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

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Shareable abstract (@ERSpublications)
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-36γ neutralising antibody or DNase I. https://bit.ly/3k9zkj4

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Abstract

Background Neutrophilic asthma (NA) is associated with increased airway interleukin (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.* Toll-like receptor 9 (TLR9)/IL-36γ signalling axis).

Methods Bacterial DNA, IL-8 and IL-36γ were measured in bronchoalveolar lavage fluid (BALF) of people with 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-36γ signalling 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-36γ increased in BALF of NA 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-36γ signalling axis.

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

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-neutrophilic asthma (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 in 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 mechanisms. 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 interferon-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-36γ in driving the inflammatory response [13]. IL-17 was shown to induce IL-36γ expression in cultured human keratinocytes [14]. IL-36γ is expressed in human airway epithelial cells and keratinocytes following exposure to various inflammatory stimuli [14, 15]. For example, rhinovirus infection increased IL-36γ in airways of healthy individuals and asthma patients [16]. IL-36γ-challenged mice demonstrated increased neutrophilic inflammation [17]. But how bacterial DNA interacts with IL-17 signalling to regulate IL-36γ production and ensuing neutrophilic inflammation has not been previously investigated.

In this study, we hypothesised that bacterial DNA in an IL-17-high environment amplifies airway neutrophilic inflammation. To define how bacterial DNA and IL-17 signalling interact, we further hypothesised that IL-36γ 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-36γ in bronchoalveolar lavage fluid (BALF) 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-36γ to the amplifying effect of IL-17 and bacterial DNA on neutrophilic inflammation.

Materials and methods

Detailed methods are provided in the supplementary material methods section.

Human subjects

BALF from healthy and asthma individuals obtained through bronchoscopy as described in our previous publication [19] was utilised 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 ($pg \cdot mL^{-1}$).

NTHi culture and DNA preparation

NTHi glycerol stock (strain R2846/12, a gift from Stephen Barenkamp at Saint Louis University School of Medicine, St Louis, MO, USA) was grown on chocolate agar plates (Hardy Diagnostics, Santa Maria, CA, USA) in a 37°C incubator. A single colony was selected and cultured in brain heart infusion broth (Sigma, St Louis, MO, USA) for 24 h at 37°C. Bacteria were centrifuged three times at 15 294×g for 5 min and resuspended in PBS to wash the bacteria. A QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used to extract DNA from bacteria according to the manufacturer's instructions. Briefly, bacterial pellet was resuspended in 200 µL of PBS containing 20 µL of proteinase K to extract DNA. To generate endotoxin-free bacterial DNA, a Pierce High-Capacity Endotoxin Removal column (Thermo Fisher Scientific, Waltham, MA, USA) was used to remove any potential endotoxin from NTHi-derived DNA as per manufacturer's instructions. A Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher

Scientific) was used to confirm endotoxin removal from NTHi-derived DNA showing <0.096 EU·mL^{-1} (0.0096 ng·mL^{-1}) of endotoxin contamination in NTHi DNA preparation, which is well below the endotoxin contamination level (0.5 EU·mL^{-1} or 0.05 ng·mL^{-1}) set by the US Food and Drug Administration (FDA) for research.

Cell culture

Airway epithelial cells and lung macrophages were stimulated with 10 ng·mL^{-1} of recombinant human or murine IL-17A for 24 h [21]. Thereafter, cells were exposed to NTHi-derived DNA at 0.3 µg·mL^{-1} [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 were: 1) C57BL/6 wild-type mouse model of IL-17 and NTHi-derived DNA treatment; 2) myeloid cell Toll-like receptor 9 (TLR9)-deficient (LysMCre^{+}TLR9$^{fl/fl}$) mouse model of IL-17 and NTHi-derived DNA treatment; and 3) recombinant human DNase I (rhDNase I) treatment in wild-type mice with IL-17 treatment and live NTHi infection. Details of experimental design and generation of LysMCre^{+}TLR9$^{fl/fl}$ mice [23, 24] are given in the full supplementary material 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, proximal airways resistance, distal airways resistance, total pulmonary elastance and distal airways elastance.

