

Early View

Original research article

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Bacterial DNA amplifies neutrophilic inflammation in IL-17-exposed airways

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Running title: Role of bacterial DNA in neutrophilic asthma

Take home message

More bacterial DNA was found in airways of asthmatics with neutrophilic inflammation. Bacterial DNA along with IL-17 amplified neutrophil chemokine production and airway neutrophil influx, which was reduced by an IL-36 γ neutralizing antibody or human DNase I.

Abstract

Background: Neutrophilic asthma (NA) is associated with increased airway IL-17 and abnormal bacterial community such as dominance of Nontypeable *Haemophilus influenzae* (NTHi), particularly during asthma exacerbations. Bacteria release various products including DNA, but whether they cooperate with IL-17 in exaggerating neutrophilic inflammation is unclear. We sought to investigate the role of bacteria-derived DNA in airway neutrophilic inflammation related to IL-17-high asthma and underlying mechanisms (e.g., TLR9/IL-36 γ signaling axis).

Methods: Bacterial DNA, IL-8 and IL-36 γ were measured in bronchoalveolar lavage fluid (BALF) of asthma and healthy subjects. The role of co-exposure to IL-17 and bacterial DNA or live bacteria in neutrophilic inflammation, and the contribution of the TLR9/IL-36 γ signaling axis were determined in cultured primary human airway epithelial cells and alveolar macrophages, and mouse models.

Results: Bacterial DNA levels were increased in asthma BALF, which positively correlated with IL-8 and neutrophil levels. Moreover, IL-36 γ increased in BALF of neutrophilic asthma patients. Bacterial DNA or NTHi infection under an IL-17-high setting amplified IL-8 production and mouse lung neutrophilic inflammation. DNase I treatment in IL-17-exposed and NTHi-infected mouse lungs reduced neutrophilic inflammation. Mechanistically, bacterial DNA-mediated amplification of neutrophilic inflammation is in part dependent on the TLR9/IL-36 γ signaling axis.

Conclusions: Bacterial DNA amplifies airway neutrophilic inflammation in an IL-17-high setting partly through the TLR9 and IL-36 γ signaling. Our novel findings may offer several potential therapeutic targets including TLR9 antagonists, IL-36 γ neutralizing antibodies and DNase I to reduce asthma severity associated with exaggerated airway neutrophilic

inflammation.

Key words: Neutrophilic asthma, IL-17, IL-36 γ , bacterial DNA, TLR9 signaling.

Introduction

Excessive airway neutrophil accumulation contributes to the development of lung diseases including asthma. In neutrophilic asthma (NA), increased airway neutrophils are associated with asthma exacerbations, airflow limitation, and reduced efficacy of corticosteroid therapy (1-3). NA patients demonstrated higher levels of inflammatory mediators including interleukin-17 (IL-17 or IL-17A) and IL-8 in sputum samples compared to non-NA (NNA) patients and healthy individuals (4). IL-17 and IL-8 may promote airway neutrophil recruitment and activation (5, 6).

The mechanisms underlying airway neutrophilic inflammation in asthma remain unclear. Recent microbiome studies suggested a role of bacterial infection in asthma (7). Among various species of bacteria identified in asthma airways, nontypeable *Haemophilus influenzae* (NTHi) has been found in both upper and lower airways. Indeed, NTHi is one of the leading pathogenic bacteria isolated in asthmatic airways especially those with NA (8). NTHi is known to evade the immune system by attaching and/or entering airway epithelial cells, which may contribute to recurrent inflammation and asthma exacerbations (9). NTHi could induce neutrophilic inflammation through IL-17 in murine models of asthma (10). However, it is unclear whether and how IL-17 induction during bacterial infection may in turn interact with bacteria-derived components such as DNA to regulate neutrophilic inflammation.

Bacteria actively or passively release DNA to exert various functions such as biofilm formation, nutrients for bacteria, repair of DNA damage and modulation of host immune response (11). The

active DNA release mechanisms may involve vesicles, prophage and lytic-independent mechanism. The passive DNA release mechanisms are related to lysis and autolysis of bacteria. Thus, both live and dead bacteria contribute to the detection of extracellular DNA from bacteria. A recent publication (12) suggests increased levels of circulating or extracellular bacterial DNA in immune deficient patients, and the bacterial DNA was biologically active as it increased the production of IFN- γ in peripheral blood mononuclear cells. Although the role of bacterial community has been studied in asthma, whether more bacterial DNA is released into the airways and subsequently affects host cell immune responses in asthma patients particularly those with IL-17-high airway environment has not been investigated. Interestingly, recent studies suggest that IL-17 may cooperate with IL-36 γ in driving the inflammatory response (13). IL-17 was shown to induce IL-36 γ expression in cultured human keratinocytes (14). IL-36 γ is expressed in human airway epithelial cells and keratinocytes following exposures to various inflammatory stimuli (14, 15). For example, rhinovirus infection increased IL-36 γ in airways of healthy individuals and asthma patients (16). IL-36 γ challenged mice demonstrated increased neutrophilic inflammation (17). But how bacterial DNA interacts with IL-17 signaling to regulate IL-36 γ production, and ensuing neutrophilic inflammation has not been previously investigated.

In this study, we hypothesized that bacterial DNA in an IL-17-high environment amplifies airway neutrophilic inflammation. To define how bacterial DNA and IL-17 signaling interact, we further hypothesized that IL-36 γ is responsible for the amplifying effect of IL-17 and bacterial DNA co-exposure on neutrophilic inflammation based on previous studies in psoriasis (14, 18). To test our hypotheses, we measured bacterial DNA, IL-8, and IL-36 γ in

bronchoalveolar lavage fluid from asthma and healthy control subjects. Further, we performed human distal (small) airway epithelial cell and alveolar macrophage cultures as well as mouse models to determine the role of co-exposure to IL-17 and bacterial DNA in airway neutrophilic inflammation, and the contribution of IL-36 γ to the amplifying effect of IL-17 and bacterial DNA on neutrophilic inflammation.

Materials and Methods

Detailed methods are in the supplemental Methods section.

Human subjects

Bronchoalveolar lavage fluid (BALF) from healthy and asthma individuals obtained through bronchoscopy as described in our previous publication (19) was utilized for the current study. The human study was approved by the National Jewish Health Institutional Review Board. De-identified donor lungs with no history of lung diseases or cigarette smoking were processed for proximal and distal airway epithelial cell isolation.

Quantification of bacterial DNA in human BALF

DNA was extracted from cell-free (centrifuged) human BALF. Bacterial DNA was quantified by real-time PCR for pan bacterial specific gene 16S rRNA (20). DNA extracted from NTHi was used to construct the standard curve to calculate BALF bacterial DNA concentration (pg/ml).

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) 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.

Cell culture

Airway epithelial cells and lung macrophages were stimulated with 10 ng/ml of recombinant human or murine IL-17A for 24 hours (21). Thereafter, cells were exposed to NTHi-derived DNA at 0.3µg/ml (22).

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

All animal procedures were approved by our Institutional Animal Care and Use Committee (IACUC). The mouse models employed in this manuscript: (1) C57BL/6 wild-type mouse model of IL-17 and NTHi-derived DNA treatment; (2) Myeloid cell TLR9 deficient (LysM-Cre⁺TLR9^{fl/fl}) mouse model of IL-17 and NTHi-derived DNA treatment; (3) rhDNase I treatment in wild-type mice with IL-17 treatment and live NTHi infection. Details of experimental design and generation of LysM-Cre⁺TLR9^{fl/fl} mice (23, 24) are in the full supplemental Methods section.

Measurement of airway hyperresponsiveness in mice

Airway hyperresponsiveness to methacholine was measured to indicate pulmonary function in wild-type C57BL/6 mice exposed to IL-17 and NTHi-derived DNA using the FlexiVent system. 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).

Statistical analysis

Data were analyzed using Graph Pad Prism software. 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.

Results

Bacterial DNA is increased in bronchoalveolar lavage fluid (BALF) of neutrophilic asthma patients

Demographic information, lung function and BALF leukocyte count data in asthma and healthy subjects are shown in Table 1. We compared pan bacterial DNA levels among non-neutrophilic asthma (NNA) and neutrophilic asthma (NA) subjects as well as normal control healthy subjects. NA in our current study was defined by $\geq 3\%$ neutrophils in BALF. This was based on the normal range of BALF neutrophils for the clinical laboratory, and our previous publication to define neutrophil-high vs. neutrophil-low refractory asthma that were positive for bacterial infection (19). NNA were defined by $< 3\%$ neutrophils in BALF. Bacterial DNA levels in BALF were significantly higher in the NA group than the NNA group and normal subjects (Figure 1a). No significant differences of bacterial DNA levels were seen between NNA and normal subjects. Significant and positive correlations of bacterial DNA levels with those of neutrophils and IL-8 in BALF were observed in asthma subjects (Figure 1b and c), but not in healthy control subjects ($r=0.4$, $p=0.6$).

Bacterial DNA amplifies IL-8 induction in IL-17-exposed human primary airway epithelial cells and alveolar macrophages

We sought to examine the direct effect of bacterial DNA on IL-8 production in a milieu mimicking a subset of asthma with higher IL-17 and neutrophil levels in the airways (25). As distal airway is the major site of airway obstruction in asthma, we used primary human small airway epithelial cells (HSAECs) grown at air-liquid interface (ALI) culture to determine if

bacterial DNA enhances IL-17-mediated IL-8 production. rhIL-17 alone (Figure 2a) induced IL-8 production as reported (26). Interestingly, NTHi-derived DNA alone did not induce IL-8. However, in cells pre-exposed to IL-17, NTHi-derived DNA significantly increased IL-8 production. As previous studies primarily utilized large airway epithelial cell culture models in asthma research, we confirmed that human tracheobronchial epithelial cells (HTBECs) also demonstrated similar responses to IL-17 and bacterial DNA (Figure 2b).

