

1

2 **Early life intranasal colonization with nontypeable *Haemophilus influenzae***
3 **exacerbates juvenile airways disease in mice**

4

5 **Running title: Early NTHi colonization exacerbates airways disease**

6

7 Jessica R. McCann^{1#}, Stanley N. Mason¹, Richard L. Auten¹, Joseph W. St. Geme, III²,
8 and Patrick C. Seed^{1,3}

9

10 ¹Duke University School of Medicine, Department of Pediatrics, Durham NC 27710

11 ²Children's Hospital of Philadelphia, Philadelphia, PA 19104

12 ³Duke University School of Medicine, Department of Molecular Genetics and
13 Microbiology, Durham NC 27710

14 #, Corresponding author email address: jessica.mccann@duke.edu

15

16

17 **ABSTRACT**

18 Accumulating evidence suggests a connection between asthma development and
19 colonization with nontypeable *Haemophilus influenzae* (NTHi). Specifically,
20 nasopharyngeal colonization of human infants with NTHi within 4 weeks of birth is
21 associated with an increased risk of asthma development later in childhood. Monocytes
22 derived from these infants have aberrant inflammatory responses to common upper
23 respiratory bacterial antigens when compared to cells derived from infants who were not
24 colonized and do not go on to develop asthma symptoms in childhood. In this study, we
25 hypothesized that early life colonization with NTHi promotes immune system
26 reprogramming and the development of atypical inflammatory responses. To address
27 this hypothesis in a highly controlled model, we tested whether colonization of mice with
28 NTHi on day of life 3 induced or exacerbated juvenile airways disease using an
29 ovalbumin (OVA) allergic model of asthma. We found that animals that were colonized
30 on day of life 3 and subjected to induction of allergy had exacerbated airways disease
31 as juveniles, defined as increased cellular infiltration into the lung, increased
32 inflammatory cytokines IL5 and IL13 in the lung lavage fluid, decreased regulatory T
33 cell-associated *FOXP3* gene expression, and increased mucus production. We also
34 found that colonization with NTHi amplified airway resistance in response to increasing
35 doses of a bronchoconstrictor following OVA immunization and challenge. Together, the
36 murine model provides evidence for early life immune programming that precedes the
37 development of juvenile airways disease and corroborates observations in human
38 children.

39

40 INTRODUCTION

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

46 Asthma has been classified traditionally as a type 1 hypersensitivity disease that is
47 characterized by a T_H2 response and the production of IL4, IL5, and IL13. These
48 cytokines play pivotal roles in mediating IgE production, mucus secretion, and airway
49 hyperreactivity (AHR). Inflammation also promotes infiltration of regulatory T cells
50 (Treg), which act to dampen IL5 and IL13 secretion by CD4⁺ CD25⁻ T_H2 cells in both
51 human and mouse models of asthma (4, 5). Along with infiltration of eosinophils, these
52 responses in the airways have been considered a defining feature of asthma. However,
53 recent studies indicate that eosinophils are prominent in only 50% of asthmatic patients
54 (2, 3, 6).

55 A subtype of non-eosinophilic asthma called neutrophilic asthma is now recognized to
56 afflict a significant number of patients and is associated with severe and persistent
57 symptoms that are refractory to treatment with corticosteroids—a main stay of moderate-
58 severe asthma treatment (6, 7). Neutrophilic asthma is characterized by sputum with an
59 increased number of neutrophils and increased levels of the proinflammatory cytokines
60 IL8 and IL17 (6, 8, 9), known neutrophil chemoattractants (10). IL17 also induces the
61 secretion of several other proinflammatory cytokines and chemokines, including IL1 β ,
62 TNF α , and IL8 (11). These cytokines are typically linked to a T_H1 inflammatory immune

63 profile, which is also associated with infiltration of macrophages and effector T cells that
64 coordinate cell mediated immunity (12, 13). Since asthma treatment is directed to
65 symptoms, it follows that differing inflammatory pathways that are invoked in
66 neutrophilic asthma may need different therapeutic approaches.

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

77 There is mounting evidence that NTHi has a role in the development and
78 exacerbation of asthma. In recent work, Bisgaard *et al.* found that children colonized
79 with NTHi within the first four weeks of life were 2-4 times more likely to develop
80 symptoms of asthma or be hospitalized for wheezing by age 5 years compared to
81 children without detectable NTHi in the nasopharynx (20). Notably, colonization at later
82 than 4 weeks of life was not associated with greater risk of asthma symptoms, indicating
83 that early exposure is required for development of asthma. This information suggests
84 that exposure during a vulnerable window may result in reprogramming of the immune
85 system. In related work, Hilty and co-workers observed that *Haemophilus* was

86 significantly more likely to be present in the lower airways of adult patients with asthma
87 than in patients with normal lung function (21). Finally, in studies examining induced
88 sputum from symptomatic or asymptomatic asthmatics, *Haemophilus* was among the
89 most common organisms isolated and was associated with the presence of increased
90 neutrophils in the airway (22, 23). Taken together, these data suggest that the presence
91 of *Haemophilus* may modify or exacerbate the immune response typically seen during
92 an inflammatory airway event such as an acute asthma attack.

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

107 We hypothesized that colonization with NTHi in early life may result in immunological
108 reprogramming that leads to induction, modification, or exacerbation of airways disease

109 in later life. To test our hypotheses, we examined whether early life colonization with
110 NTHi either modified or induced asthma-like disease in a mouse model of allergic
111 airways hypersensitivity. We found that early life colonization with NTHi in 3 day-old
112 mice led to more severe airways disease in juveniles. More severe airways disease
113 post-colonization was accompanied by increased infiltration of immune effector cells
114 (neutrophils and eosinophils) and inflammatory cytokines into the airways, coupled with
115 decreased expression of Treg-associated FOXP3. We also observed that previously
116 NTHi colonized, allergen sensitized mice had increased secretion of mucus into the
117 airways and heightened sensitivity to aerosolized bronchoconstrictor medication.