Statistical analysis

Data were analysed using Graph Pad Prism software. For parametric data, a paired 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 the Kruskal–Wallis test for multiple group comparisons. Mouse airway hyperresponsiveness data were analysed 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 BALF of NA 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 NNA and NA subjects as well as normal control healthy subjects. NA in our current study was defined by ⩾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 *versus* neutrophil-low refractory asthma that were positive for bacterial infection [19]. NNA subjects were defined as those with <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 in 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 culture to determine if bacterial DNA enhances IL-17-mediated IL-8 production. Recombinant human IL-17 (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 utilised large airway epithelial cell culture models in asthma research, we confirmed that human tracheobronchial epithelial cells 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

TABLE 1 Characteristics of healthy and asthma subjects

	Healthy subjects	Non-neutrophilic asthma (NNA)	Neutrophilic asthma (NA)	p-value
Subjects n	20	14	16	
Age years	33.6±2.0	42.2±3.7	51.8±3.9	<0.01 NA *versus* healthy, >0.05 NA *versus* NNA
Sex M/F n	7/13	6/8	8/8	0.33 NA *versus* healthy and NNA
FEV_1 % predicted	96.0±2.7	75.4±5.5	61.2±5.0	<0.01 NA *versus* healthy, 0.07 NA *versus* NNA
BMI kg·m^{-2}	29.7±1.3	27.5±1.5	29.3±1.5	0.55 NA *versus* healthy and NNA
F_{ENO} ppb	23.2±3.3	36.3±5.9	52.7±13.1	0.02 NA *versus* healthy, >0.05 NA *versus* NNA
ACT score	N/A	17.5±1.3	17.1±1.6	0.84 NA *versus* NNA
Inhaled corticosteroid use	None	High dose (3), low dose (11)	High dose (9), low dose (5), medium dose (1), unknown (1)	0.11 NA *versus* NNA
Neutrophils % in BALF	1.9±0.3	1.4±0.2	15.3±4.6	<0.01 NA *versus* healthy and NNA
Eosinophils % in BALF	0.5±0.2	1.8±0.7	3.0±0.9	<0.01 NA *versus* healthy, >0.05 NA *versus* NNA
Macrophages % in BALF	91.5±1.0	90.4±2.7	72.4±4.9	<0.01 NA *versus* healthy and NNA
Lymphocytes % in BALF	6.1±0.8	6.4±1.3	9.9±1.7	0.11 NA *versus* healthy and NNA
IL-8 pg·mL^{-1} in BALF	24.8±1.5	41.8±5.8	138.5±42.7	<0.01 NA *versus* healthy and NNA

Data expressed as mean±SEM unless otherwise stated. All subjects were nonsmokers. M: male; F: female; FEV_1: forced expiratory volume in 1 s; BMI: body mass index; F_{ENO}: fractional exhaled nitric oxide; ACT: asthma control test; BALF: bronchoalveolar lavage fluid; IL-8: interleukin-8; N/A: not applicable.

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 recombinant mouse IL-17A (rmIL-17A) for 24 h and then exposed to NTHi-derived DNA for 24 h. 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 supplementary figure S2, 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.

