative of those from two replicate experiments. Abbreviations and symbols: BAL, bronchoalveolar lavage fluid; NTHi+OVA, NTHi-colonized, OVA-sensitized mice; Saline+OVA, saline-treated, OVA-sensitized mice; Saline, saline-treated mice without OVA sensitization; NTHi, NTHi-colonized mice without OVA sensitization; horizontal bars, means; error bars, standard deviations. The statistical significance of the differences between NTHi-colonized, OVA-sensitized mice and saline-treated, OVA-sensitized mice was determined using the Mann-Whitney nonparametric test.

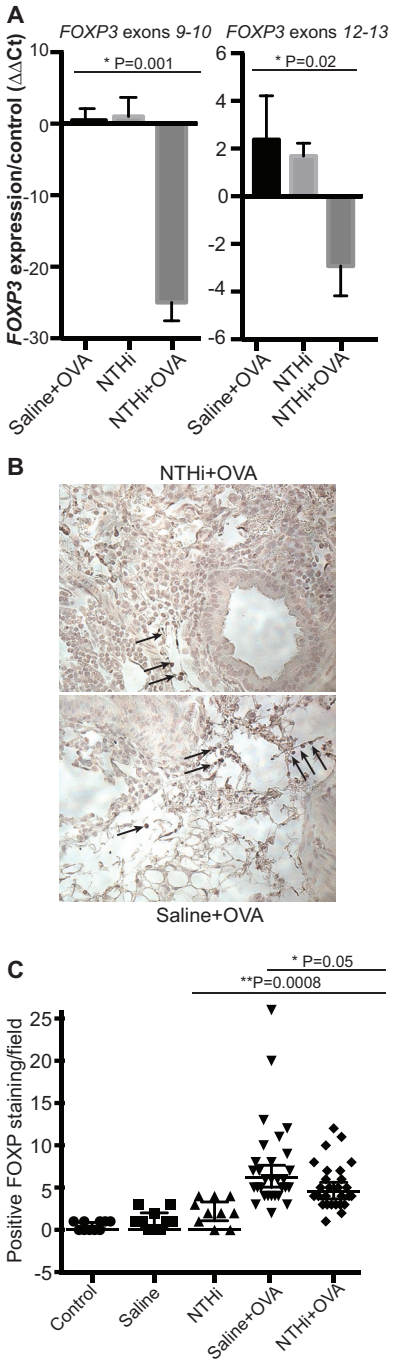

FIG 5 NTHi-colonized, OVA-sensitized mice (NTHi+OVA) have lower levels of *FOXP3* expression in lung tissue than saline-treated, OVA-sensitized mice (Saline+OVA). (A) Lung *FOXP3* transcript levels from NTHi-colonized, OVA-sensitized mice, saline-treated, OVA-sensitized mice, and animals colonized with NTHi only were measured by qPCR using the transcript level of the GAPDH gene as an internal control. Transcript levels were normalized to lung-derived transcript levels from mice treated with saline only. Error bars, means and standard deviations from one experiment. Data are representative of those from two replicate experiments. The statistical significance of the difference between NTHi-colonized, OVA-sensitized mice and saline-treated, OVA-sensitized mice was determined using a Student *t* test. Ct, threshold cycle. (B) Lungs from mice that had undergone OVA aerosol challenge were sectioned, and sections were immunostained for FOXP3 and then counterstained with hematoxylin. Arrows, instances of positive staining. (C) Cells that stained positively for FOXP3 in ≥5 fields on ≥5 sections per experimental group were counted under ×40 magnification. Error bars, 95% confidence intervals of the geometric means for each data group. The statistical significance of the difference between the NTHi-colonized, OVA-sensitized and the saline-treated, OVA-sensitized groups and the group colonized with NTHi only and the NTHi-colonized, OVA-sensitized group was determined using the Mann-Whitney nonparametric test.