118

119 **METHODS**

120 **Bacterial growth and preparation for inoculation.** Nontypeable *Haemophilus*
121 *influenzae* (NTHi) strain TN106.P2 was used for all the described studies. This strain is
122 a spontaneous streptomycin-resistant variant of a strain originally isolated from a patient
123 with pneumonia (26). The strain expresses the HMW1/HMW2 and Hap adhesins.
124 Bacteria were streaked from freezer stocks onto chocolate agar (BD), grown overnight
125 at 37°C in 5% CO₂, and inoculated into BHI broth supplemented with hemin and NAD to
126 OD₆₀₀ 0.2 as described previously (27). Cultures were grown with shaking to OD₆₀₀ 0.8,
127 at which point bacteria were pelleted, washed in PBS, and resuspended in PBS to a
128 final concentration of 2x10⁸ CFU/ml. Aliquots of this suspension were diluted and
129 plated to determine the actual inoculating dose for each experiment.

130 **Animal infections.** All animal procedures were performed according to protocols
131 approved by the Duke Institutional Animal Care and Use Committee (IACUC). BALB/c

132 mice were bred in the Duke DLAR breeding facility. Confirmed pregnant females were
133 transferred from the breeding facility into BSL-2 caged housing. Three-day-old (~1.5 g)
134 BALB/c mice were held supine with gentle hand restraint and inoculated intranasally
135 with 5×10^5 to 1×10^6 colony forming units of streptomycin-resistant NTHi strain TN106.P2
136 in 5 μ l of PBS. Control animals received an intranasal dose of 5 μ l of PBS alone.
137 Subgroups of animals inoculated with NTHi were euthanized on days 1, 2, 3, 5, 14, 20,
138 49, and 53 post inoculation. Lung, intestine, liver, spleen, and nasopharyngeal tissue
139 was harvested, homogenized in sterile PBS, and plated on supplemented BHI agar
140 containing 200 μ g/ml streptomycin to measure bacterial burden in each organ or site.
141 Data were analyzed using the Graphpad Prism software package.

142 **Induction of allergic airways disease.** We used an ovalbumin (OVA) allergy model to
143 induce airways disease in mice essentially as described (8, 28, 29). OVA (Sigma, Cat
144 #A5378 lot SLBB770V) was absorbed to alum over an hour-long incubation at room
145 temperature with mixing. The absorbed protein-alum conjugate was pelleted and
146 resuspended in PBS. One dose absorbed to a final concentration of 1 mg alum was
147 injected intraperitoneally at either a sub-sensitizing (16 μ g, or Low OVA) (30) or
148 sensitizing (50 μ g, or High OVA) concentration one to two weeks after weaning, or at 4-
149 5 weeks of age. Control animals received alum alone. Seven days later, sensitized
150 animals were subjected to OVA by aerosol in a Plexiglas™ pie chamber (Braintree
151 Scientific) nebulized by a Shuco Model S5000 air compressor at a low dose (0.1% in
152 PBS) or high dose (1% in PBS) for 15 min each day for 3 days. Control animals were
153 exposed to aerosolized PBS only. On the fourth day, one group of animals were
154 sacrificed for collection of lavage and tissue samples were collected, and another group

155 of animals were subjected to dynamic compliance and total respiratory system
156 resistance measurements using a Flexivent apparatus (SciReq, Montreal, Canada).
157 Briefly, animals subjected to Flexivent measurement were anesthetized with ketamine
158 (100 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally. A 19 gauge tracheal
159 cannula was placed by cutdown just below the cricothyroid membrane and secured with
160 5-0 silk. Throughout the procedure, animals were monitored by ECG and received
161 thermal support with a water blanket. Methacholine at the indicated doses was delivered
162 via nebulizer directly into the trachea. Approximately 1 minute following each aerosol
163 dose delivery, pulmonary function testing was performed by the forced oscillometry
164 technique (31, 32). Following the final measurement, the anesthetized animals were
165 euthanized by intraperitoneal injection with Euthasol™. Lungs were lavaged, removed,
166 sampled, and fixed as described below.

167 **Sample collection and preparation.** Following induction of allergic airways disease,
168 lungs were inflated at 30 cm of pressure with 10% formalin for 20 minutes. The cardiac
169 lobe was then submerged in 10% formalin for 24 hours. Organs were then moved to
170 70% ethanol, embedded in paraffin, and sectioned for staining. Duplicate 5 µm sections
171 were mounted onto charged slides. Alternatively, prior to inflation with fixative, the left
172 lobe of the lung was tied off with 5-0 silk at the bronchial branch point, removed,
173 minced, and placed immediately in 1 ml Trizol (Invitrogen). In other experiments, groups
174 of animals were lavaged with 3 serial 1 ml volumes of ice cold PBS via a 20 g cannula
175 inserted into the trachea. Volumes from each lung were pooled, and infiltrating cells
176 were harvested by centrifugation. Cells were then counted using a hemacytometer,
177 deposited in equal total numbers on charged slides by cytopsin, and stained using

178 Wright's differential stain. At least 200 cells from each sample were then counted by a
179 blinded analyst and categorized based on staining pattern. The remaining lavage fluid
180 was stored at -80°C.

181 **Immunohistochemistry** Paraffin embedded lung tissue sections were stained for
182 FOXP3 expression essentially as described (33). Briefly, 5 µm sections were mounted
183 onto charged slides, cleared in HistoClear (National Diagnostics) and rehydrated.
184 Sections were then heated to just below boiling in 10 mM citrate buffer with 0.05%
185 Tween 20 for 10 minutes, washed in water, and quenched in 0.3% hydrogen peroxide
186 for 10 min. After blocking in 0.2% Roche Blocking Reagent (Sigma) in PBS 0.05%
187 Tween 20 for 1 h at room temperature, slides were incubated with purified anti-FOXP3
188 (eBioscience) antibody or isotype control (Pierce) at 1:200 in blocking buffer overnight
189 at 4°C. Slides were then washed repeatedly in PBS 0.05% Tween, incubated in 1:200
190 biotinylated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) for 30
191 min at room temperature in blocking buffer, then 1:200 streptavidin linked horseradish
192 peroxidase (Biorad) for 30 min, also in blocking buffer. Signal was detected with 3,3-
193 Diaminobenzidine (DAB) substrate (Sigma) and sections were counterstained with Gill's
194 hematoxylin (Polysciences, Inc.). Slides were then dehydrated, cleared, and mounted
195 with Permount (Thermo-Fisher). Positively stained cells in at least 5 fields at 40x
196 magnification from at least 5 samples per experimental group were counted by a
197 blinded analyst.

