Supplemental Information

Materials and Methods

Human subjects

With the approval of National Jewish Health Institutional Review Board (IRB), bronchoalveolar lavage fluid (BALF) from healthy and asthmatic individuals was obtained through bronchoscopy as we previously described (1). Healthy controls were recruited from the community who had normal lung function, and did not have history of respiratory diseases. Asthma diagnosis was based on the American Thoracic Society criteria (2). Current or past smokers were excluded from this study. Human subject recruitment and inclusion followed the policy of National Institutes of Health (NIH) regarding gender, race, ethnicity and age. De-identified donor lungs from nonsmokers with no history of lung diseases were obtained from the National Disease Research Interchange (Philadelphia, Pennsylvania, USA), the International Institute for the Advancement of Medicine (Edison, New Jersey, USA) or Donor Alliance of Colorado. The collected lungs were donated for medical research and were approved by our IRB. Human small airway epithelial cells (HSAEC) were obtained from the distal lung using a 2 mm bronchoscopy brush (Conmed Greenwood Village, CO) as we previously described (3). Human tracheobronchial epithelial cells (HTBEC) were isolated from the trachea and proximal parts of the main bronchi as we previously reported (4).

Nontypeable *Haemophilus influenzae* (NTHi) culture and DNA preparation

NTHi glycerol stock (strain R2846/12, a gift from Dr. Stephen Barenkamp at Saint Louis University School of Medicine, Saint Louis, MO, USA) was grown on chocolate agar plates (Hardy Diagnostics, Santa Maria, California, USA) in a 37˚C incubator. A single colony was

selected and cultured in brain heart infusion broth (Sigma, Saint Louis, MO, USA) supplemented with 10µg/ml of hemin and 2µg/ml nicotinamide adenine dinucleotide (NAD) for 24 hours at 37˚C. Bacteria were centrifuged three times at 12,000 *rpm* for 5 minutes and resuspended in PBS to wash the bacteria. A QIAamp DNA Mini Kit (Qiagen, Germany) was used to extract DNA from bacteria according to manufacturer's instructions. Briefly, bacterial pellet was resuspended in 200µl of PBS containing 20µl of proteinase K to extract DNA. To generate endotoxin-free bacterial DNA, a Pierce High-Capacity Endotoxin Removal column (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to remove any potential endotoxin from NTHi-derived DNA per manufacturer's instruction. A Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, USA) was used to confirm endotoxin removal from NTHi-derived DNA showing less than 0.096EU/ml (0.0096ng/ml) of endotoxin contamination in NTHi DNA preparation, which is well below the endotoxin contamination level (0.5EU/ml or 0.05ng/ml) set by the FDA for research.

HSAEC and HTBEC culture

Freshly isolated HSAEC were expanded on irradiated NIH 3T3 fibroblasts in the presence of a Rho kinase inhibitor Y-27632 (5, 6). Air-liquid interface (ALI) culture of HSAEC was performed as previously described (4). Briefly, cells were seeded onto collagen-coated 12-well transwell plates (Transwell 2460, Corning Incorporated, Corning, New York, USA) and maintained in F6 medium (4). After ~7 days of submerge culture, cells were shifted to ALI culture for 21 days to induce mucociliary differentiation. On day 21 of ALI, cells were stimulated with 10ng/ml of recombinant human IL-17A (Peprotech, Rocky Hill, New Jersey, USA) for 24 hours (7). Then, cells were exposed to 0.3µg/ml (8) of NTHi-derived DNA using

FuGENE® HD transfection reagent (Promega™, Madison, Wisconsin, USA) for 16 hours. Next, media ± DNA was removed. Cells were then washed with PBS and added with fresh media ± rhIL-17A at 10ng/ml. Basolateral supernatants were collected after 24 and 48 hours of NTHi DNA treatment. Cells were harvested after 48 hours and lysed with RLT buffer for RNA extraction. The doses of bacterial DNA and rhIL-17A used in this study were chosen based on physiological relevance and our dose optimization. We chose the bacterial DNA concentration at 0.3 µg/ml based on: (1) bacterial DNA levels in BAL fluid of neutrophilic asthma subjects. We typically instill a total of 120 ml (60 ml x 2 times) saline for lavaging the human lungs trough bronchoscopy. Given the epithelial lining fluid volume recovered in heathy human subjects through BAL procedure is about 1.2 ml [2], the loss (>70%) of small quantity of DNA during DNA extraction in cell-free fluid [3] and about 2% of the entire lung surface area covered by 120 ml instilled saline [4], we estimated that airway epithelial cells and alveolar macrophages in human neutrophilic asthmatics may access about 1000 times higher bacterial DNA concentration at epithelial lining fluid than the bacterial DNA concentration (median [interquartile range], 185 [74 to 398] pg/ml) we measured in BALF from neutrophilic asthma subjects. Thus, it is likely that human lung cells with neutrophilic asthma may be exposed to bacterial DNA at up to 200 to 300 pg/ml x 1000 times (200 to 300 ng/ml or 0.2 to 0.3 µg/ml). This would support the dose of 0.3 µg/ml bacterial DNA we used in our cell culture experiments. (2) The dose response study we performed. With different doses of rhIL-17A (10 and 20ng/ml) and bacteria-derived DNA (0.3 and 0.6 µg/ml) tested, we found that cells exposed to 10ng/ml of rhIL-17A and 0.3µg/ml of bacterial DNA already demonstrated a significant increase of IL-8 compared with IL-17 or bacterial DNA treatment alone (Supplemental Figure 1).