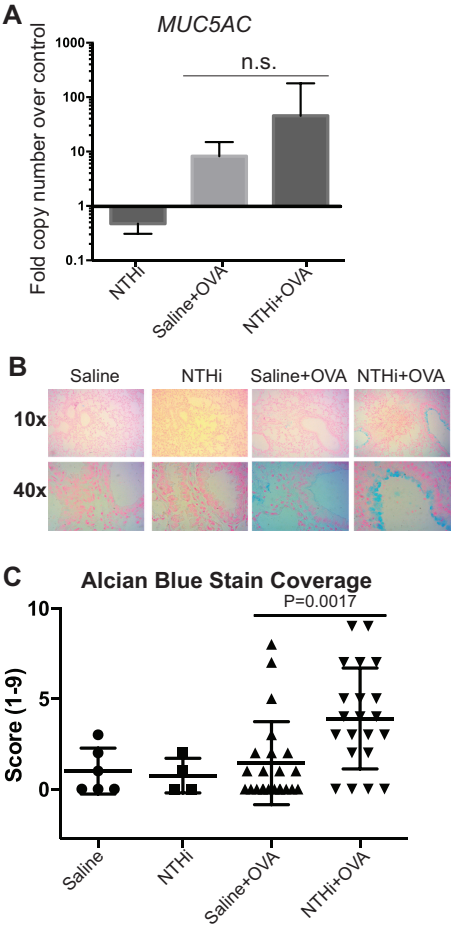

FIG 6 There is more visible mucus in the lungs of NTHi-colonized, OVA-sensitized mice (NTHi+OVA) after allergic airway disease induction than in the lungs of saline-treated, OVA-sensitized mice (Saline+OVA). (A) *MUC5AC* transcript levels in the lungs of NTHi-colonized, OVA-sensitized mice, saline-treated, OVA-sensitized mice, and animals colonized with NTHi only were directly measured against a standard curve. Transcript levels were normalized to lung-derived transcript levels from mice treated with saline only. Error bars, means and standard deviations from one experiment. Data are representative of those from three replicate experiments. n.s., not significant. (B) Lungs from mice that had undergone OVA aerosol challenge were sectioned, stained for acidic mucins with alcian blue, and counterstained with nuclear fast red. (C) Two independent images from a ×10 magnification for each animal were coded, imaged, and scored as described in Materials and Methods. The total number of points was averaged and compared between experimental groups. Bars, means and standard deviations for all scores from each experimental group. The statistical significance of the differences between the NTHi-colonized, OVA-sensitized and the saline-treated, OVA-sensitized groups was determined using the Mann-Whitney nonparametric test.

DISCUSSION

The objective of this study was to test whether early-life colonization with an opportunistic pathogen and common resident of the upper respiratory tract (URT) changes long-term immune responses to allergens and the manifestations of airway hyperre-

sponsiveness. The association between acute upper respiratory microbial colonization and the development of allergic disease is complex. There is emerging evidence that early-life exposure to microbes and environmental antigens has lifelong consequences that impact immune system development (40–45). Early-life respiratory tract infections with viral agents, such as respiratory syncytial virus, rhinovirus, and influenza virus, increase the odds ratio for the development of inflammatory diseases like asthma later in childhood (46). In contrast, the long-term consequences of nasopharyngeal colonization with specific bacteria during the neonatal period have yet to be as well delineated. Skewing the microbial milieu during early life is likely to durably change the host immune response (47–49). Thus, early antibiotic exposure would affect the microbiome, as would domination by particular species. New therapies that abrogate or delay colonization by specific taxa in the upper respiratory tract, in the gut, and on the skin may have long-lasting benefits, particularly in those individuals with genetic backgrounds that contribute to a higher for risk for asthma and allergy.

We found that juvenile animals colonized with NTHi as neonates had significantly less resistance to an inhaled bronchoconstrictor drug, methacholine. Airway constriction signaling in asthma involves IL-13. This cytokine induces phosphoinositide 3-kinase γ (PI3Kγ) activity, which in turn directly regulates airway smooth muscle contraction (50). We observed elevated levels of IL-13 in mice colonized as neonates following induction of allergic airway disease. Thus, the elevated levels of IL-13 in the lungs may have had direct or indirect effects on the immune response in NTHi-colonized animals after administration of methacholine.

We also found that induction of allergic disease in the juvenile period resulted in the infiltration of more eosinophils and more neutrophils into the lungs of mice that were colonized with NTHi as neonates than into those of animals that were exposed to saline. This infiltration with inflammatory cells occurred even though there were no detectable bacteria in the nasopharyngeal passage or the lungs at the time of OVA exposure. We did a semiquantitative initial measurement of lung lavage fluid and found that IL-5 and IL-13 levels were increased in NTHi-colonized, OVA-sensitized mice compared with those in the mice in the other experimental groups. Neutrophil chemoattractants, such as IL-8 and IL-17, and the downstream effector cytokines IL-1β and TNF-α were not seen in measurable quantities in our initial screen, but future experiments will include detailed investigations of the host immune pathways that lead to increased neutrophil infiltration in the absence of acute infection. NTHi has been implicated in the induction of a T_H17 response in a mouse model of airway disease. Essilfie et al. (8) found that neutrophil, T_H17 cell, and IL-17 levels were elevated in the lungs of animals with induced airway disease and exposure to NTHi compared to their levels in the lungs of animals with induced airway disease and no NTHi exposure.