198 **Cytokine ELISA.** Cytokine levels in bronchoalveolar lavage fluid were measured using
199 multi- and single analyte ELISArray plates (Qiagen) or eBioscience ELISA kits for IL5
200 and IL13, following the manufacturer's protocol. In brief, 50 or 100 µl of undiluted

201 bronchoalveolar lavage fluid was added to precoated capture ELISA plates and tested
202 according to the manufacturer's instructions.

203 **Alcian blue-mucus staining quantification.** Lungs from mice that had undergone
204 OVA aerosol challenge were inflated, fixed, embedded, sectioned, and stained for acidic
205 mucins with Alcian blue and counter stained nuclear fast red (Sigma) (34). Two
206 independent images from a 10x magnification for each animal were coded, imaged by a
207 blinded analyst, and set against a 9-square grid. Sections were given 1 point for each
208 square on the grid that contained Alcian blue-stained mucus. The number of points per
209 image were graphed and used to quantify total visible mucus secretion.

210 **Quantitative PCR.** The left lobe from each lung was homogenized in 1 ml Trizol
211 (Invitrogen), and RNA was extracted according to the manufacturer's instructions. To
212 measure *IL5* and *IL13* transcript, 3 µg of total RNA from lung tissue were transcribed to
213 cDNA using the iScript reverse transcriptase kit (BioRad) in a 40 µl total volume.
214 Resulting cDNA was diluted 1:1 in nuclease free water, and 2 µl of this dilution was
215 used in each qPCR reaction. *GAPDH* transcript levels were used as internal controls.
216 *FOXP3*, *GAPDH*, *IL5*, and *IL13* mouse specific validated primer sets were obtained
217 from Integrated DNA Technologies (Coralville, IA). To measure *MUC5AC* and *FOXP3*
218 transcript, 2 µg of total RNA was transcribed to cDNA using oligo₍₂₀₎dT primer and
219 Superscript III reverse transcriptase according to the manufacturer's instructions
220 (Invitrogen). Two µl of cDNA was then used directly in each qPCR reaction. The
221 following cycling conditions were used for all qPCR assays: One cycle of 1 min at 95°C
222 and 40 cycles of 15 s at 95°C and 60 s at 60°C. Actual transcript was measured by
223 EvaGreen (Biotium Inc., Hayward, CA) incorporation into amplified DNA in a

224 StepOnePlus (Thermo-Fisher) thermocycler. Negative qPCR controls included no
225 template and no reverse transcriptase (straight RNA) samples. *MUC5AC* primers used
226 are described in Ehre *et al.* (35). *MUC5AC* transcript was directly quantified against a
227 standard curve. To generate template DNA for the *MUC5AC* standard curve, an intron-
228 spanning portion of *MUC5AC* was directly synthesized (Genewiz, South Plainfield, NJ)
229 and ligated into pUC57-amp.

230 **Statistical Analysis.** All data were analyzed in Graph Pad Prism Version 6.0. Statistical
231 significance was determined using Sidak-Bonferroni, Mann-Whitney nonparametric, and
232 student's t-tests where appropriate.

233 RESULTS

234 Previous studies have demonstrated that respiratory tract colonization with NTHi in
235 immunocompetent adult mice lasts approximately 72 hours and is then cleared (36, 37).
236 In contrast, neonatal colonization with NTHi has not been tested. Accordingly, we first
237 sought to determine whether NTHi could establish sustained colonization of neonatal
238 mice. As shown in Figure 1A, inoculation of 1×10^6 colony-forming units (CFU) of NTHi
239 directly into the nares of 3-day old mice resulted in sustained nasal colonization in all
240 mice for 7 days and in the majority of mice for 14 days post inoculation. Conversely,
241 this dose and route of inoculation produced only transient colonization of the lungs, with
242 clearance by day 5 in almost all animals (Fig 1B). NTHi was never recovered by culture
243 from the blood, spleens, intestines, or livers at any time point post colonization (data not
244 shown), indicating that the bacteria did not spread beyond the respiratory tract.
245 Furthermore, we found that colonization with NTHi resulted in no detectable differences
246 in the growth of the animals relative to uncolonized controls (weight at 5 d post infection:

247 control, 6.5 +/- 0.4 g; NTHi, 5.9 +/- 0.7 g. Weight at 46 d post infection: control, 21.5 +/-
248 2.1 g; NTHi, 19.31 +/- 1.2 g. n≥10 mice per group).

249 We then inoculated neonatal mice with ~10⁶ CFU of NTHi or vehicle alone at 3 days
250 of life, chosen to allow for reproducible introduction of bacteria into the small caliber
251 nasal passage of the neonatal mouse. NTHi-colonized and non-colonized control
252 animals were sensitized according to the timeline in Figure 2A. Mice were injected with
253 either a sensitizing or sub-sensitizing (as described in the Methods section) dose of
254 ovalbumin (OVA) by intraperitoneal injection at 4 weeks of age. A subset of the NTHi-
255 colonized and a subset of the non-colonized animals received alum only as an
256 additional control. One week later, these mice were challenged with aerosolized OVA at
257 0.1% (low) or 1% (high) on three consecutive days. By using either low or high doses of
258 OVA, we could test whether NTHi colonization might act as adjuvant to sub sensitizing
259 doses (low OVA) of antigen or as an accelerant to sensitizing doses (high OVA) of
260 antigen. Approximately 24 hours after the last aerosol challenge, the respiratory system
261 resistance of each animal was measured following administration of increasing doses of
262 the bronchoconstrictor methacholine.

263 As shown in Figure 2B, when airways disease was induced using sensitizing doses of
264 OVA, animals with early life exposure to NTHi had increased airways resistance starting
265 at 10 mg/ml methacholine, and further increased airways resistance at 25 mg/ml when
266 compared with animals not colonized in early life (Fig. 2B). There was no significant
267 difference between NTHi-colonized and non-colonized animals when low doses of OVA
268 were administered (Fig 2C). These data suggest that NTHi exposure in early life primed

269 for more severe airways disease in the juvenile period, but did not act as an adjuvant for
270 what would otherwise be sub-sensitizing antigen exposure.