As most investigators used proximal airway epithelial cells for cell culture studies, we also compared the responses of paired distal and proximal airway epithelial cells. HTBECs were expanded, cultured and treated in the same manner as HSAECs.

To inhibit DNA signaling in HSAECs, a broad inhibitor of DNA signaling was employed. Inhibitory CpG oligodeoxynucleotides (ODN) (A151) and negative control of A151 (Thermo Fisher Scientific, USA) were utilized. HSAECs in submerged culture were stimulated with 10ng/ml of recombinant human IL-17A (Peprotech, USA) for 24 hours. Thereafter, cells were exposed to 0.3µg/ml of NTHi-derived DNA ± 3µM of A151 or 3µM of negative control of A151 using FuGENE® HD transfection reagent (Promega™, Madison, Wisconsin, USA) for 16 hours. Next, media ± DNA was removed, washed with PBS, and refreshed with media ± 10ng/ml rhIL-17A.

Human alveolar macrophage culture

Bronchoalveolar lavage (BAL) was done on the right middle lobe of the donor lungs by lavaging the lobe three times with balanced salt solution and EDTA, and then three times with the salt solution alone. The lavage fluid was collected and centrifuged to obtain cells including alveolar macrophages (AM). BAL cells were either used fresh or frozen in 90% FBS and 10% DMSO. As our previous established protocol (9), after using RBC lysis buffer and macrophage enrichment, BAL cells were seeded into 24-well plates at 5×10^5 cells/well in D10 media at 37 °C in 5% CO_2 for 2 hours and then washed to remove non-adherent cells. After 48 hours, the adherents AMs were stimulated with 10ng/ml of rhIL-17A (Peprotech) for 24 hours, and then exposed to 0.3 µg/ml of NTHi-derived DNA. Next day, cells ± DNA were washed with PBS, and fresh media ± 10 ng/ml rhIL-17A was added. Supernatants were collected after 24 and 48 hours.

Generation of stimulator of interferon genes (STING) knockout (SKO) HTBEC

SKO HTBEC were generated using the CRISPR/Cas9 system as we previously described (10). A single guide (sg) RNA (sgRNA sequence: 5′ GCTGGGACTGCTTAAACG 3′) was designed to target exon 3 of human STING, while a scrambled sgRNA was used for the control CRISPR (11, 12). STING or scrambled sgRNA was cloned into an 'all in one' pLenti-CRISPR vector co-expressing the scaffold RNA and Cas9 nuclease accompanied with a puromycin resistance gene. The construct was sequenced to confirm the presence of sgRNA sequence, and then packaged into lentivirus by co-transfection with VSV-G and psPAX2 plasmids in 293FT cells. The packaged lentivirus in cell culture supernatant was transduced into ~70% confluent HTBEC. After 48 hours of recovery, the transduced cells were seeded onto irradiated, puromycin-resistant 3T3 fibroblasts for expansion and selection with puromycin (1µg/mL) for 7 days. STING knockout was confirmed by western blot. Cells were then seeded onto collagen-coated 24-well plates with BronchiaLife^{TM} media (StemCell) under the submerged condition for 48 hours, and were stimulated with 10ng/ml of rhIL-17A (Peprotech) for 24 hours, followed by exposure to 0.3µg/ml of NTHi-derived DNA, and harvested in the same manner as HSAEC.

Mouse tracheal epithelial cell (mTEC) culture

Tracheas isolated from TLR9 knockout (KO) mice and wild-type littermate control mice on C57BL/6 background were digested with 0.1% protease, and the released cells were expanded on irradiated NIH 3T3 fibroblasts (5, 11). ALI culture of mTEC was performed in a similar fashion as the human cells described above.