Since macrophages are involved in asthma (27), and respond to various danger signals (28, 29), we investigated whether human alveolar macrophages (HAMs), like epithelial cells, responded to bacterial DNA in an IL-17-high milieu. IL-17 alone in HAMs, unlike airway epithelial cells, did not increase IL-8 (Figure 2c). However, NTHi-derived DNA alone in HAMs increased IL-8. Importantly, combinational treatment of IL-17 and NTHi-derived DNA amplified IL-8 response in HAMs (Figure 2c).

Bacterial DNA enhances neutrophilic inflammation in mouse lungs exposed to IL-17

To investigate the *in vivo* effects of bacterial DNA in IL-17 mediated inflammation, wild-type C57BL/6 mice were intranasally inoculated with rmIL-17A for 24 hrs, and then exposed to NTHi-derived DNA for 24 hrs. As previously reported (30), rmIL-17A increased airway neutrophilic inflammation (Figure 3a and b). NTHi-derived DNA also induced lung neutrophilic inflammation. Importantly, levels of neutrophils and neutrophil chemoattractant LIX (CXCL5, Figure 3c) were further increased in the combinational treatment group.

We then determined if IL-17 and DNA may increase airway hyperresponsiveness in mice. As shown in Supplemental Figure 2, combinational IL-17 and DNA treatment resulted in an increase in the distal airway resistance, total pulmonary elastance, and distal airway elastance

after methacholine challenges. However, proximal airway resistance was similar among different treatment groups. Our data suggest that distal, but not proximal, airway obstruction occurred following the combinational treatment.

Role of Toll-like receptor 9 (TLR9) signaling in amplification of airway neutrophilic inflammation by bacterial DNA and IL-17

To determine if TLR9 mediates airway epithelial IL-8 production, HSAECs stimulated with both IL-17 and bacterial DNA were treated with A151 (a synthetic oligonucleotide initially identified as a TLR9 antagonist) or its negative control. A151 reduced IL-8 induction (Figure 4a). Because A151 was recently found to inhibit the cGAS/STING signaling axis involved in bacterial infection (31), we determined if STING signaling contributes to bacterial DNA-mediated pro-inflammatory response using STING knockout (SKO) human airway epithelial cells. Interestingly, there was no significant difference in IL-8 production between control and SKO cells with the combinational treatment (Figure 4b). Our results suggest a role of TLR9 signaling in airway epithelial responses to bacterial DNA.

To further confirm the role of TLR9 signaling in neutrophil mediator production, mouse tracheal epithelial cells (mTEC) and bone marrow-derived macrophages (BMDM) from TLR9 KO and wild-type mice were utilized. TLR9 KO (vs. wild-type) mTEC and BMDM with combinational IL-17 and DNA treatment significantly decreased the production of neutrophil chemoattractants LIX/CXCL5 and KC/CXCL1 (Figure 4c and d). Notably, KC was not induced in BMDM exposed to mammalian (mTEC-derived) DNA (Figure 4d), suggesting a role of bacterial DNA, but not host DNA, in the pro-inflammatory response.

To investigate the *in vivo* role of TLR9 signaling in myeloid cell (e.g., macrophage) responses to bacterial DNA, a mouse model of Cre-mediated TLR9 deletion in myeloid cells was utilized. As compared to the control mice (LysMCre⁻TLR9^{fl/fl}), TLR9 conditional knockout mice (LysMCre⁺TLR9^{fl/fl}) had significantly lower levels of neutrophils and LIX following IL-17 and NTHi-derived DNA co-treatment or DNA treatment alone (Figure 5).

IL-36 γ regulates neutrophilic inflammation induced by bacterial DNA and IL-17

As TLR9 signaling is in part involved in DNA/IL-17-mediated neutrophilic inflammation, we further determined the role of other signaling pathways such as IL-36 γ . IL-17 is a potent inducer of IL-36 γ during bacterial and fungal infections (15, 32). Activation of IL-36 γ signaling increases pro-neutrophilic mediators in the airways (15, 33).

We measured IL-36 γ in BALF from NA and NNA subjects and healthy subjects. Notably, NA subjects demonstrated significantly higher levels of IL-36 γ protein than the other two groups (Figure 6a). In HSAECs, IL-36 γ was slightly induced by IL-17, but was significantly increased by both IL-17 and bacterial DNA (Figure 6b). To demonstrate the role of IL-36 γ in IL-8 production, an IL-36 γ neutralizing antibody was employed in HSAECs. The IL-36 γ neutralizing antibody (vs. an isotype control IgG) significantly reduced the induction of IL-8 by the combinational treatment (Figure 6c).

DNase I treatment reduces neutrophilic inflammation in IL-17-exposed and NTHi-infected mice

To determine the role of DNA signaling in exaggerating the inflammatory response to IL-17 and live bacteria, we treated mice exposed to IL-17 and live NTHi with recombinant human DNase I.

Similar to our NTHi-derived DNA mouse model, NTHi infection in IL-17-treated mice significantly increased lung neutrophil influx. Importantly, DNase I treatment effectively reduced lung neutrophilic inflammation in NTHi-infected mice with or without IL-17 exposure (Figure 7a and b). Moreover, IL-17-challenged mice showed significantly higher levels of NTHi in the lung, which were also decreased by DNase I treatment (Figure 7c). Additionally, combinational IL-17 and NTHi treatment further increased IL-36 γ release into the airways, which was reduced by DNase I (Figure 7d).

Discussion

Our understanding about the role of bacterial products such as DNA in asthma is very limited. In the present study, we found increased bacterial DNA in asthmatic airways with high levels of neutrophils. Importantly, bacterial DNA or live bacteria under an IL-17-high setting is able to amplify lung neutrophilic inflammation. Mechanistically, we have identified the involvement of the TLR9/IL-36 γ signaling axis in bacterial DNA-mediated neutrophilic inflammation.

Although lung microbiome studies have demonstrated the presence of bacterial DNA in healthy subjects and its dysregulation in asthmatic airways (34), it has been challenging to study the collective functions of various strains of bacteria. In the current study, we demonstrated that bacterial DNA or live bacteria in an IL-17-high environment enhanced the production of pro-neutrophilic cytokines (e.g., IL-8 or LIX) and recruitment of neutrophils into the lung. Data from our cell culture and mouse models are supportive of our previous clinical studies showing increased IL-17 in airways of asthma subjects with neutrophilic inflammation (19). Importantly, our current study further demonstrated increased bacterial DNA in asthmatic airways, which significantly correlated with IL-8 and neutrophil levels. It remains unclear which types of cells in the lung respond to bacterial DNA and contribute to neutrophilic inflammation. As distal lung is the major site of airway obstruction in asthma, we chose to study two major types of cells, small airway epithelial cells and lung macrophages, which are critical in the distal lung response to pathogens and inflammatory mediators. Our cell culture experiments clearly demonstrated that both cell types enhanced IL-8 production following DNA and IL-17 co-exposure although each cell type responded differently to DNA alone or IL-17 alone. While airway epithelial cells responded to IL-17, lung macrophages did not. The potential explanation may be related to

different levels of IL-17 receptor (IL-17RA) in these cells. Our preliminary data suggest that IL-17 increased IL-17RA mRNA expression (about 2-fold) in airway epithelial cells, but not in macrophages (Supplemental Figure 3). Whether other types of lung cells such as alveolar epithelial cells are involved in bacterial DNA sensing under the IL-17 setting warrants further studies. Nonetheless, our mouse models of bacterial DNA and live bacterial infection also demonstrated the enhancing effect of bacterial DNA signaling on neutrophilic inflammation in IL-17-high environment. Defining the *in vivo* contribution of each cell type is beyond the scope of this manuscript, but it will be pursued to uncover the contribution of structural versus immune cells to the host response to bacterial DNA.

One of the key questions is about how bacterial DNA interacts with IL-17-mediated signaling cascade to amplify neutrophilic inflammation. Previous studies suggest that unmethylated bacterial DNA primarily utilizes TLR9 signaling (35). Here, we utilized TLR9 deficient mTEC and BMDM cells to study the role of TLR9. Based on LIX and KC data, TLR9 signaling contributed to 40% and 90% effects of bacterial DNA and IL-17 in mTEC and BMDM, respectively. By using the TLR9 conditional knockout mouse model, we were able to further demonstrate a critical role of myeloid cell (e.g., macrophages) TLR9 signaling in neutrophilic inflammation amplified by both bacterial DNA and IL-17. In the future, we will consider using TLR9 conditional knockout in airway epithelium to reveal the *in vivo* contribution of epithelial TLR9 signaling to the pro-inflammatory effect of bacterial DNA and IL-17. In addition, DNA may utilize STING and inflammasome pathways to induce inflammatory responses (36-38). Our data in STING KO cells suggests that STING signaling does not contribute to IL-8 production in airway epithelial cells exposed to bacterial DNA. However, we cannot exclude the involvement

of STING signaling in pro-neutrophilic mediator production in macrophages or in mouse models. The specific role of inflammasome in bacterial DNA and IL-17 induced inflammation will be considered in future studies.