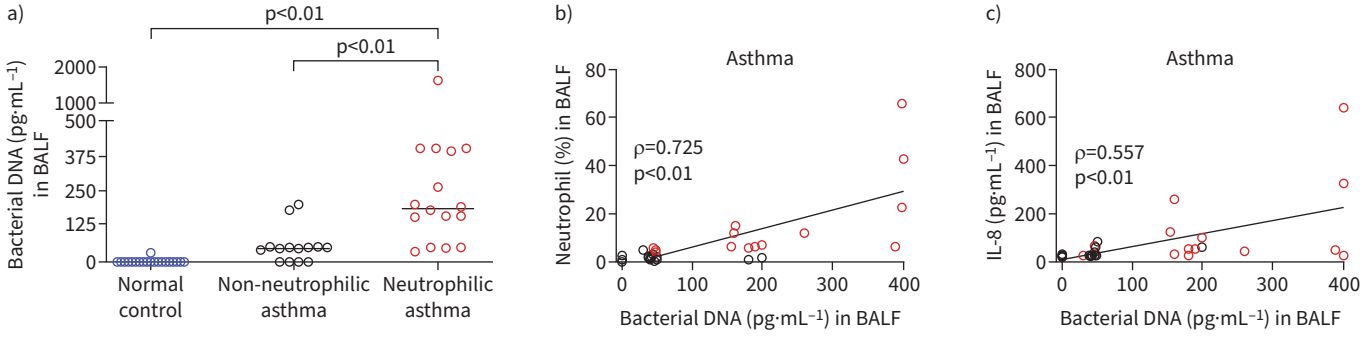

FIGURE 1 Increased bacterial DNA in bronchoalveolar lavage fluid (BALF) of neutrophilic asthma subjects. a) Bacterial DNA levels in BALF of normal subjects, non-neutrophilic asthma subjects and neutrophilic asthma subjects. b) Correlations between bacterial DNA and neutrophils and c) interleukin (IL)-8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

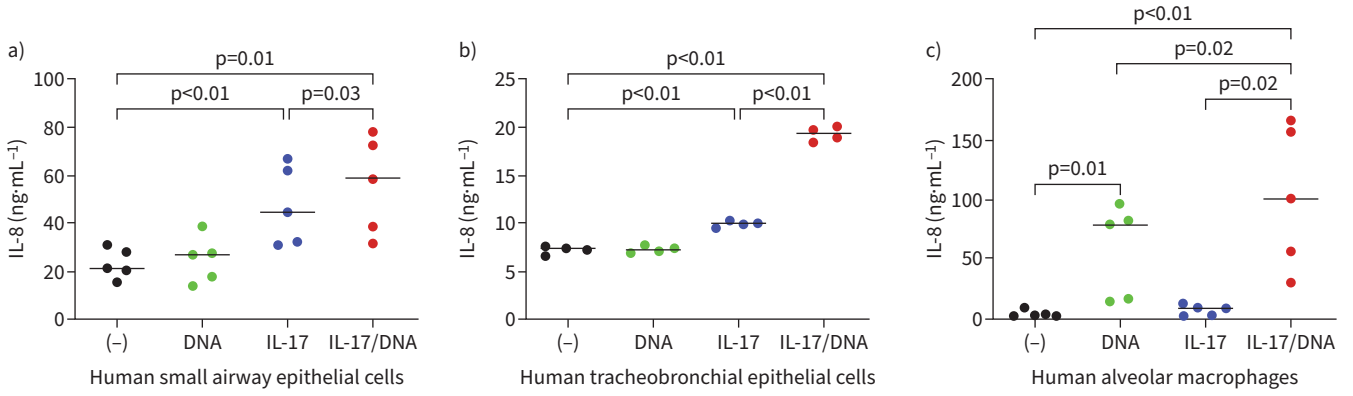

FIGURE 2 Bacterial DNA amplified interleukin (IL)-8 production in IL-17-exposed human airway epithelial cells and lung macrophages. a) Primary normal human small airway epithelial cells (n=5 subjects) grown at air–liquid interface. b) Primary normal human tracheobronchial epithelial cells under submerged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

Role of TLR9 signalling 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 cyclic GMP-AMP synthase (cGAS)/stimulator of interferon genes (STING) signalling axis involved in bacterial infection [31], we determined if STING signalling 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 signalling in airway epithelial responses to bacterial DNA.