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

281 Cellular infiltration into the lung is driven by cytokine chemoattractants. With this
282 information in mind, we performed an initial multianalyte ELISA on a limited number of
283 NTHi-colonized-allergic and sham-colonized-allergic animals to screen for upregulation
284 of several different cytokines involved in T_H1 , T_H2 and T_H17 immune responses (data
285 not shown). The T_H1 cytokines screened were IL2, IL10, IL12, $IFN\gamma$, and $TNF\alpha$, while
286 the T_H2 -related cytokines were IL2, IL4, IL5, IL10, and IL13. Cytokines IL6, IL17A, IL23,
287 $TNF\alpha$, and $TGF\beta1$ are associated with T_H17 cells. In this initial assay, we observed
288 measurable differences in only IL5 and IL13, cytokines that are involved in recruiting
289 eosinophils to the lung (38). Thus, we tested the bronchoalveolar lavage fluid for the
290 presence of these cytokines by quantitative, single-analyte sandwich ELISA. We found
291 that lung lavage fluid from NTHi colonized-OVA sensitized animals had significantly

292 higher levels of both IL13 and IL5 (Figs 4A and 4B) than the control OVA sensitized
293 animals. These data were reflected in the IL5 and IL13 gene expression data, in which
294 animals colonized at day of life 3 subsequently had higher but not significantly higher
295 IL5 and IL13 transcript levels (Fig 4C) following induction of airways disease compared
296 to animals that were not colonized as neonates.

297 Regulatory T cells (Treg) can dampen inflammation in the lung in mouse models of
298 allergic airways disease, restricting IL5 and IL13 production that is reflected in lower
299 measured levels in lavage fluid (4). We hypothesized lung tissue from NTHi colonized,
300 OVA sensitized mice may have fewer functional Treg cells and measured expression of
301 *FOXP3*, a marker of Treg abundance and activity (39). We found that NTHi colonized,
302 OVA sensitized mice had significantly less *FOXP3* expression in lung tissue than all
303 other experimental groups (Fig 5A). To determine if we could observe fewer Treg cells
304 in lung tissue of NTHi colonized, OVA sensitized mice in comparison to control groups,
305 we immunostained for FOXP3 protein in paraffin embedded, sectioned lungs. We found
306 that saline treated, OVA sensitized mice had more detectable FOXP3+ cells when
307 compared with NTHi-OVA sensitized groups (Fig 5B), suggesting a greater number of
308 Treg cells had infiltrated the airways of the saline-OVA sensitized mice.

309 As another approach to quantify the level of airway obstruction during allergic
310 airways disease, we measured mucus secreted into the airway and the upregulation of
311 *MUC5AC*, the major driver of mucus production in mice (35). As show in in Figure 6A,
312 we found that *MUC5AC* transcript levels trended higher but not significantly higher in
313 juvenile animals that were colonized as neonates and then sensitized to OVA (Fig 6A).
314 Consistent with higher levels of *MUC5AC* transcript, mice that were colonized with NTHi

315 as neonates had more visible mucus in the major airways than did mice that were not
316 previously colonized by NTHi (Fig 6B +C).

317 **DISCUSSION**

318 The objective of this study was to test whether early life colonization with an
319 opportunistic pathogen and common resident of the upper respiratory tract (URT)
320 changes long term immune responses to allergens and the manifestation of airway
321 hyperresponsiveness. The association between acute upper respiratory microbial
322 colonization and the development of allergic disease is complex. There is emerging
323 evidence that early life exposure to microbes and environmental antigens has lifelong
324 consequences that impact immune system development (40-45). Early life respiratory
325 tract viral infections with agents such as with respiratory syncytial virus, rhinovirus, and
326 influenza virus increase the odds ratio of developing inflammatory diseases like asthma
327 later in childhood (46). In contrast, the long-term consequences of nasopharyngeal
328 colonization with specific bacteria during the neonatal period has yet to be as well
329 delineated. Skewing the microbial milieu during early life is likely to durably change the
330 host immune response (47-49). Thus, early antibiotic exposure would affect the
331 microbiome, as would domination by particular species. New therapies that abrogate or
332 delay colonization by specific taxa in the upper respiratory tract, in the gut, and on the
333 skin may have long-lasting benefits, particularly in those individuals with genetic
334 backgrounds that contribute to a higher for risk for asthma and allergy.

335 We found that juvenile animals colonized as neonates with NTHi had significantly
336 less resistance to an inhaled bronchoconstrictor drug, methacholine. Airway
337 constriction signaling in asthma involves IL13. This cytokine induces phosphoinositide

338 3-kinase γ (PI3K γ) activity, which in turn directly regulates airway smooth muscle
339 contraction (50). We observed elevated levels of IL13 in mice colonized as neonates
340 following induction of allergic airways disease. Thus, the elevated levels of IL13 in the
341 lungs may have had direct or indirect effects on the immune response in NTHi-
342 colonized animals after administration of methacholine.

343 We also found that that induction of allergic disease in the juvenile period resulted in
344 more eosinophils and more neutrophils infiltrating the lung in mice that were colonized
345 with NTHi as neonates than in animals that were exposed to saline. This infiltration with
346 inflammatory cells occurred even though there were no detectable bacteria in the
347 nasopharyngeal passage or the lungs at the time of OVA exposure. We did a
348 semiquantitative initial measurement on lung lavage fluid and found that IL5 and IL13
349 were increased in NTHi colonized, OVA-sensitized mice when compared with other
350 experimental groups. Neutrophil chemoattractants such as IL8 and IL17 and the
351 downstream effector cytokines IL1 β and TNF α were not seen in measurable quantities
352 in our initial screen, but future experiments will include detailed investigations of the
353 host immune pathways that lead to increased neutrophil infiltration in the absence of
354 acute infection. NTHi has been implicated in the induction of a T_H17 response in a
355 mouse model of airways disease. Essilfie *et al.* (8) found that neutrophils, T_H17 cells,
356 and IL17 were elevated in the lungs of animals with induced airways disease and
357 exposure to NTHi compared to mice with induced airways disease and no NTHi
358 exposure.

359 It has been recently hypothesized that early life exposure via the gut to commensal
360 organisms may regulate airway immune responses via regulatory T cell and dendritic

361 cell production of immunosuppressive cytokines such as TGF β and IL10 (51, 52).
362 Indeed, early life and later life exposure to gut commensal organisms ameliorates OVA-
363 induced allergic airways disease phenotypes via a Treg-dependent suppression of
364 inflammation (53). Notably, colonizing adults did not elicit the suppressive effects on
365 asthma-like symptoms associated with early life commensal colonization (53), pointing
366 yet again to a critical window of immune programming susceptibility early in life. We
367 observed that early life upper respiratory tract exposure to NTHi appeared to have the
368 opposite effect on Treg mediated immune suppression (Fig 5), as there was significantly
369 less *FOXP3* expressing cells in the NTHi+OVA mice when compared to the
370 Saline+OVA group. These data suggest as of yet undelineated mechanisms in early life
371 exposure to this potential pathogen that lead to dysregulation of appropriate control
372 mechanisms required to limit pathologic inflammatory responses later in life.