We further explored whether combination of DNA and IL-17 may enhance IL-36 γ signaling pathway. In our preliminary bulk RNA sequencing studies in human small airway epithelial cells, we observed an increase (about 5-fold, n=3) of IL-36 γ mRNA expression by IL-17, but not by DNA treatment (Supplemental Figure 4). Our data suggest that IL-17 increased IL-36 γ protein production especially in the presence of bacterial DNA, leading to a significant increase of IL-8. The role of IL-36 γ in asthma was further supported by the fact that IL-36 γ protein levels were significantly higher in airways of patients with neutrophilic asthma. Importantly, for the first time, we demonstrated a synergistic effect of bacterial DNA on IL-17-mediated IL-36 γ protein production, which may contribute to IL-8 production in airway epithelial cells. Previous studies showed that IL-36 γ facilitates TLR9 translocation from ER to lysosomes and potentiates TLR9 activation (39). Interestingly, both IL-36 γ and TLR9 signaling pathways are mediated by MyD88/IRAK signaling to activate NF- κ B signaling. Thus, TLR9 and IL-36 γ may cooperate in pro-neutrophilic inflammation amplified by IL-17 and bacterial DNA.

An exciting aspect of our current study is to translate our discovery into the therapeutic potential of DNase I in attenuating excessive neutrophilic inflammation induced by bacteria and IL-17. Although DNase I treatment was effective in reducing lung inflammation in a mouse model of rhinovirus infection, its focus was on the role of DNase in endogenous (host) DNA released from leukocytes (e.g., neutrophils) (40). By using an NTHi infection mouse model, we demonstrated

that DNase I treatment reduced airway IL-36 γ and neutrophil levels in mouse lungs exposed to both IL-17 and live bacteria. We speculate that the therapeutic effect of DNase is in part through degradation of DNA released from the bacteria, but we cannot exclude the possibility that DNase also degraded endogenous DNA released from neutrophils and other types of cells.

One of the interesting findings in this study was that IL-17 challenged mice showed higher lung NTHi load. Although this is in line with the observation of increased airway bacterial load in patients with neutrophilic asthma (4), it seems to be contradictory to previous beliefs on the protective role of IL-17 in bacterial clearance (41, 42). The exact mechanisms for this observation remain unclear, but this may be related to the effect of IL-17 on IL-36 γ as IL-36 γ may promote lung bacterial (e.g., *Pseudomonas aeruginosa*) infection (43). In addition, we observed more lung neutrophil recruitment in mice infected with live NTHi than in mice treated with NTHi-derived DNA in the absence or presence of IL-17. This may be attributed to the fact that live bacteria utilize multiple mechanisms to induce the inflammatory response. For example, lipooligosaccharide (LOS), a major surface antigen of NTHi, is able to induce the pro-inflammatory response (44). Thus, bacterial DNA likely serves as one of the components or mechanisms by which bacteria enhance neutrophilic inflammation in IL-17-high environment. We found more airway eosinophils in patients with neutrophilic asthma compared to healthy controls, but not patients with non-neutrophilic asthma. This may support the concept of heterogeneity of airway inflammation in asthma. There is a subset of patients with mixed-granulocytic asthma, who had higher levels of both neutrophils and eosinophils (45, 46). Notably, DNA sensing by the cyclic GMP–AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway may activate transcription factor STAT6 and subsequently increase the

expression of cytokines such as eosinophil chemoattractant CCL26 (eotaxin-3) (47, 48). We speculate that increased bacterial DNA in neutrophilic asthma patients may also utilize the cGAS-STING pathway to induce the recruitment of eosinophils into the airway. The neutrophil asthma group in our current study appeared to have more patients on high dose inhaled corticosteroids (ICS) compared to the non-neutrophilic group although the difference did not reach the statistically significant level. The use of high dose ICS has been associated with bacterial infection and airway neutrophilia (49, 50). Whether ICS affect airway bacterial DNA levels and associated neutrophilic inflammation in patients with neutrophilic asthma remains unclear, but warrants future studies.

There are several limitations to the current study. First, our studies focus on the acute treatment of bacterial DNA or bacterial infection. Although our models are relevant to address acute asthma exacerbations, it will be important to determine the role of bacterial DNA in neutrophilic inflammation in chronic asthma. Second, the mechanisms for the *in vivo* beneficial effects of DNase I treatment in IL-17 and NTHi exposed mice remain unclear. We observed reduced bacterial load in the lungs of mice treated with DNase I in the absence or presence of IL-17. Our finding is consistent with a previous study showing less bacterial load, biofilm formation and viability after DNase I treatment in a bacterial culture model (51). This may suggest that the therapeutic effect of DNase I in our mouse models of NTHi infection with or without IL-17 treatment could be in part through degradation of DNA released from the bacteria. However, given the ability of DNase to reduce the formation of neutrophil extracellular traps (NETs) and associated inflammatory response (52), we cannot exclude the possible effect of DNase-mediated degradation of DNA released from host cells such as neutrophils during bacterial infection. At

present, there is no DNase that specifically degrades bacteria-derived DNA. Therefore, it would be technically difficult to discern if the *in vivo* beneficial effect of DNase I treatment comes from the degradation of DNA from the bacteria and/or the host. Future research efforts are warranted to engineer bacteria-specific DNase to determine the contribution of bacterial DNA in lung inflammation during bacterial infection and IL-17 treatment. Third, the therapeutic effect of DNase on airway neutrophilic inflammation in human asthma was not addressed, but will be considered in our future clinical studies aimed to determine if DNase may prevent the frequency of asthma exacerbations associated with bacterial infections. Finally, the molecular mechanisms by which bacterial DNA or infection cooperates with IL-17 in exaggerating neutrophilic inflammation needs to be further investigated.

In conclusion, we have demonstrated a role of bacterial DNA in amplifying airway neutrophilic inflammation in an IL-17-high setting. The interplay of TLR9 and IL-36 γ signaling may serve as one of the mechanisms to exaggerate airway neutrophilic inflammation (Figure 8). We anticipate that our findings may have implications in developing several potential therapeutic approaches, including TLR9 antagonists, IL-36 γ neutralizing antibodies and DNase I, to attenuate asthma exacerbations in patients who are insensitive to corticosteroid therapy.

Figure legends

Figure 1. Increased bacterial DNA in bronchoalveolar lavage fluid (BALF) of neutrophilic asthma subjects. (a) bacterial DNA levels in BALF of normal subjects, non-neutrophilic asthma subjects and neutrophilic asthma subjects. Correlations between bacterial DNA and neutrophils (b) and IL-8 (c) in BALF of non-neutrophilic asthma subjects (black open circles) and neutrophilic asthma subjects (red open circles). The horizontal bars represent medians.

Figure 2. Bacterial DNA amplified IL-8 production in IL-17-exposed human airway epithelial cells and lung macrophages. (a) Primary normal human small airway epithelial cells (n=5 subjects) grown at air-liquid interface. (b) Primary normal human tracheobronchial epithelial cells under submerged culture (4 subjects). (c) Primary normal human alveolar macrophages (n=5 subjects). Cells were stimulated with or without IL-17 for 24 hours and then treated with or without bacterial DNA for 48 hours. The horizontal bars represent medians.

Figure 3. Bacterial DNA increased neutrophilic inflammation in IL-17 exposed mouse lungs. Wild-type C57BL/6 mice (n=10 to 12 mice/group) from two independent experiments were intranasally challenged with IL-17 (3µg/mouse) for 24 hours, followed by DNA (1µg/mouse) derived from Nontypeable *Haemophilus influenzae*. After 24 hours, bronchoalveolar lavage (BAL) fluid was analyzed for % of neutrophils (a), total numbers of neutrophils (b), and neutrophil chemoattractant LIX (c). The horizontal bars represent medians.

Figure 4. Role of DNA signaling in bacteria DNA-mediated amplification of neutrophil chemokines. (a) Primary normal human small airway epithelial cells (n=4 subjects) under

submerged culture were treated with IL-17 for 24 hours, and then exposed to bacterial DNA±A151, or A151 negative control (ctrl) for 48 hours. **(b)** Human tracheobronchial epithelial cells transduced with lentivirus containing the STING sgRNA or scramble (SCR) control sgRNA plasmid construct were treated with IL-17 for 24 hours, and then exposed to bacterial DNA under the submerged condition for 48 hours. KO = knockout. **(c)** Mouse tracheal epithelial cells (mTEC) isolated from wild-type (C57BL/6) and TLR9 KO mice were grown at air-liquid interface and stimulated with or without IL-17 for 24 hours and then treated with/without bacterial DNA for 48 hours. **(d)** Bone marrow derived macrophages from wild-type and TLR9 KO mice were cultured and stimulated with or without IL-17 and exposed to either bacterial DNA (bDNA) or mammalian DNA (mDNA) extracted from mTEC. N=3 to 4 replicates. The horizontal bars represent means.

Figure 5. TLR9 conditional knockout in myeloid cells down-regulates airway neutrophilic inflammation in mice exposed to IL-17 and bacterial DNA. $\text{LysMCre}^{+}\text{TLR9}^{\text{fl/fl}}$ and $\text{LysMCre}^{-}\text{TLR9}^{\text{fl/fl}}$ mice were challenged with IL-17 (3 $\mu\text{g}/\text{mouse}$) in 0.01% BSA or 0.01% BSA ([−] control) for 24 hours, followed by bacterial DNA (1 $\mu\text{g}/\text{mouse}$) challenge or Tris-EDTA buffer via intranasal inoculation. After 24 hours of DNA exposure, bronchoalveolar lavage (BAL) fluid was collected and analyzed for neutrophils **(a, b)**, and neutrophil chemoattractant LIX **(c)**. The horizontal bars represent medians.