To further confirm the role of TLR9 signalling in neutrophil mediator production, mouse tracheal epithelial cells (mTEC) and bone marrow-derived macrophages (BMDM) from TLR9 knockout (KO) and wild-type mice were utilised. TLR9 KO (*versus* 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 signalling in myeloid cell (*e.g.* macrophage) responses to bacterial DNA, a mouse model of Cre-mediated TLR9 deletion in myeloid cells was utilised. As compared to the

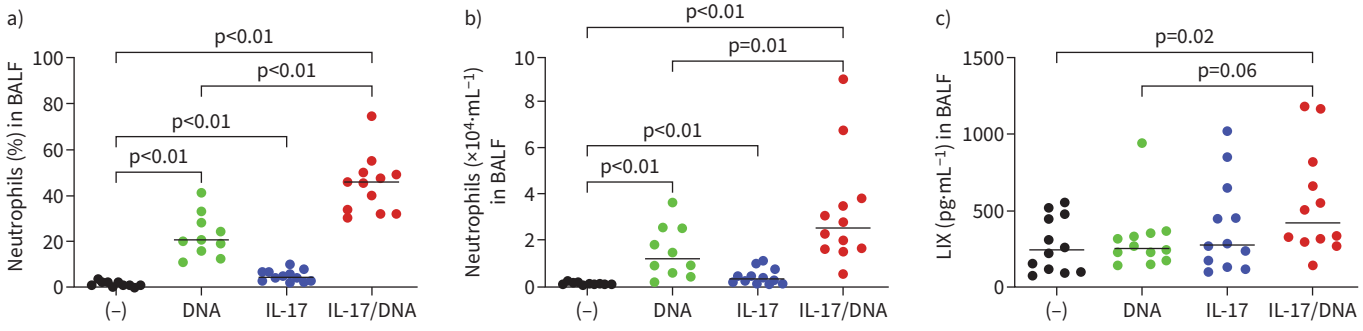

FIGURE 3 Bacterial DNA increased neutrophilic inflammation in interleukin (IL)-17-exposed mouse lungs. Wild-type C57BL/6 mice (n=10 to 12 mice per group) from two independent experiments were intranasally challenged with IL-17 (3 µg/mouse) for 24 h, followed by DNA (1 µg/mouse) derived from nontypeable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