It has recently been hypothesized that early-life exposure to commensal organisms via the gut may regulate airway immune responses via regulatory T cell and dendritic cell production of immunosuppressive cytokines, such as TGF-β and IL-10 (51, 52). Indeed, early-life and later-life exposure to gut commensal organisms ameliorates OVA-induced allergic airway disease phenotypes via a Treg-dependent suppression of inflammation (53). Notably, colonization of adults did not elicit the suppressive effects on asthma-like symptoms associated with early-life commensal colonization (53), pointing yet again to a critical window of immune programming susceptibility early in life. We observed that early-life upper respiratory tract exposure to NTHi appeared to have the opposite effect on Treg-mediated immune suppression (Fig. 5), as there were significantly fewer *FOXP3*-expressing cells in the NTHi-colonized, OVA-sensitized mice than the saline-treated, OVA-sensitized group. These data suggest as of yet undelineated mechanisms in early-life exposure to this potential pathogen that lead to the dysregulation of the appropriate control mechanisms required to limit pathological inflammatory responses later in life.

In our mouse model, we demonstrated that early-life colonization with NTHi leads to greater mucus secretion in mice after the induction of airway disease. Mucus secretion, like airway constriction, is dependent in part on IL-13 signaling (54), and the increased mucus secretion that we see in our airway disease model may be due to the higher IL-13 levels seen in the lung lavage fluid following early-life NTHi colonization. It has been demonstrated that NTHi can directly stimulate MUC5AC production in the mouse via signaling downstream of Toll-like receptor 2 in a model of bacterial exposure to NTHi purified surface protein (55). However, the heightened IL-13 response in the juvenile animals is not likely directly secondary to persistent NTHi lung colonization, since NTHi was not recovered from the lungs of almost all the colonized animals at later than 7 days postinoculation.

The immune system components that undergo enduring changes as a consequence of early-life microbial exposure and lead to the differences that we observed in the current study are unclear. The description of the infant respiratory microbiome is only just beginning (45, 56, 57), but a recent study found an association between early-life *H. influenzae* nasopharyngeal colonization and microbial population perturbations brought on by viral infection and antibiotic treatment (49). A long-term study performed in Denmark showed that children who exhibited signs of asthma and wheezing by age 7 years had an abnormal immune response to *H. influenzae* at age 6 months, as measured by cytokine levels and T-cell responses in stored peripheral blood mononuclear cells (PBMCs) obtained from the study participants. PBMCs from these samples were stimulated with *H. influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* and expressed aberrant levels of IL-5 and IL-13 and decreased levels of the type 1 cytokines IFN-γ and TNF-α compared to PBMCs from samples from control, age-matched children who did not develop asthma symptoms (58).

The interaction of microbe-associated molecular pattern receptors with their ligands may be developmentally specific and therefore underlie the ability of particular organisms to program later immune responses. Interestingly, recent work by Siegel et al. (59) demonstrates that following pneumococcal colonization macrophages from infant mice undergo altered trafficking to the nasopharynx compared with macrophages from animals first colonized as adults. This leads to delayed clearing of colonizing *Streptococcus pneumoniae* cells from the upper respiratory tract in infants compared to the rates of bacterial clearance in adult animals. Further research will be needed to understand the different types of bacteria and the host factors that interact and potentiate lifelong exacerbated or atypical immune responses.

The ability of NTHi colonization to drive later airway hyperresponsiveness may be shared among several organisms that stimulate similar outcomes. It is also possible that the mechanism is not due to NTHi colonization *per se* but instead is a consequence

of an NTHi-induced dysbiosis of the typical nasal microbiota. Our tractable murine model should be amenable for use for the dissection of these nonexclusive mechanisms and further identification of the links between assembly of the early-life nasopharyngeal-respiratory tract microbiota and later inflammatory disorders.

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