Figure 6. IL-36 γ in human bronchoalveolar lavage fluid (BALF) and cultured airway epithelial cells. **(a)** Representative western blot images and densitometry of IL-36 γ protein in BALF from healthy control subjects, non-neutrophilic asthma (NNA) and neutrophilic asthma

(NA) subjects. Each dot represented one subject. (b) IL-36 γ western blot images and densitometry in supernatants of cultured primary normal human small airway epithelial cells (n=5 subjects) exposed to IL-17 for 24 hrs, and then bacterial DNA for 48 hrs. (c) Primary normal human small airway epithelial cells were stimulated with or without IL-17 for 24 hours and then treated with/without bacterial DNA in the presence of an IgG isotype control or an IL-36 γ neutralizing antibody for 48 hrs. The horizontal bars represent medians.

Figure 7. Recombinant human DNase I reduces lung neutrophilic inflammation in IL-17-challenged and nontypeable *Haemophilus influenzae* (NTHi)-infected mice. Wild-type C57BL/6 mice were challenged with IL-17 (3 μ g/mouse), NTHi (10⁷ CFU/mouse), and DNase I (5 μ g/mouse) via intranasal inoculation. After 24 hours, bronchoalveolar lavage (BAL) fluid was collected for analyzing total numbers of neutrophils (a), neutrophil chemoattractant LIX (b), count of colony forming units (CFU) in the right lung tissue homogenate (c), and IL-36 γ protein using western blot (d). The horizontal bars represent medians.

Figure 8. Proposed mechanisms by which bacterial DNA and IL-17 cooperate to amplify airway neutrophilic inflammation. IL-17 binds to its receptor and induces IL-36 γ expression. IL-36 γ binds to its heterodimeric receptor complex, subsequently recruits MyD88, activates MAPK and NF- κ B signaling cascades, and induces IL-8 and neutrophilic inflammation. Bacterial DNA binds to TLR9, leading to activation of MAPK and NF- κ B signaling pathways. Activation of IL-36 γ signaling also results in TLR9 translocation, leading to further activation of TLR9 signaling and amplification of neutrophilic inflammation. MyD88 = adaptor protein myeloid differentiated protein 88. IRAK1 = Interleukin-1 receptor-associated kinase 1. MAPK =

mitogen-activated protein kinase. NF- κ B = nuclear transcription factor kappa B. TLR9 = Toll-like receptor 9.

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Author's contribution

NM, design of the work, the acquisition, analysis and interpretation of data, and draft of the manuscript; RJM, RA and CJW, conception of the work, review and edits on the manuscript; NS, KGD, NP CK and LZ, contribution to experiments, review and edits on the manuscript. HWC, conception and design of the work, analysis and interpretation of data, draft and edits of the manuscript.

Table 1. Characteristics of healthy and asthma subjects

	Healthy subjects (n=20)	Non-neutrophilic asthma (NNA) (n=14)	Neutrophilic asthma (NA) (n=16)	P value
Age (yrs)	33.6±2.0	42.2±3.7	51.8±3.9*	*<0.01 vs. healthy, >0.05 NA vs. NNA
Gender	Male (7) Female (13)	Male (6) Female (8)	Male (8) Female (8)	0.33
FEV ₁ (% predicted)	96.±2.7	75.4±5.5*	61.2±5.0*	*<0.01 vs. healthy 0.07, NA vs. NNA
BMI	29.7±1.3	27.5±1.5	29.3±1.5	0.55
FeNO (parts per billion)	23.2±3.3	36.3±5.9	52.7±13.1*	*0.02 vs. healthy, p>0.05 NA vs. NNA
ACT score	N/A	17.5±1.3	17.1±1.6	0.84, NA vs. NNA
Inhaled corticosteroid (CS) use	None	High dose (3), low dose (11)	High dose (9), Low dose (5), medium dose (1), unknown (1)	0.11
Neutrophils (%) in BAL	1.9±0.3	1.4±0.2	15.3±4.6	*<0.01 vs. healthy and NNA
Eosinophils (%) in BAL	0.5±0.2	1.8±0.7	3.0±0.9*	*<0.01 vs. healthy, p>0.05 NA vs. NNA
Macrophages (%) in BAL	91.5±1.0	90.4±2.7	72.4±4.9*	*<0.01 vs. healthy and NNA
Lymphocytes (%) in BAL	6.1±0.8	6.4±1.3	9.9±1.7	0.11
IL-8 (pg/ml) in BAL	24.8±1.5	41.8±5.8	138.5±42.7*	*<0.01 vs. healthy and NNA

Data expressed as means±SEM.

FEV₁ = forced expiratory volume in one second; BMI = body mass index; FeNO = fractional exhaled nitric oxide; ACT = asthma control test.

All subjects were non-smokers.

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Figure 1

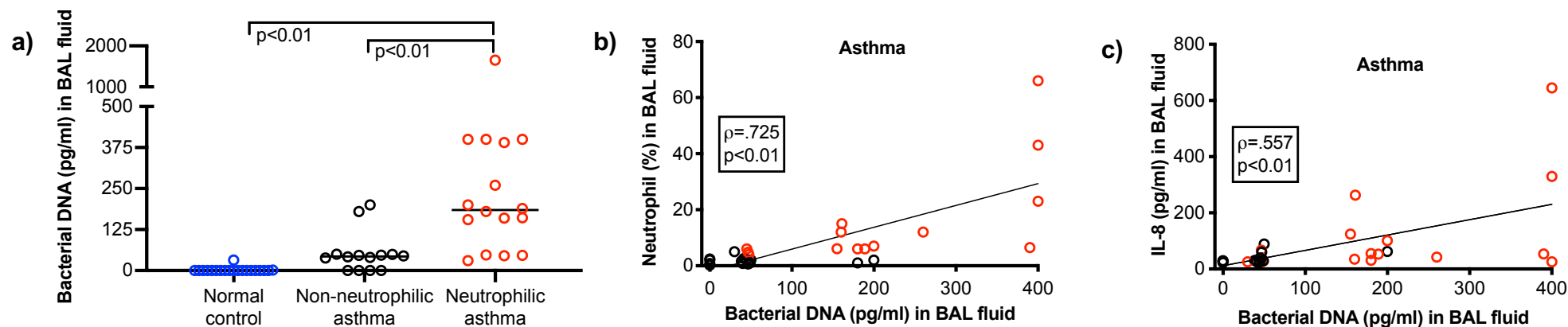


Figure 1. Increased bacterial DNA in bronchoalveolar lavage fluid (BALF) of neutrophilic asthma subjects. (a) bacterial DNA levels in BALF of normal subjects, non-neutrophilic asthma subjects and neutrophilic asthma subjects. Correlations between bacterial DNA and neutrophils (b) and IL-8 (c) in BALF of non-neutrophilic asthma subjects (black open circles) and neutrophilic asthma subjects (red open circles) . The horizontal bars represent medians.

Figure 2

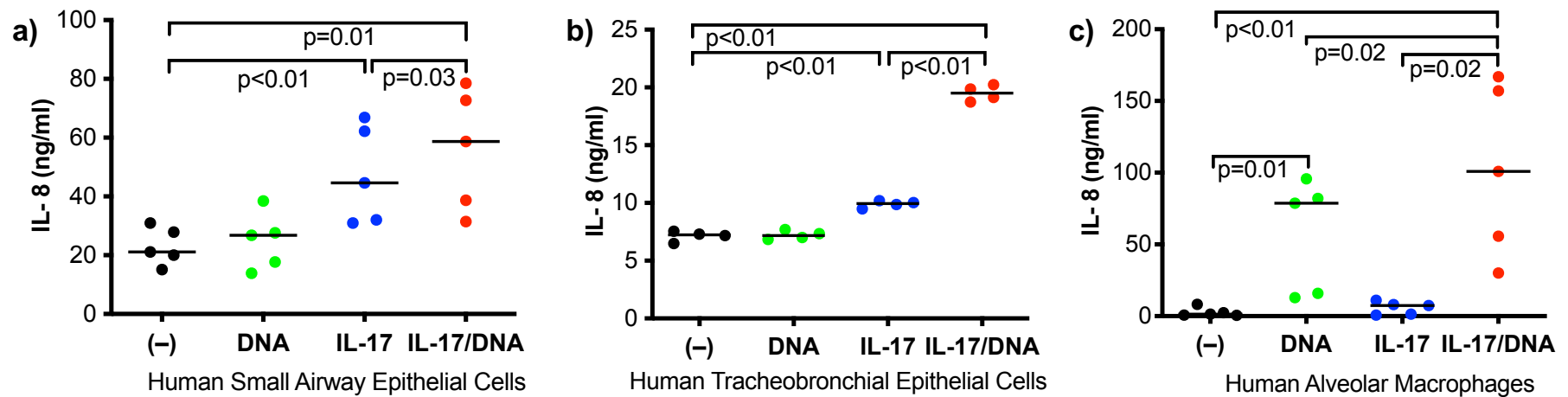


Figure 2. Bacterial DNA amplified IL-8 production in IL-17-exposed human airway epithelial cells and lung macrophages. (a) Primary normal human small airway epithelial cells (n=5 subjects) grown at air-liquid interface. (b) Primary normal human tracheobronchial epithelial cells under submerged culture (4 subjects). (c) Primary normal human alveolar macrophages (n=5 subjects). Cells were stimulated with or without IL-17 for 24 hours and then treated with or without bacterial DNA for 48 hours. The horizontal bars represent medians.