373 In our mouse model, we demonstrated that early life colonization with NTHi leads to
374 greater mucus secretion in mice after the induction of airways disease. Mucus
375 secretion, like airway constriction, is dependent in part on IL13 signaling (54), and the
376 increased mucus secretion we see in our airways disease model may be due to the
377 higher IL13 seen in the lung lavage fluid following early life NTHi colonization. It has
378 been demonstrated that NTHi can directly stimulate MUC5AC production in mouse via
379 signaling downstream of TLR2 in a model of bacterial exposure to NTHi purified surface
380 protein (55). However, the heightened IL13 response in the juvenile animals is not likely
381 direct secondary to persistent NTHi lung colonization since NTHi was not recovered
382 from the lungs of almost all the colonized animals later than 7 days post inoculation.

383 The immune system components that would undergo enduring changes as a
384 consequence of early life microbial exposure and lead to the differences we observed in
385 current study are unclear. The description of the infant respiratory microbiome is only
386 just beginning (45, 56, 57), but a recent study found an association between early life *H.*
387 *influenzae* nasopharyngeal colonization and microbial population perturbations brought
388 on by viral infection and antibiotic treatment (49). A long-term study performed in
389 Denmark showed that children who exhibited signs of asthma and wheeze by age
390 seven years had an abnormal immune response to *H. influenzae* at age 6 months, as
391 measured by cytokine levels and T-cell responses in stored PBMCs obtained from the
392 study participants. PBMC from these samples were stimulated with *H. influenzae*, *M.*
393 *catarrhalis* and *S. pneumoniae* at and expressed aberrant levels of IL5 and IL13 with
394 decreased type 1 cytokines IFN γ , and TNF α when compared to control, age matched
395 samples from children who did not develop asthma symptoms (58).

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

406 The ability of NTHi colonization to drive later airway hyperresponsiveness may be
407 shared among several organisms that stimulate similar outcomes. It is also possible
408 that the mechanism is not due to NTHi colonization *per se*, but instead is a
409 consequence of an NTHi-induced dysbiosis of the typical nasal microbiota. Our
410 tractable murine model should be amenable to dissecting these non-exclusive
411 mechanisms and further identify the links between assembly of the early life
412 nasopharyngeal-respiratory tract microbiota and later inflammatory disorders.

413

414 **FUNDING STATEMENT**

415 This project was fully or in part by a Children's Miracle Network grant (J.R.M.), NIH
416 grant GM108494 (P.C.S.), NIH grant AI121742 (P.C.S.), and NIH grant DC02873
417 (J.W.S.). The funders had no role in study design, data collection and interpretation, or
418 the decision to submit the work for publication.

419 **ACKNOWLEDGEMENTS**

420 Author contributions: J.R.M., J.W.S, and P.C.S. designed experiments, J.R.M., S.N.M,
421 and R.L.A. performed experiments, J.R.M., J.W.S, and P.C.S. wrote the paper, and all
422 authors provided critical feedback on the manuscript. We thank Barbara Theriot, Julie
423 Sproule, and Félix Araujo-Perez for help with sample preparation and data collection.

424

425

426

427

428 LITERATURE CITED

- 429 1. **CDC.** Jan 11 2012 2012. Asthma. <http://www.cdc.gov/asthma/asthmadata.htm>.
430 Accessed 11/2015
- 431 2. **Busse WW, Lemanske RF, Jr.** 2001. Asthma. *N Engl J Med* **344**:350-362.
- 432 3. **Umetsu DT, McIntire JJ, Akbari O, Macaubas C, DeKruyff RH.** 2002. Asthma:
433 an epidemic of dysregulated immunity. *Nature Immunol* **3**:715-720.
- 434 4. **Kearley J, Barker JE, Robinson DS, Lloyd CM.** 2005. Resolution of airway
435 inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory
436 T cells is interleukin 10 dependent. *J Exp Med* **202**:1539-1547.
- 437 5. **Robinson DS.** 2009. Regulatory T cells and asthma. *Clin Exp Allergy* **39**:1314-
438 1323.
- 439 6. **Pavord ID, Brightling CE, Woltmann G, Wardlaw AJ.** 1999. Non-eosinophilic
440 corticosteroid unresponsive asthma. *Lancet* **353**:2213-2214.
- 441 7. **Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID.**
442 2002. Analysis of induced sputum in adults with asthma: identification of
443 subgroup with isolated sputum neutrophilia and poor response to inhaled
444 corticosteroids. *Thorax* **57**:875-879.
- 445 8. **Essilfie AT, Simpson JL, Horvat JC, Preston JA, Dunkley ML, Foster PS,**
446 **Gibson PG, Hansbro PM.** 2011. *Haemophilus influenzae* infection drives IL-17-
447 mediated neutrophilic allergic airways disease. *PLoS Pathogens* **7**:e1002244.
- 448 9. **Gibson PG, Simpson JL, Saltos N.** 2001. Heterogeneity of airway inflammation
449 in persistent asthma : evidence of neutrophilic inflammation and increased
450 sputum interleukin-8. *Chest* **119**:1329-1336.
- 451 10. **Monteseirin J.** 2009. Neutrophils and asthma. *Journal of investigational*
452 *allergology & clinical immunology : official organ of the International Association*
453 *of Asthmology* **19**:340-354.
- 454 11. **Cosmi L, Liotta F, Maggi E, Romagnani S, Annunziato F.** 2011. Th17 cells:
455 new players in asthma pathogenesis. *Allergy* **66**:989-998.
- 456 12. **Del Prete G.** 1992. Human Th1 and Th2 lymphocytes: their role in the
457 pathophysiology of atopy. *Allergy* **47**:450-455.
- 458 13. **Mosmann TR, Coffman RL.** 1989. TH1 and TH2 cells: different patterns of
459 lymphokine secretion lead to different functional properties. *Ann Rev Immunol*
460 **7**:145-173.
- 461 14. **De Serres G, Lampron N, La Forge J, Rouleau I, Bourbeau J, Weiss K,**
462 **Barret B, Boivin G.** 2009. Importance of viral and bacterial infections in chronic
463 obstructive pulmonary disease exacerbations. *J Clin Virol* **46**:129-133.
- 464 15. **Hardy GG, Tudor SM, St Geme JW, 3rd.** 2003. The pathogenesis of disease
465 due to nontypeable *Haemophilus influenzae*. *Methods Mol Med* **71**:1-28.
- 466 16. **Rao VK, Krasan GP, Hendrixson DR, Dawid S, St Geme JW, 3rd.** 1999.
467 Molecular determinants of the pathogenesis of disease due to non-typable
468 *Haemophilus influenzae*. *FEMS Microbiol Rev* **23**:99-129.
- 469 17. **St Geme JW, 3rd.** 1996. Progress towards a vaccine for nontypable
470 *Haemophilus influenzae*. *Ann Med* **28**:31-37.
- 471 18. **St Geme JW, 3rd.** 2000. The pathogenesis of nontypable *Haemophilus*
472 *influenzae* otitis media. *Vaccine* **19 Suppl 1**:S41-50.