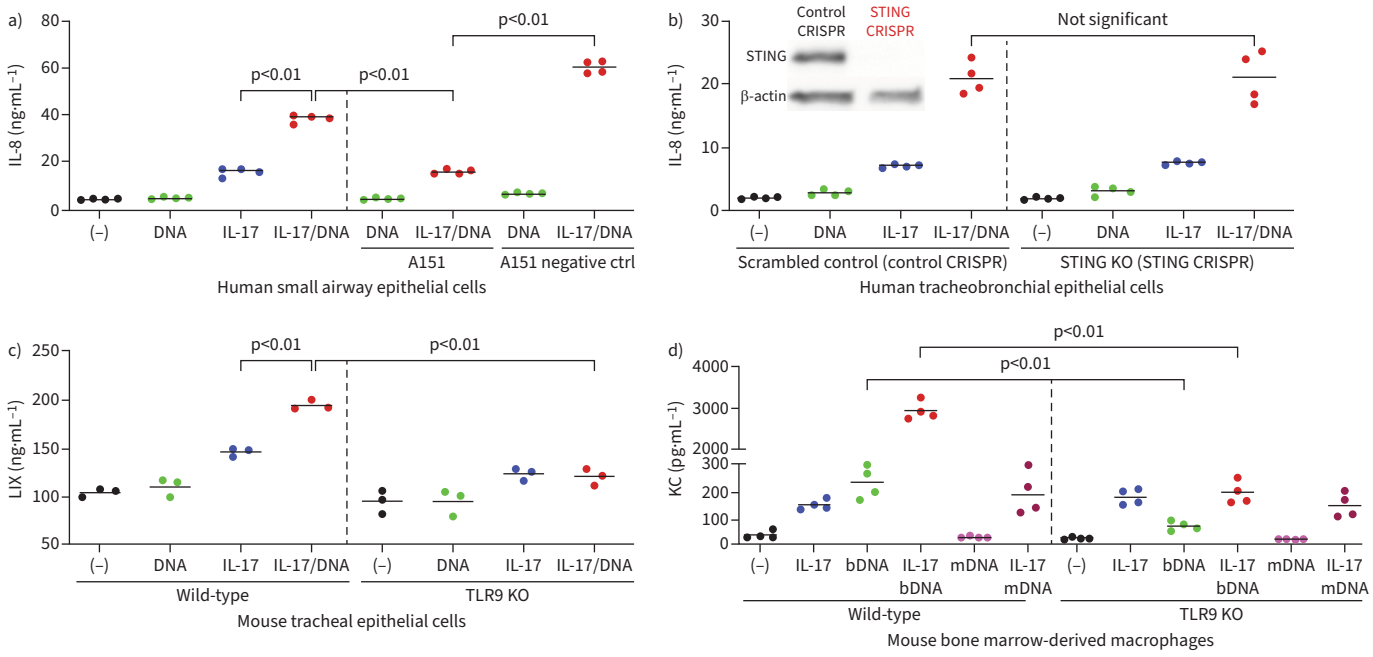

FIGURE 4 Role of DNA signalling in bacterial DNA-mediated amplification of neutrophil chemokines. a) Primary normal human small airway epithelial cells (n=4 subjects) under submerged culture were treated with interleukin (IL)-17 for 24 h, and then exposed to bacterial DNA±A151 or A151 negative control (ctrl) for 48 h. 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 h, and then exposed to bacterial DNA under the submerged condition for 48 h. c) Mouse tracheal epithelial cells (mTEC) isolated from wild-type (C57BL/6) and Toll-like receptor 9 (TLR9) knockout (KO) mice were grown at air–liquid interface and stimulated with or without IL-17 for 24 h and then treated with/without bacterial DNA for 48 h. 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. (–) indicates controls. The horizontal bars represent means.

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

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

We measured IL-36γ in BALF from NA and NNA subjects and healthy subjects. Notably, NA subjects demonstrated significantly higher levels of IL-36γ protein than the other two groups (figure 6a). In HSAECs, IL-36γ 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-36γ in IL-8 production, an IL-36γ neutralising antibody was employed in HSAECs. The IL-36γ neutralising antibody (*versus* 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 signalling 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-36γ release into the airways, which was reduced by DNase I (figure 7d).