Figure 3

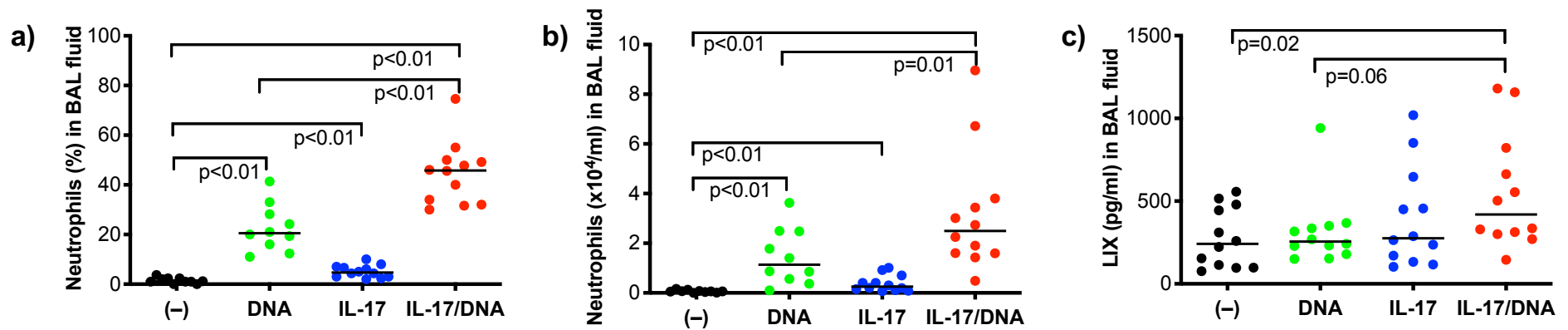


Figure 3. Bacterial DNA increased neutrophilic inflammation in IL-17 exposed mouse lungs. Wild-type C57BL/6 mice (n=10 to 12 mice/group) from two independent experiments were intranasally challenged with IL-17 (3 μ g/mouse) for 24 hours, followed by DNA (1 μ g/mouse) derived from Nontypeable *Haemophilus influenzae*. After 24 hours, bronchoalveolar lavage (BAL) fluid was analyzed for % of neutrophils (a), total numbers of neutrophils (b), and neutrophil chemoattractant LIX (c). The horizontal bars represent medians.

Figure 4

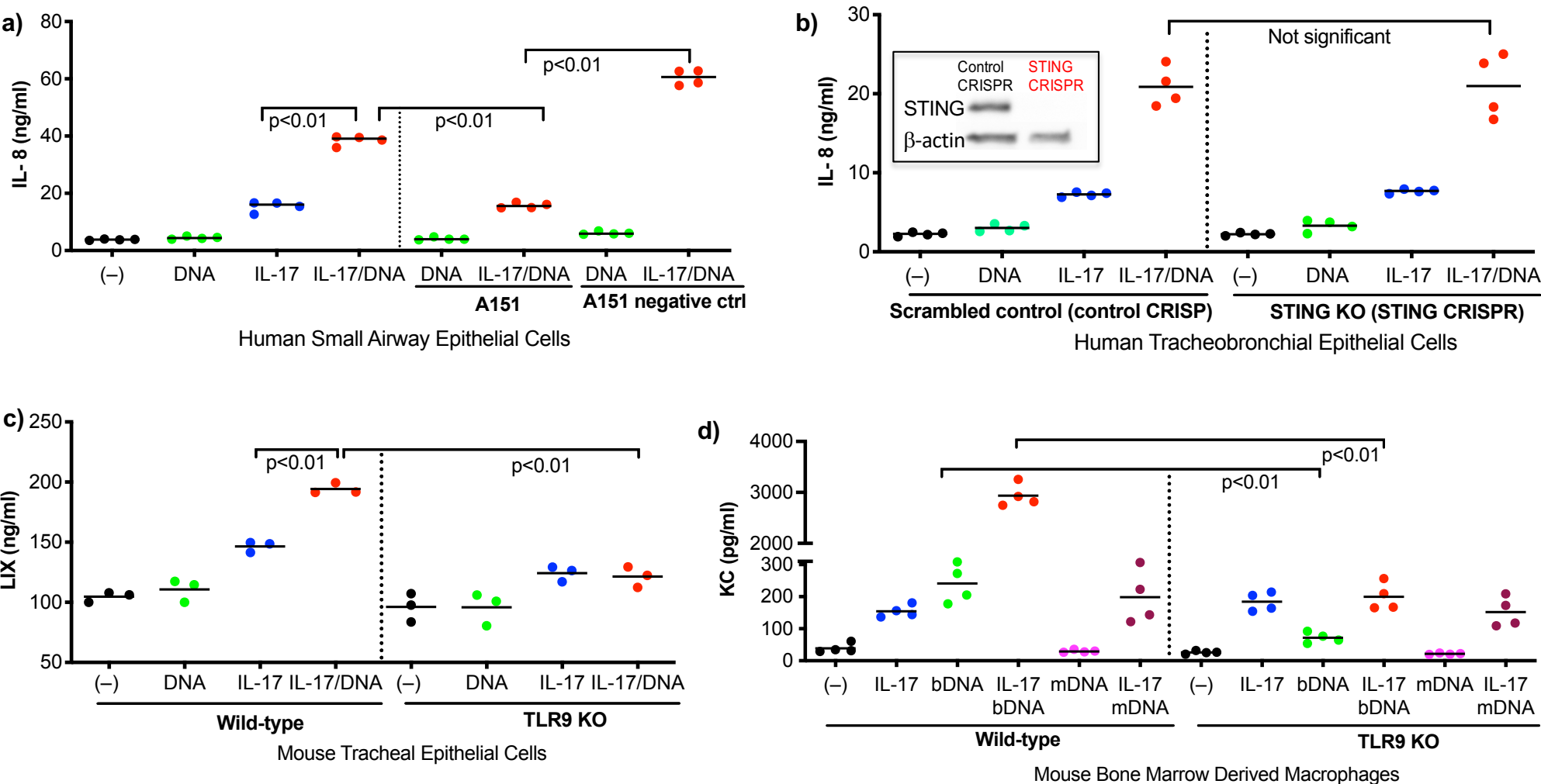


Figure 4. Role of DNA signaling in bacteria DNA-mediated amplification of neutrophil chemokines. (a) Primary normal human small airway epithelial cells (n=4 subjects) under submerged culture were treated with IL-17 for 24 hours, and then exposed to bacterial DNA ± A151, or A151 negative control (ctrl) for 48 hours. (b) Human tracheobronchial epithelial cells transduced with lentivirus containing the STING sgRNA or scramble (SCR) control sgRNA plasmid construct were treated with IL-17 for 24 hours, and then exposed to bacterial DNA under the submerged condition for 48 hours. KO = knockout. (c) Mouse tracheal epithelial cells (mTEC) isolated from wild-type (C57BL/6) and TLR9 KO mice were grown at air-liquid interface and stimulated with or without IL-17 for 24 hours and then treated with/without bacterial DNA for 48 hours. (d) Bone marrow derived macrophages from wild-type and TLR9 KO mice were cultured and stimulated with or without IL-17 and exposed to either bacterial DNA (bDNA) or mammalian DNA (mDNA) extracted from mTEC. N = 3 to 4 replicates. The horizontal bars represent means.