- 473 19. **Marti-Llitas P, Regueiro V, Morey P, Hood DW, Saus C, Sauleda J, Agusti**
474 **AG, Bengoechea JA, Garmendia J.** 2009. Nontypeable *Haemophilus*
475 *influenzae* clearance by alveolar macrophages is impaired by exposure to
476 cigarette smoke. *Infect Immun* **77**:4232-4242.
- 477 20. **Bisgaard H, Hermansen MN, Buchvald F, Loland L, Halkjaer LB,**
478 **Bonnelykke K, Brasholt M, Heltberg A, Vissing NH, Thorsen SV, Stage M,**
479 **Pipper CB.** 2007. Childhood asthma after bacterial colonization of the airway in
480 neonates. *N Engl J Med* **357**:1487-1495.
- 481 21. **Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, Davies J, Ervine**
482 **A, Poulter L, Pachter L, Moffatt MF, Cookson WO.** 2010. Disordered microbial
483 communities in asthmatic airways. *PLoS One* **5**:e8578.
- 484 22. **Simpson JL, Grissell TV, Douwes J, Scott RJ, Boyle MJ, Gibson PG.** 2007.
485 Innate immune activation in neutrophilic asthma and bronchiectasis. *Thorax*
486 **62**:211-218.
- 487 23. **Wood LG, Simpson JL, Hansbro PM, Gibson PG.** 2010. Potentially pathogenic
488 bacteria cultured from the sputum of stable asthmatics are associated with
489 increased 8-isoprostane and airway neutrophilia. *Free Radical Res* **44**:146-154.
- 490 24. **Finkelman FD, Wills-Karp M.** 2008. Usefulness and optimization of mouse
491 models of allergic airway disease. *J Allergy Clin Immunol* **121**:603-606.
- 492 25. **Kelada SN, Wilson MS, Tavarez U, Kubalanza K, Borate B, Whitehead GS,**
493 **Maruoka S, Roy MG, Olive M, Carpenter DE, Brass DM, Wynn TA, Cook DN,**
494 **Evans CM, Schwartz DA, Collins FS.** 2011. Strain-dependent genomic factors
495 affect allergen-induced airway hyperresponsiveness in mice. *Am J Respir Cell*
496 *Mol Biol* **45**:817-824.
- 497 26. **Cutter D, Mason KW, Howell AP, Fink DL, Green BA, St Geme JW, 3rd.**
498 2002. Immunization with *Haemophilus influenzae* Hap adhesin protects against
499 nasopharyngeal colonization in experimental mice. *J Infect Dis* **186**:1115-1121.
- 500 27. **Anderson P, Johnston RB, Jr., Smith DH.** 1972. Human serum activities
501 against *Hemophilus influenzae*, type b. *The Journal of clinical investigation*
502 **51**:31-38.
- 503 28. **Nials AT, Uddin S.** 2008. Mouse models of allergic asthma: acute and chronic
504 allergen challenge. *Dis Model Mech* **1**:213-220.
- 505 29. **Pichavant M, Goya S, Hamelmann E, Gelfand EW, Umetsu DT.** 2007. Animal
506 models of airway sensitization. *Curr Protoc Immunol* **Chapter 15**:Unit 15 18.
- 507 30. **Fedulov AV, Leme A, Yang Z, Dahl M, Lim R, Mariani TJ, Kobzik L.** 2008.
508 Pulmonary exposure to particles during pregnancy causes increased neonatal
509 asthma susceptibility. *Am J Respir Cell Mol Biol* **38**:57-67.
- 510 31. **Gueders MM, Paulissen G, Crahay C, Quesada-Calvo F, Hacha J, Van Hove**
511 **C, Tournoy K, Louis R, Foidart JM, Noel A, Cataldo DD.** 2009. Mouse models
512 of asthma: a comparison between C57BL/6 and BALB/c strains regarding
513 bronchial responsiveness, inflammation, and cytokine production. *Inflamm Res*
514 **58**:845-854.
- 515 32. **Hantos Z, Collins RA, Turner DJ, Janosi TZ, Sly PD.** 2003. Tracking of airway
516 and tissue mechanics during TLC maneuvers in mice. *J Appl Physiol* (1985)
517 **95**:1695-1705.