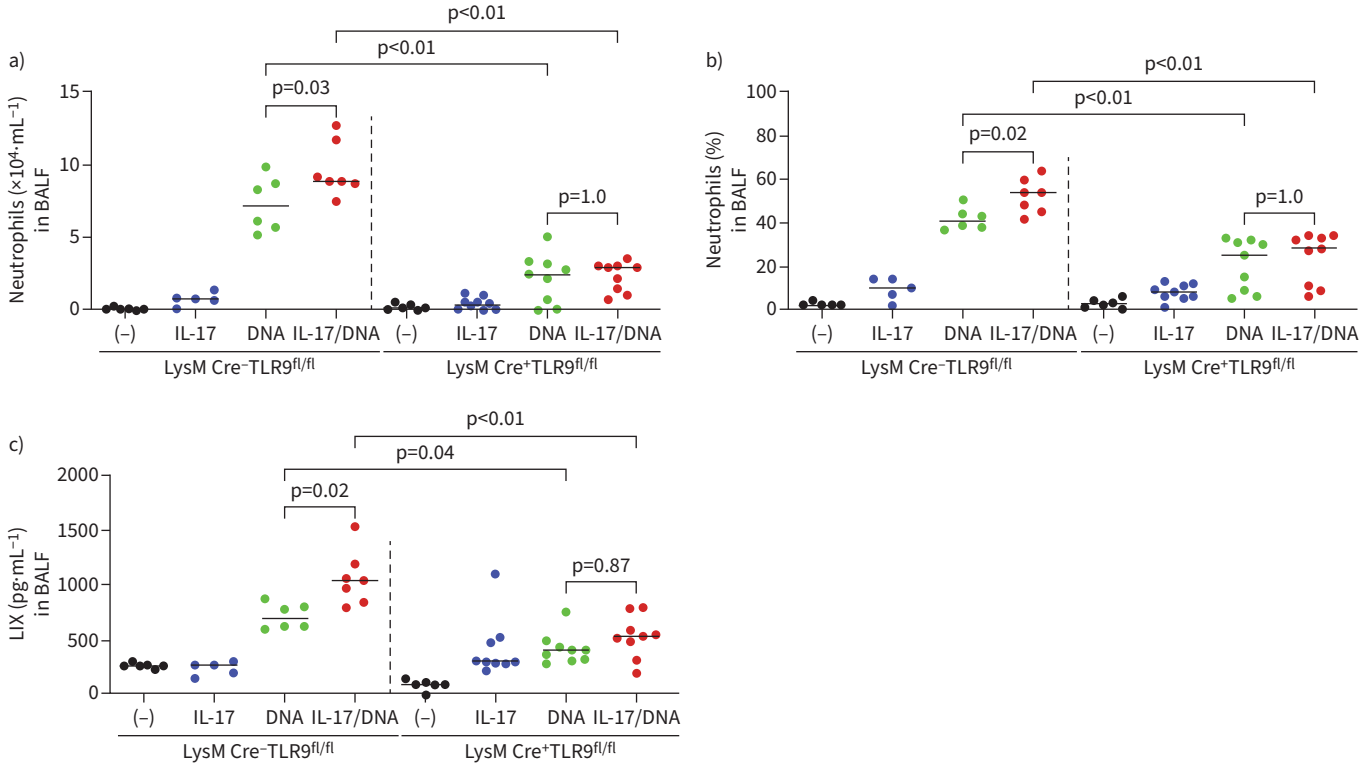

FIGURE 5 Toll-like receptor 9 (TLR9) conditional knockout in myeloid cells downregulates airway neutrophilic inflammation in mice exposed to interleukin (IL)-17 and bacterial DNA. LysMCre^{+}TLR9$^{fl/fl}$ and LysMCre^{-}TLR9$^{fl/fl}$ mice were challenged with IL-17 (3 µg/mouse) in 0.01% bovine serum albumin (BSA) or 0.01% BSA (control) for 24 h, followed by bacterial DNA (1 µg/mouse) challenge or Tris-EDTA buffer *via* intranasal inoculation. After 24 h of DNA exposure, bronchoalveolar lavage fluid (BALF) was collected and analysed for a,b) neutrophils and c) neutrophil chemoattractant LIX. (–) indicates controls. The horizontal bars represent medians.

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-36γ signalling 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 two-fold) in airway epithelial cells, but not in macrophages (supplementary figure S3). 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