Figure 5

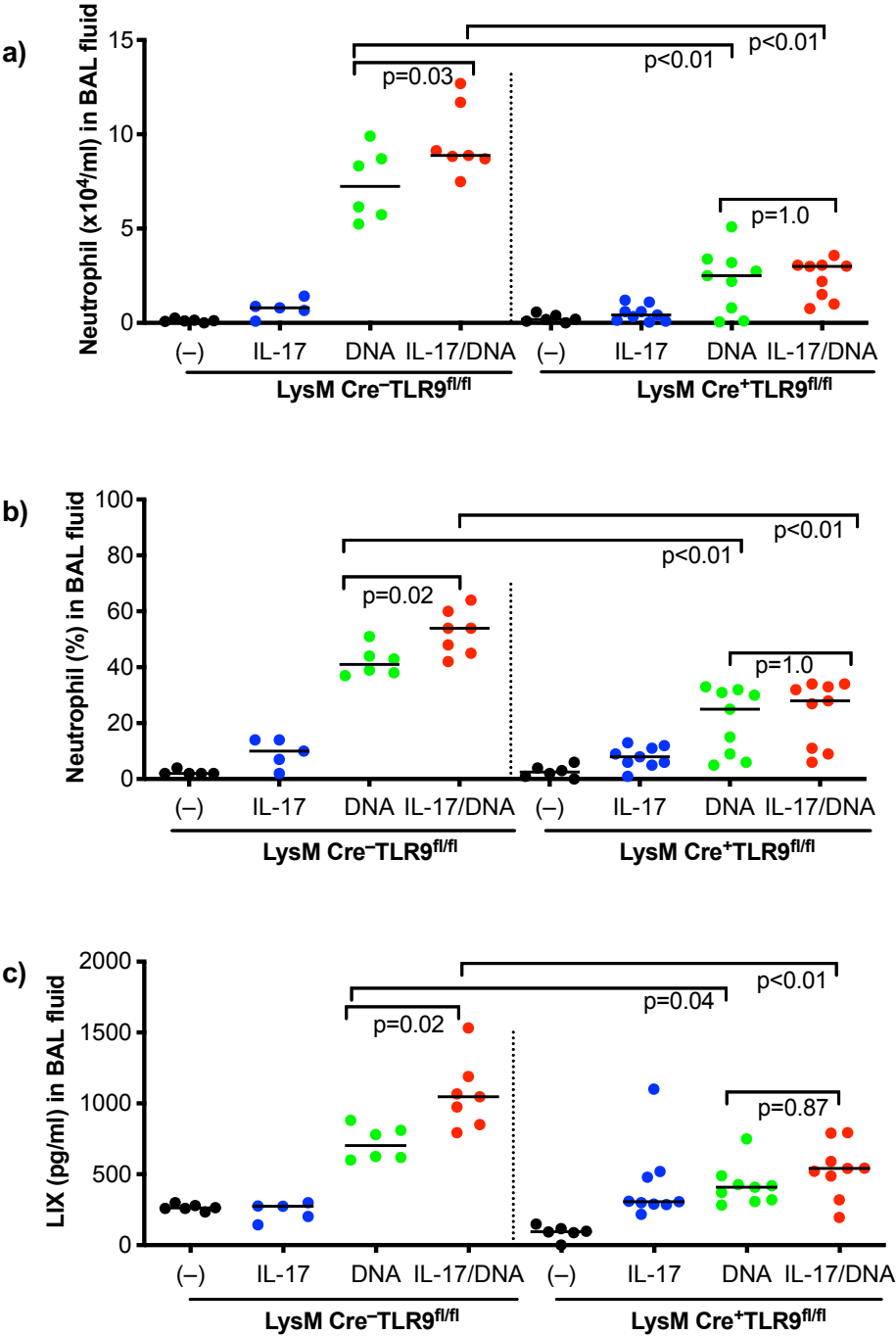


Figure 5. TLR9 conditional knockout in myeloid cells down-regulates airway neutrophilic inflammation in mice exposed to IL-17 and bacterial DNA. LysMCre⁺TLR9^{fl/fl} and LysMCre⁻TLR9^{fl/fl} mice were challenged with IL-17 (3 $\mu\text{g}/\text{mouse}$) in 0.01% BSA or 0.01% BSA ([–] control) for 24 hours, followed by bacterial DNA (1 $\mu\text{g}/\text{mouse}$) challenge or Tris-EDTA buffer via intranasal inoculation. After 24 hours of DNA exposure, bronchoalveolar lavage (BAL) fluid was collected and analyzed for neutrophils (**a**, **b**), and neutrophil chemoattractant LIX (**c**). The horizontal bars represent medians.

Figure 6

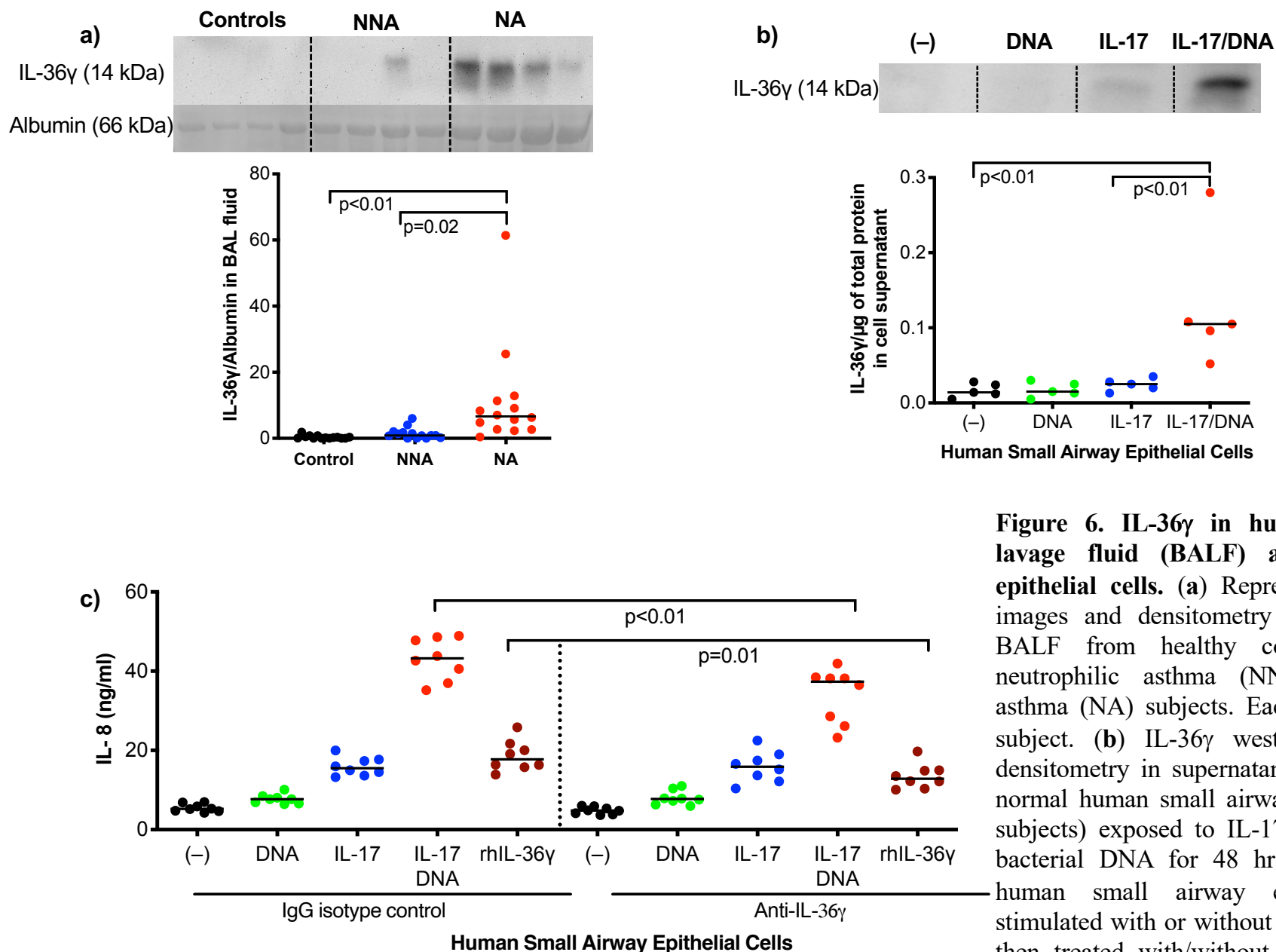


Figure 6. IL-36γ in human bronchoalveolar lavage fluid (BALF) and cultured airway epithelial cells. (a) Representative western blot images and densitometry of IL-36γ protein in BALF from healthy control subjects, non-neutrophilic asthma (NNA) and neutrophilic asthma (NA) subjects. Each dot represented one subject. (b) IL-36γ western blot images and densitometry in supernatants of cultured primary normal human small airway epithelial cells (n=5 subjects) exposed to IL-17 for 24 hrs, and then bacterial DNA for 48 hrs. (c) Primary normal human small airway epithelial cells were stimulated with or without IL-17 for 24 hours and then treated with/without bacterial DNA in the presence of an IgG isotype control or an IL-36γ neutralizing antibody for 48 hrs. The horizontal bars represent medians.