Mouse bone marrow derived macrophages (BMDM) culture

Bone marrow from femurs of TLR9 KO mice and wild-type C57BL/6 mice was used to generate macrophages (BMDM) (13). Bone marrow cells were seeded at a density of $1x10^6$/ml in D10 media supplemented with 25ng/ml M-CSF (Peprotech) in 100mm dishes (Corning, New York, USA). After ~7 days in culture, differentiated macrophages were seeded on to 24-well plates at $5x10^5$ cells/well in D10 media. After 48 hours, cells were stimulated with 10ng/ml of recombinant murine IL-17A for 24 hours and then with 0.3µg/ml of NTHi-derived DNA. Cell supernatants were collected after 24 and 48 hours.

Mouse models of airway inflammation induced by IL-17 and NTHi-derived DNA or live NTHi

Mice were maintained and bred in the animal facility in the Biological Resources Center at National Jewish Health. All animals and procedures were approved by our Institutional Animal Care and Use Committee (IACUC).

(1) *Wild-type mouse model of IL-17 and NTHi-derived DNA treatment*: Wild-type (WT) C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). To determine whether NTHi-derived DNA exaggerates IL-17-induced airway inflammation, mice were administered intranasally with rmIL-17A (Peprotech, Rocky Hill, New Jersey, USA) at 3µg/mouse prepared in 0.01% bovine serum albumin (BSA) or 0.01% BSA (control) for 24 hrs (14), followed by NTHi-derived DNA at 1µg/mouse (15, 16) or 50µL Tris-EDTA (TE) buffer (17). Our chosen dose of bacterial DNA at 1µg/mouse was based on the fact that about 50% of the dose in 50 µl administered to the nose likely entered the lung [5], the actual dose given in mouse lung is about 0.5 µg. The lung volume of a typical 20-gram mouse is about 1 ml. Thus,

the bacterial DNA we gave to mice is also about 0.5 μg/ml, which is close to the human cell culture model and the upper range of bacterial DNA concentration in human neutrophilic asthma subjects.

Mice were sacrificed after 24 hours of DNA administration. BALF was collected by lavaging the lung with 1ml 0.9% saline solution for leucocyte counts using the Diff-Quick staining on cytospin slides and cytokine measurement using the ELISA.

(2) *Myeloid cell TLR9 deficient mouse model of IL-17 and NTHi-derived DNA treatment*: LysM-Cre ER^{T2} (tamoxifen-inducible Cre expression) mice on C57BL/6 background were purchased from the Jackson Laboratory. TLR9 floxed mice on C57BL/6 background were generated as described previously (18, 19). In summary, heterogeneous $TLR9^{fl/wt}$ male and female mice (a kind gift from Dr. Mark Shlomchik in the Department of Immunology at University of Pittsburg) were crossed to generate homozygous mice carrying floxed TLR9 gene ($TLR9^{fl/fl}$), which was confirmed using a PCR-based genotyping protocol (Forward: 5'-CAAGGAGAA-TCCAGGAGGCTAGTG-3', Reverse: 5'-GGAGAACCTGTGAGAGCCAGG-3'). LysM-Cre ER^{T2} and $TLR9^{fl/fl}$ mice were bred to generate LysM-Cre^{+} or LysM-Cre^{-} (control) $TLR9^{fl/fl}$ mice that were first treated with tamoxifen chow for 7 days, and then transferred to normal chow for 7 more days before they were used for the treatment with IL-17±NTHi derived DNA as described above for the wild-type mice. mTEC and lung macrophages were isolated to confirm TLR9 deficiency in macrophages, but not in mTEC using RT-PCR. Our data demonstrated that there was no significant difference of TLR9 expression in mTECs between $TLR9^{fl/fl}$ LysM-Cre^{+}/LysM-Cre^{-} mice ($p=0.6$). Meanwhile, there was about 7-fold decrease (or about 86%

reduction, $p<0.05$) of TLR9 expression in macrophages isolated from lungs of LysM-Cre^{+} TLR9$^{fl/fl}$ mice compared with LysM-Cre^{-} TLR9$^{fl/fl}$ mice.

(3) *Recombinant human DNase I treatment in wild-type mice with IL-17 treatment and live NTHi infection*: To determine whether DNA signaling plays a key role in bacteria-mediated exaggeration of inflammation of IL-17-exposed airways, DNase I treatment was carried out. Wild-type mice were intranasally inoculated with 10^7 colony forming units (CFU) of NTHi (20) and 3μg of rmIL-17A or BSA (control) in the presence or absence of 5μg recombinant human DNase I (Abcam, Cambridge, United Kingdom) (21). Mice were sacrificed after 24 hours of the treatment. BALF was collected for leucocyte counts and pro-inflammatory mediators. To quantify lung bacterial load, the entire right lung was homogenized and cultured on chocolate agar plates (Hardy Diagnostics) to count CFU.