- 518 33. **McBride A, Konowich J, Salgame P.** 2013. Host defense and recruitment of
519 Foxp3(+) T regulatory cells to the lungs in chronic *Mycobacterium tuberculosis*
520 infection requires toll-like receptor 2. *PLoS Pathog* **9**:e1003397.
- 521 34. **Glasser SW, Senft AP, Whitsett JA, Maxfield MD, Ross GF, Richardson TR,**
522 **Prows DR, Xu Y, Korfhagen TR.** 2008. Macrophage dysfunction and
523 susceptibility to pulmonary *Pseudomonas aeruginosa* infection in surfactant
524 protein C-deficient mice. *J Immunol* **181**:621-628.
- 525 35. **Ehre C, Worthington EN, Liesman RM, Grubb BR, Barbier D, O'Neal WK,**
526 **Sallenave JM, Pickles RJ, Boucher RC.** 2012. Overexpressing mouse model
527 demonstrates the protective role of Muc5ac in the lungs. *Proc Natl Acad Sci U S*
528 *A* **109**:16528-16533.
- 529 36. **Gawronski JD, Wong SM, Giannoukos G, Ward DV, Akerley BJ.** 2009.
530 Tracking insertion mutants within libraries by deep sequencing and a genome-
531 wide screen for *Haemophilus* genes required in the lung. *Proc Natl Acad Sci U S*
532 *A* **106**:16422-16427.
- 533 37. **Liu DF, Mason KW, Matri M, Pazirandeh M, Cutter D, Fink DL, St Geme JW,**
534 **3rd, Zhu D, Green BA.** 2004. The C-terminal fragment of the internal 110-
535 kilodalton passenger domain of the Hap protein of nontypeable *Haemophilus*
536 *influenzae* is a potential vaccine candidate. *Infection and immunity* **72**:6961-
537 6968.
- 538 38. **Pope SM, Brandt EB, Mishra A, Hogan SP, Zimmermann N, Matthaei KI,**
539 **Foster PS, Rothenberg ME.** 2001. IL-13 induces eosinophil recruitment into the
540 lung by an IL-5- and eotaxin-dependent mechanism. *J Allergy Clin Immunol*
541 **108**:594-601.
- 542 39. **Hori S, Nomura T, Sakaguchi S.** 2003. Control of regulatory T cell development
543 by the transcription factor Foxp3. *Science* **299**:1057-1061.
- 544 40. **Cahenzli J, Koller Y, Wyss M, Geuking MB, McCoy KD.** 2013. Intestinal
545 microbial diversity during early-life colonization shapes long-term IgE levels. *Cell*
546 *Host Microbe* **14**:559-570.
- 547 41. **Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, Glickman JN,**
548 **Siebert R, Baron RM, Kasper DL, Blumberg RS.** 2012. Microbial exposure
549 during early life has persistent effects on natural killer T cell function. *Science*
550 **336**:489-493.
- 551 42. **Pflughoeft KJ, Versalovic J.** 2012. Human microbiome in health and disease.
552 *Annu Rev Pathol* **7**:99-122.
- 553 43. **Smith MI, Yatsunenko T, Manary MJ, Trehan I, Mkakosya R, Cheng J, Kau**
554 **AL, Rich SS, Concannon P, Mychaleckyj JC, Liu J, Hout E, Li JV, Holmes**
555 **E, Nicholson J, Knights D, Ursell LK, Knight R, Gordon JI.** 2013. Gut
556 microbiomes of Malawian twin pairs discordant for kwashiorkor. *Science*
557 **339**:548-554.
- 558 44. **Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI.** 2009.
559 The effect of diet on the human gut microbiome: a metagenomic analysis in
560 humanized gnotobiotic mice. *Sci Transl Med* **1**:6ra14.
- 561 45. **Biesbroek G, Tsivtsivadze E, Sanders EA, Montijn R, Veenhoven RH,**
562 **Keijser BJ, Bogaert D.** 2014. Early respiratory microbiota composition

- determines bacterial succession patterns and respiratory health in children. *Am J Respir Crit Care Med* **190**:1283-1292.
46. **Feldman AS, He Y, Moore ML, Hershenson MB, Hartert TV.** 2015. Toward primary prevention of asthma. Reviewing the evidence for early-life respiratory viral infections as modifiable risk factors to prevent childhood asthma. *Am J Respir Crit Care Med* **191**:34-44.
47. **Bassis CM, Erb-Downward JR, Dickson RP, Freeman CM, Schmidt TM, Young VB, Beck JM, Curtis JL, Huffnagle GB.** 2015. Analysis of the upper respiratory tract microbiotas as the source of the lung and gastric microbiotas in healthy individuals. *MBio* **6**.
48. **Noverr MC, Noggle RM, Toews GB, Huffnagle GB.** 2004. Role of antibiotics and fungal microbiota in driving pulmonary allergic responses. *Infect Immun* **72**:4996-5003.
49. **Teo SM, Mok D, Pham K, Kusel M, Serralha M, Troy N, Holt BJ, Hales BJ, Walker ML, Hollams E, Bochkov YA, Grindle K, Johnston SL, Gern JE, Sly PD, Holt PG, Holt KE, Inouye M.** 2015. The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host Microbe* **17**:704-715.
50. **Jiang H, Xie Y, Abel PW, Toews ML, Townley RG, Casale TB, Tu Y.** 2012. Targeting phosphoinositide 3-kinase gamma in airway smooth muscle cells to suppress interleukin-13-induced mouse airway hyperresponsiveness. *J Pharmacol Exp Ther* **342**:305-311.
51. **Christensen HR, Frokiaer H, Pestka JJ.** 2002. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. *J Immunol* **168**:171-178.
52. **Karimi K, Inman MD, Bienenstock J, Forsythe P.** 2009. *Lactobacillus reuteri*-induced regulatory T cells protect against an allergic airway response in mice. *Am J Respir Crit Care Med* **179**:186-193.
53. **Feleszko W, Jaworska J, Rha RD, Steinhausen S, Avagyan A, Jaudszus A, Ahrens B, Groneberg DA, Wahn U, Hamelmann E.** 2007. Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. *Clin Exp Allergy* **37**:498-505.
54. **Laoukili J, Perret E, Willems T, Minty A, Parthoens E, Houcine O, Coste A, Jorissen M, Marano F, Caput D, Tournier F.** 2001. IL-13 alters mucociliary differentiation and ciliary beating of human respiratory epithelial cells. *J Clin Invest* **108**:1817-1824.
55. **Chen R, Lim JH, Jono H, Gu XX, Kim YS, Basbaum CB, Murphy TF, Li JD.** 2004. Nontypeable *Haemophilus influenzae* lipoprotein P6 induces MUC5AC mucin transcription via TLR2-TAK1-dependent p38 MAPK-AP1 and IKKbeta-IkappaBalpha-NF-kappaB signaling pathways. *Biochem Biophys Res Comm* **324**:1087-1094.
56. **Sakwinska O, Bastic Schmid V, Berger B, Bruttin A, Keitel K, Lepage M, Moine D, Ngom Bru C, Brussow H, Gervaix A.** 2014. Nasopharyngeal microbiota in healthy children and pneumonia patients. *J Clin Microbiol* **52**:1590-1594.