Figure 7

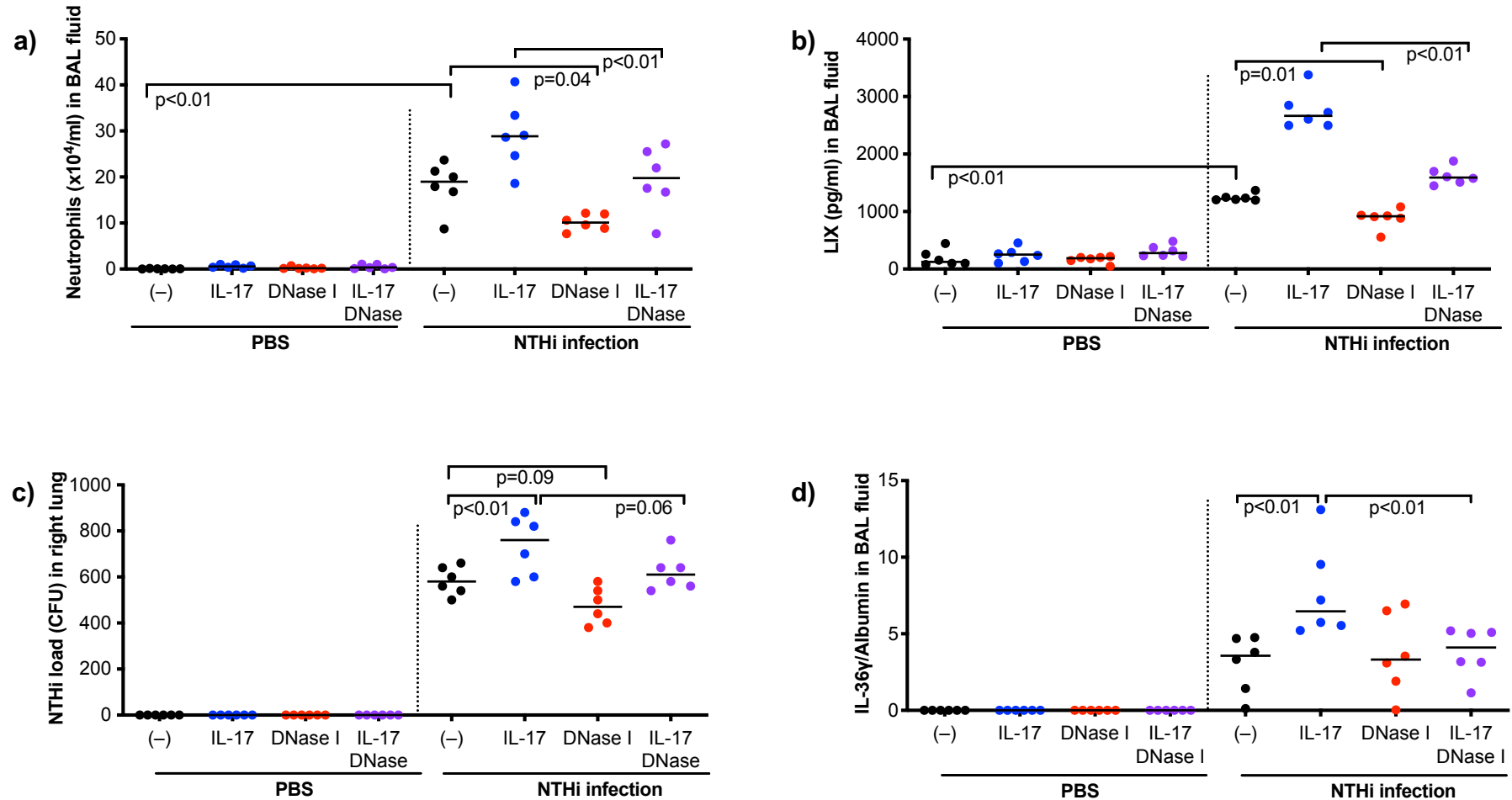


Figure 7. Recombinant human DNase I reduces lung neutrophilic inflammation in IL-17-challenged and nontypeable *Haemophilus influenzae* (NTHi)-infected mice. Wild-type C57BL/6 mice were challenged with IL-17 (3 $\mu\text{g}/\text{mouse}$), NTHi (10⁷ CFU/mouse), and DNase I (5 $\mu\text{g}/\text{mouse}$) via intranasal inoculation. After 24 hours, bronchoalveolar lavage (BAL) fluid was collected for analyzing total numbers of neutrophils (a), neutrophil chemoattractant LIX (b), count of colony forming units (CFU) in the right lung tissue homogenate (c), and IL-36 γ protein using western blot (d). The horizontal bars represent medians.

Figure 8

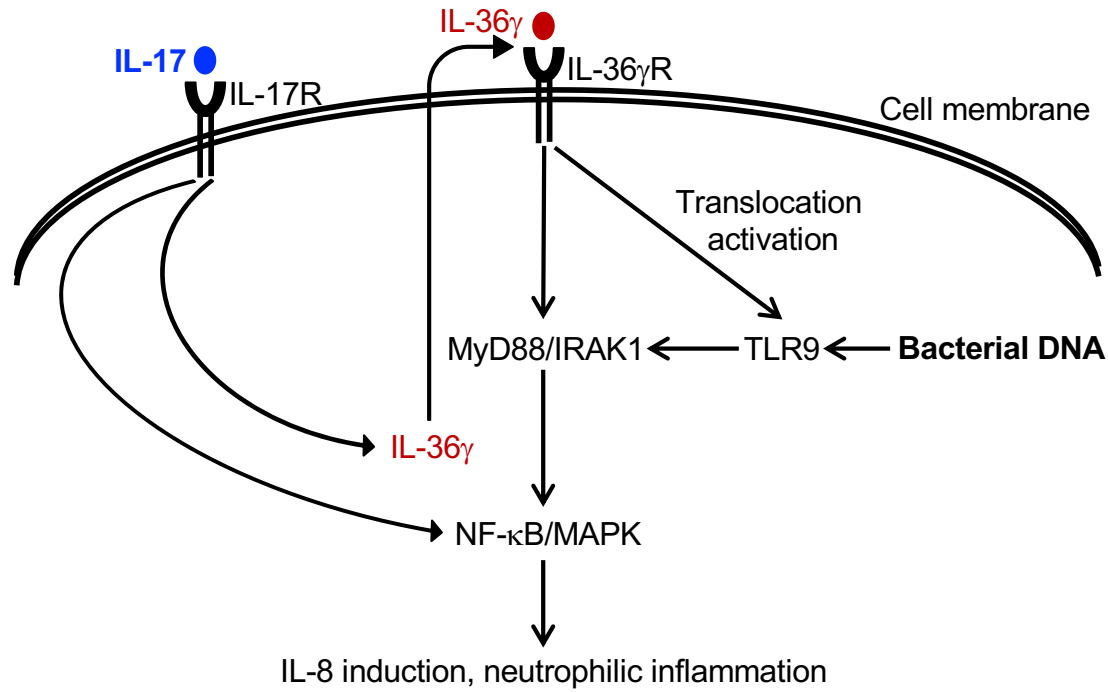


Figure 8. Proposed mechanisms by which bacterial DNA and IL-17 cooperate to amplify airway neutrophilic inflammation. IL-17 binds to its receptor and induces IL-36 γ expression. IL-36 γ binds to its heterodimeric receptor complex, subsequently recruits MyD88, activates MAPK and NF- κ B signaling cascades, and induces IL-8 and neutrophilic inflammation. Bacterial DNA binds to TLR9, leading to activation of MAPK and NF- κ B signaling pathways. Activation of IL-36 γ signaling also results in TLR9 translocation, leading to further activation of TLR9 signaling and amplification of neutrophilic inflammation. MyD88 = adaptor protein myeloid differentiated protein 88. IRAK1 = Interleukin-1 receptor-associated kinase 1. MAPK = mitogen-activated protein kinase. NF- κ B = nuclear transcription factor kappa B. TLR9 = Toll-like receptor 9.

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 \pm DNA was removed. Cells were then washed with PBS and added with fresh media \pm 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 μ g/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 through bronchoscopy. Given the epithelial lining fluid volume recovered in healthy 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 μ g/ml). This would support the dose of 0.3 μ g/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 μ g/ml) tested, we found that cells exposed to 10ng/ml of rhIL-17A and 0.3 μ g/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 \pm 3µM of A151 or 3µM of negative control of A151 using FuGENE® HD transfection reagent (Promega™, Madison, Wisconsin, USA) for 16 hours. Next, media \pm DNA was removed, washed with PBS, and refreshed with media \pm 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% CO₂ for 2 hours and then washed to remove non-adherent cells. After 48 hours, the adherent 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 \pm DNA were washed with PBS, and fresh media \pm 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 BronchiaLifeTM 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 1×10^6 /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 5×10^5 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 0.3 μ g/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 3 μ g/mouse prepared in 0.01% bovine serum albumin (BSA) or 0.01% BSA (control) for 24 hrs (14), followed by NTHi-derived DNA at 1 μ g/mouse (15, 16) or 50 μ L Tris-EDTA (TE) buffer (17). Our chosen dose of bacterial DNA at 1 μ g/mouse was based on the fact that about 50% of the dose in 50 μ L administered to the nose likely entered the lung [5], the actual dose given in mouse lung is about 0.5 μ g. 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 cytopsin 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 3 µg of rmIL-17A or BSA (control) in the presence or absence of 5 µg 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 ($\Delta\Delta CT$) method was utilized with the housekeeping gene 18S as an internal control (23, 24).