Measurement of airway hyperresponsiveness

Measurements of respiratory mechanics in response to methacholine were made using the FlexiVent system (Scireq, Montreal, Canada) in wild-type C57BL/6 mice exposed to IL-17 and NTHi-derived DNA. Mice were paralyzed with 1 mg/kg pancuronium bromide 5 minutes before starting the experiment. Mice anesthetized with a cocktail of 100 mg/kg of ketamine and 15 mg/ml of xylazine, were intubated with an 18-g cannula secured to the trachea with a suture. Mouse cannula was attached to FlexiVent on the default ventilation pattern. Measurements of respiratory mechanics were made by the forced oscillation technique. Response to increasing doses of aerosolized methacholine (0, 6.25, 12.5, 25, and 50 mg/ml) was determined by resistance and elastance measurements every 30 seconds for 5 minutes, ensuring the parameters

calculated had peaked. Lungs were inflated to estimate total lung capacity after each dose to maintain airway patency. The resistance (Rrs, Rn, and G) or elastance (Ers and H) measurements were then averaged at each dose per treatment group and percent change over the baseline was graphed. Data were reported as total pulmonary resistance (Rrs), proximal airways resistance (Rn), distal airways resistance (G), total pulmonary elastance (Ers), and distal airways elastance (H).

Quantification of bacterial DNA in human BALF

DNA was extracted from cell-free (centrifuged) human BALF using a DNA mini kit (Qiagen, Hilden, Germany). Whole cells were spined down (centrifuged) to remove cells for collecting cell-free BALF. Bacterial DNA was quantified by real-time PCR for pan bacterial specific gene 16S rRNA (Forward: 5'-TCCTACGGGAGGCAGCAGT-3'; Reverse: 5'-GGACTACCAGGG-TATCTA-ATCCTGTT-3' and Probe: 5'-CGTATTACCGCGGCTGCTGGCAC-3') (22). NTHi-derived DNA was used to construct the real-time PCR standard curves to calculate bacterial DNA concentration in human BALF, which was expressed as pg/ml.

Quantitative real-time reverse transcription PCR (qRT-PCR)

RNA was extracted from human and mouse cells using the GenCatch total RNA Extraction System (Epoch Life Sciences) and was reversely transcribed to cDNA. Taqman RT-PCR assays of genes of interest were obtained from Applied Biosystems (Foster City, California). To calculate relative mRNA expression levels, the comparative cycle of threshold (ΔΔCT) method was utilized with the housekeeping gene 18S as an internal control (23, 24).

Western blot

To measure IL-36γ in airway epithelial lining fluid, Amicon ultra centrifugal filters (Millipore, Burlington, Massachusetts, USA) were used to concentrate human (10-fold) and mouse (5-fold) BALF samples. The concentrated samples were boiled and then loaded on SDS-PAGE and transferred onto nitrocellulose membranes and were probed with an antibody against human IL-36γ (1:1000, R&D Systems, Minneapolis, Minnesota, USA) or anti-mouse IL-36γ (1:1000, Abcam), followed by horseradish peroxidase-conjugated secondary IgG (1:3000; EMD Millipore, Burlington, Massachusetts, USA) and Pierce® ECL Western blotting substrate. Densitometry was performed using the National Institutes of Health's Image-J software to quantify target protein expression. IL-36 gamma can be found in human serum samples [16]. In order to reduce the impact of IL-36γ from the serum on the levels of lung cells-derived IL-36γ, IL-36γ levels in BALF were normalized the levels of albumin. Similar approach to use albumin to normalize BALF data of proteins found in serum/plasma has been previously reported [17].

ELISA

Mouse KC/CXCL1 and LIX/CXCL5, and human IL-8/CXCL8 protein levels were measured using Duoset ELISA kits from R&D systems.

Statistical analysis

Data were analyzed using Graph Pad Prism software (GraphPad Software Inc., San Diego, CA, USA). For parametric data, a paired Student's *t*-test was performed for two-group comparisons or two-way ANOVA followed by the Tukey's multiple comparison test. For non-parametric data, comparisons were done using the Mann-Whitney test for two group comparisons or using

the Kruskal-Wallis test for multiple group comparisons. Mouse airway hyperresponsiveness data was analyzed using a mixed-effects model with repeated measures two-way ANOVA. Correlation was assessed using the Spearman coefficient. A p value of <0.05 was considered to be statistically significant.

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