- 609 57. **Laufer AS, Metlay JP, Gent JF, Fennie KP, Kong Y, Pettigrew MM.** 2011.
610 Microbial communities of the upper respiratory tract and otitis media in children.
611 MBio **2**:e00245-00210.
- 612 58. **Larsen JM, Brix S, Thyssen AH, Birch S, Rasmussen MA, Bisgaard H.** 2014.
613 Children with asthma by school age display aberrant immune responses to
614 pathogenic airway bacteria as infants. J Allergy Clin Immunol **133**:1008-1013.
- 615 59. **Siegel SJ, Tamashiro E, Weiser JN.** 2015. Clearance of Pneumococcal
616 Colonization in Infants Is Delayed through Altered Macrophage Trafficking. PLoS
617 Pathog **11**:e1005004.
- 618
- 619

620

621

622 **FIGURE LEGENDS**

623

624 **Figure 1. The majority of neonatal animals remained colonized with *H. influenzae***
625 **until at least 14 days post infection.** Groups of 3-day old mice were intranasally
626 inoculated with 10^6 CFU of nontypeable *H. influenzae* (NTHi). Horizontal solid lines
627 represent the geometric means. Error bars represent the standard deviations of the
628 mean, and dashed lines indicate the limit of detection. Each symbol represents total
629 CFU sample from an individual. **A)** NTHi CFU counts from nasal washes on the
630 indicated day post inoculation. **B)** NTHi CFU counts from homogenized lungs on the
631 indicated day post inoculation.

632

633 **Figure 2. Airway hypersensitivity increased in animals colonized with *H.***
634 ***influenzae* early in life. A)** Sensitization timeline. Neonatal mice were colonized on
635 day of life 3, sensitized, then challenged with aerosolized ovalbumin (OVA) for three
636 consecutive days one week following sensitization. Airway hyperreactivity and immune

637 factors were then measured as described in the text. **B)** Animals were given sensitizing
638 doses of OVA by IP injection, then challenged with aerosolized OVA. Data shown are
639 representative of triplicate experiments. Statistical significance was determined using the
640 Sidak-Bonferroni method. *, $P=0.004$; ** $P=0.0004$. **C)** Animals were given sub-
641 sensitizing doses of OVA by IP injection and then challenged by aerosolized OVA a
642 week later. Results shown are representative of duplicate experiments. Abbreviations:
643 OVA, ovalbumin; IP, intraperitoneal; IN, intranasal; AER, aerosol; AHR, airway
644 hyperreactivity; BALF, bronchoalveolar lavage fluid. NTHi+OVA, *Haemophilus* colonized
645 and OVA sensitized; Saline+OVA, saline treated, OVA sensitized; Saline, saline treated
646 without OVA sensitization; NTHi, *Haemophilus* colonized without OVA sensitization.

647

648 **Figure 3. Lungs of mice colonized by NTHi as neonates prior to induction of**
649 **airways disease had greater eosinophil and neutrophil infiltration than lungs of**
650 **mice treated with saline. A)** Cells were isolated from bronchoalveolar lavage fluid and
651 categorized based on differential staining pattern. NTHi+OVA, *Haemophilus* colonized
652 and OVA sensitized; Saline+OVA, saline treated, OVA sensitized; Saline, saline treated
653 without OVA sensitization; NTHi, *Haemophilus* colonized without OVA sensitization.
654 Statistical significance between NTHi+OVA and Saline+OVA groups was determined
655 using the Mann-Whitney nonparametric test.

656

657 **Figure 4. Animals colonized on day of life 3 had higher IL5 and IL13 cytokine**
658 **infiltration in the lung lavage fluid than non-colonized animals after induction of**
659 **airways disease.** Bronchoalveolar lavage fluid was collected from each animal

660 following approximately 24 hours after airways induction tested by sandwich ELISA for
661 the presence of cytokine IL5 (A) or IL13 (B). C) *IL5* and *IL13* transcript levels following
662 induction of airways disease were internally normalized to *GAPDH* and expressed as
663 fold over the levels in Saline only mice. Data shown are representative of 2 replicate
664 experiments. NTHi+OVA, *Haemophilus* colonized and OVA sensitized; Saline+OVA,
665 saline treated, OVA sensitized; Saline, saline treated without OVA sensitization; NTHi,
666 *Haemophilus* colonized without OVA sensitization. Horizontal bars indicate the means.
667 Error bars indicate the standard deviations. Statistical significance between NTHi+OVA
668 and Saline+OVA was determined using the Mann-Whitney nonparametric test.

669
670 **Figure 5. NTHi+OVA mice have lower levels of *FOXP3* expression than**
671 **Saline+OVA mice in lung tissue. A)** Lung *FOXP3* transcript levels from NTHi+OVA ,
672 Saline+OVA, and NTHi only animals were measured by qPCR using *GAPDH* transcript
673 as an internal control. Transcript levels were normalized to lung-derived transcript from
674 Saline only mice. Error bars represent mean and standard deviation from one
675 experiment. Data are representative of two replicate experiments. Statistical
676 significance between NTHi+OVA and Saline+OVA was determined using a student's t
677 test. **B)** Lungs from mice that had undergone OVA aerosol challenge were sectioned,
678 and sections were immunostained for FOXP3 then counter stained with hematoxylin. **C)**
679 Cells that stained positively for FOXP3 were counted in ≥ 5 fields at 40x magnification on
680 ≥ 5 sections per experimental group. Arrows point to instances of positive staining. Error
681 bars represent the 95% confidence interval of the geographic mean for each data

682 group. Statistical significance between the NTHi+OVA/Saline+OVA and
683 NTHi/NTHi+OVA groups was determined using the Mann-Whitney nonparametric test.

684

685 **Figure 6. There is more visible mucus in HA lungs post allergic airways disease**

686 **induction than in non-colonized allergic (SA) lungs. A)** Lung *MUC5AC* transcript

687 levels from NTHi+OVA , Saline+OVA, and NTHi only animals were directly measured

688 against a standard curve. Transcript levels were normalized to lung-derived transcript

689 from Saline only mice. Error bars represent mean and standard deviation from one

690 experiment. Data are representative of three replicate experiments. **B)** Lungs from mice

691 that had undergone OVA aerosol challenge were sectioned, and stained for acidic

692 mucins with Alcian blue and counter stained with nuclear fast red. **C)** Two independent

693 images from a 10x magnification for each animal were coded, imaged, and scored as

694 described in Methods. Total points were averaged and compared between experimental

695 groups. Bars indicate means and standard deviations for all scores from each

696 experimental group. Statistical significance between the NTHi+OVA and Saline+OVA

697 groups was determined using the Mann-Whitney nonparametric test.

698