Western blot

To measure IL-36 γ 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-36 γ (1:1000, R&D Systems, Minneapolis, Minnesota, USA) or anti-mouse IL-36 γ (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-36 γ from the serum on the levels of lung cells-derived IL-36 γ , IL-36 γ 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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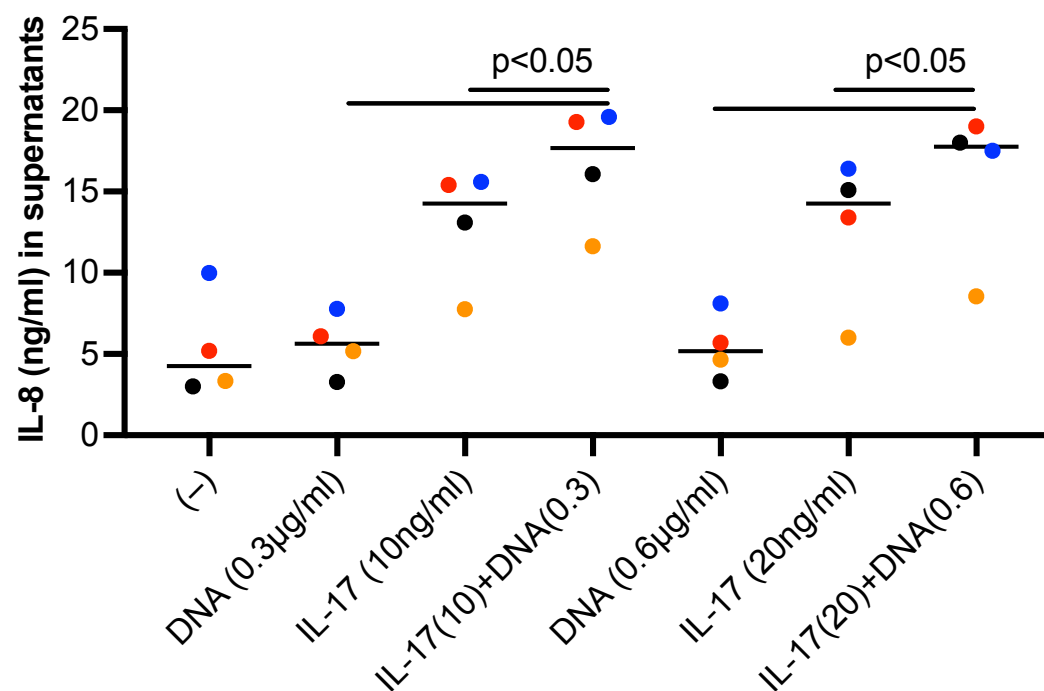
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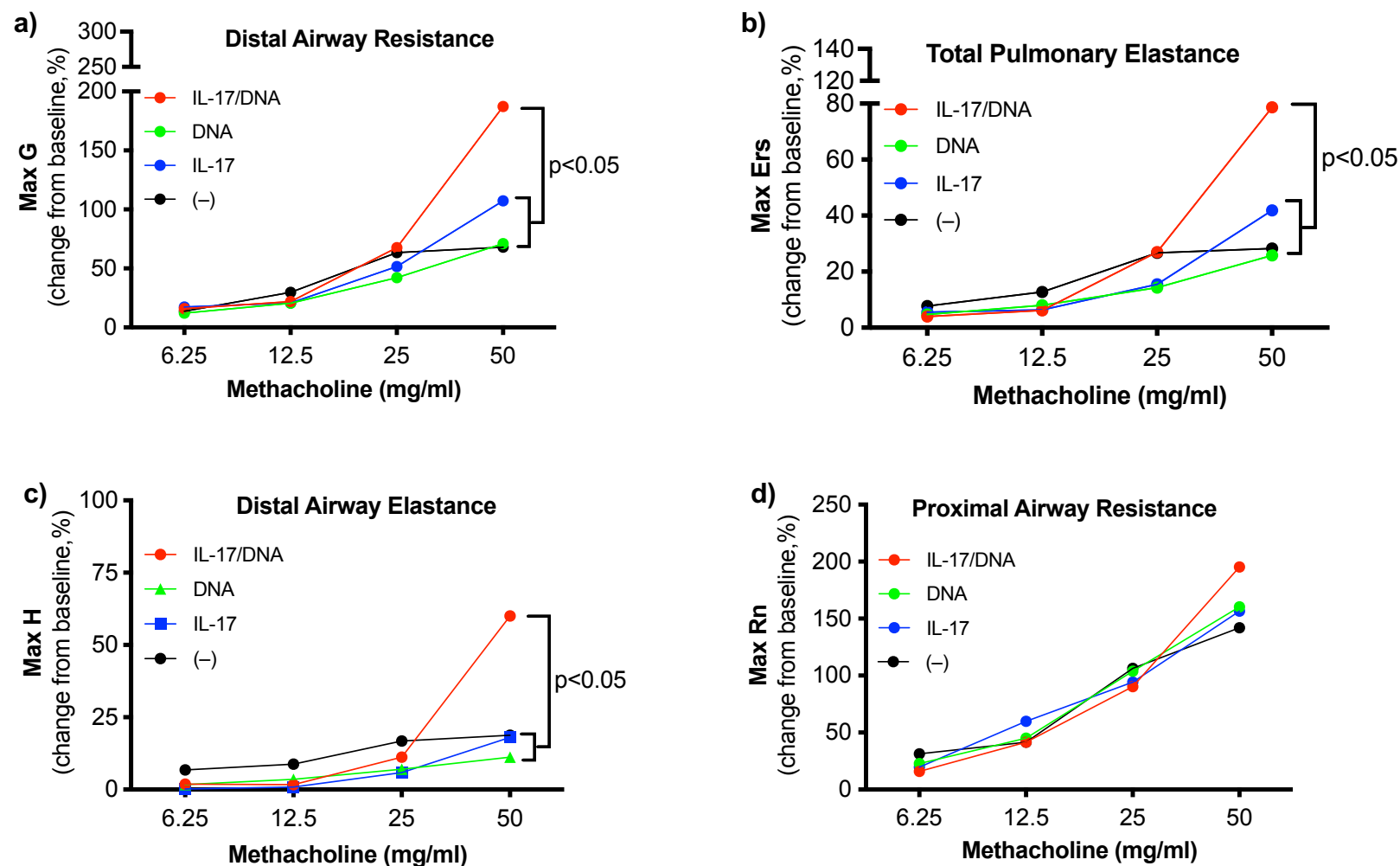
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Supplemental Figure 1



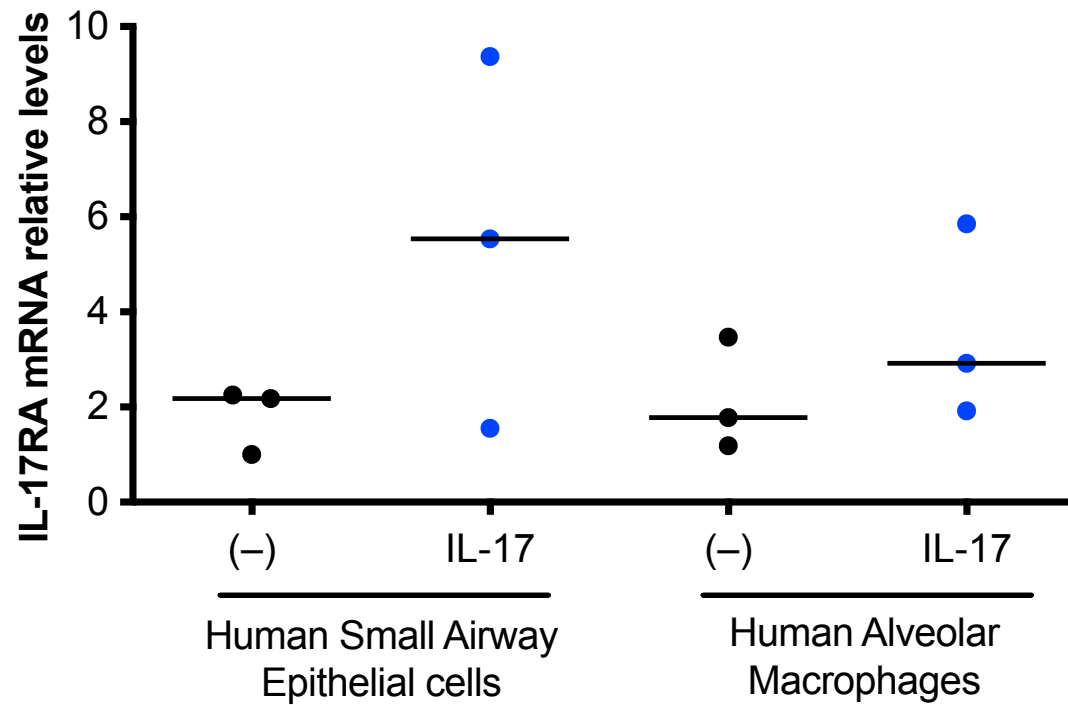
Supplemental Figure 1. Dose response study of NTHi-derived DNA and IL-17 stimulation in cultured human airway epithelial cells. Combination of bacterial DNA at 0.3 µg/ml and IL-17 at 10 ng/ml already amplified IL-8 production significantly in primary normal human small airway epithelial cells (n=4 subjects) grown under submerged conditions. Cells were stimulated with or without IL-17 for 24 hours and then treated with or without bacterial DNA for 24 hours, or or left untreated (medium control, (-)) for 48 hours. The horizontal bars represent medians.

Supplemental Figure 2



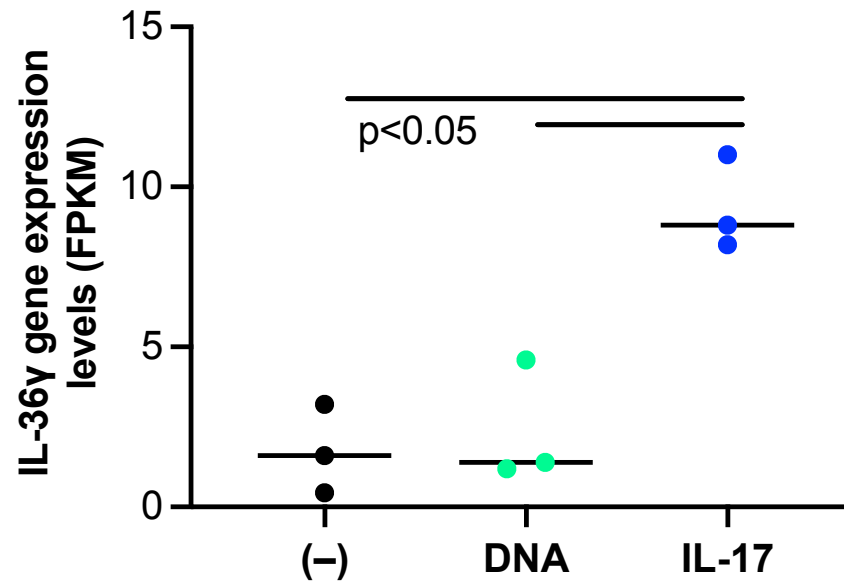
Supplemental Figure 2. Bacterial DNA in IL-17-challenged mice impaired distal airway function. Wild-type C57BL/6 mice (n = 10 to 12 mice/group) from two independent experiments were intranasally challenged with IL-17 (3 μ g/mouse) in 0.01% bovine serum albumin (BSA) or 0.01% BSA (-) for 24 hours, followed by DNA (1 μ g/mouse) derived from Nontypeable *Haemophilus influenzae* or Tris-EDTA buffer (-) used to prepare DNA. After 24 hours, airway function was assessed by exposing mice to increasing doses of methacholine. Data were reported as distal airway resistance (Max G) (a), total pulmonary elastance (Max Ers) (b), distal airways elastance (Max H) (c), and proximal airway resistance (Max Rn) (d). Mean values were presented.

Supplemental Figure 3



Supplemental Figure 3. Impact of IL-17 on IL-17RA expression. Primary normal human small airway epithelial cells grown at air-liquid interface, and alveolar macrophages cultured under submerged conditions were stimulated with or without IL-17 for 48 hours. N=3 subjects.

Supplemental Figure 4



Supplemental Figure 4. Impact of IL-17 on IL-36 γ gene expression. Primary normal human small airway epithelial cells grown at air-liquid interface were stimulated with or without IL-17 or bacterial DNA (NTHi-derived DNA) for 48 hours. FPKM stands for fragments per kilobase of transcript per million mapped reads indicating relative expression of a gene (IL-36 γ) proportional to the number of cDNA fragments of origin. N=3 subjects.