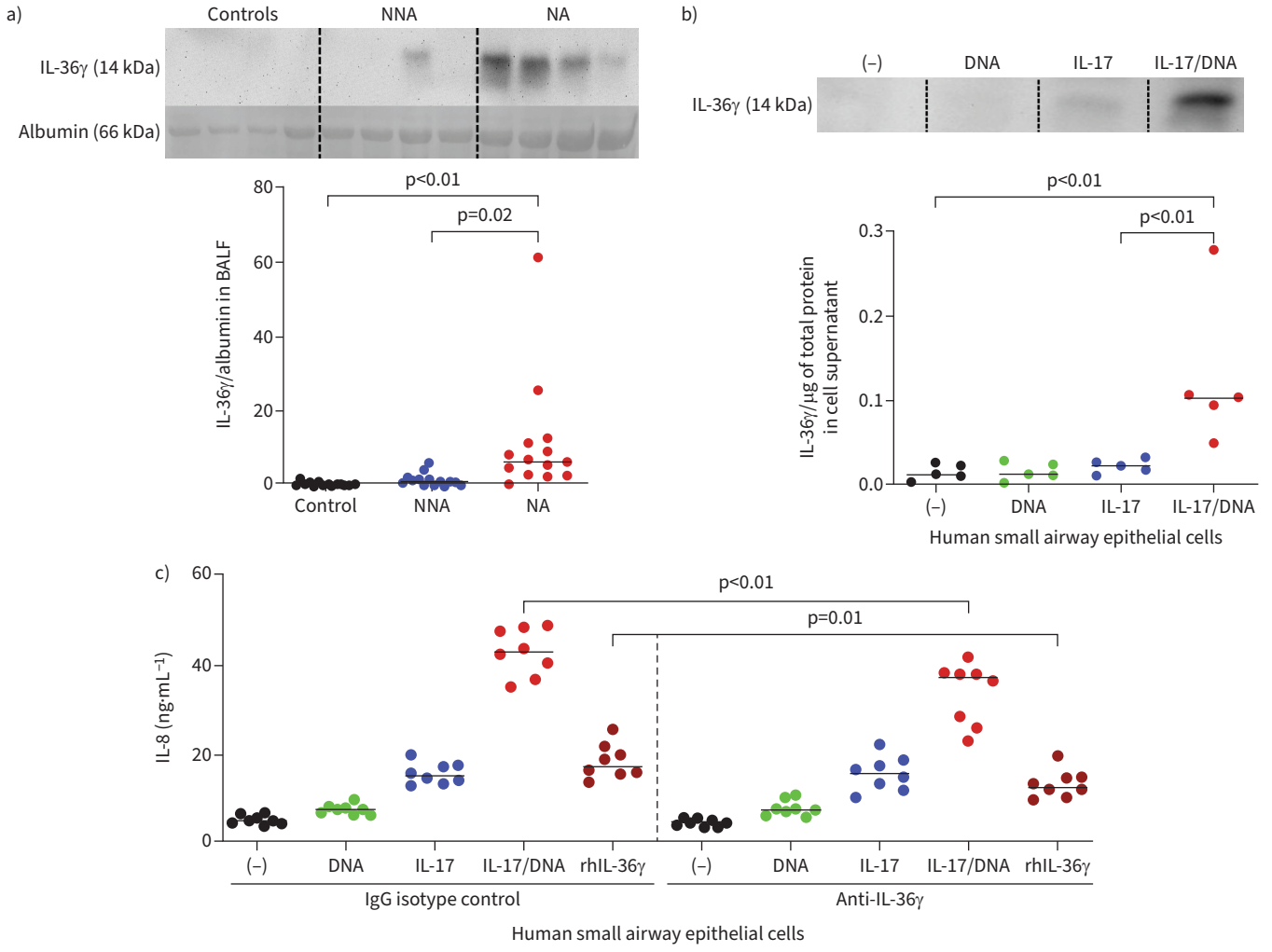

FIGURE 6 Interleukin (IL)-36γ in human bronchoalveolar lavage fluid (BALF) and cultured airway epithelial cells. a) Representative Western blot images and densitometry of IL-36γ protein in BALF from healthy control subjects, non-neutrophilic asthma (NNA) and neutrophilic asthma (NA) subjects. Each dot represents 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 h, and then bacterial DNA for 48 h. c) Primary normal human small airway epithelial cells were stimulated with or without IL-17 for 24 h and then treated with/without bacterial DNA in the presence of an IgG isotype control or an IL-36γ neutralising antibody for 48 h. (–) indicates controls. The horizontal bars represent medians.

infection also demonstrated the enhancing effect of bacterial DNA signalling on neutrophilic inflammation in the 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 the IL-17-mediated signalling cascade to amplify neutrophilic inflammation. Previous studies suggest that unmethylated bacterial DNA primarily utilises TLR9 signalling [35]. Here, we utilised TLR9-deficient mTEC and BMDM cells to study the role of TLR9. Based on LIX and KC data, TLR9 signalling 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.* macrophage) TLR9 signalling 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 signalling to the pro-inflammatory effect of bacterial DNA and IL-17. In addition, DNA may utilise STING and inflammasome pathways to induce inflammatory responses [36–38]. Our data in STING KO cells suggest that STING signalling does not contribute to IL-8 production in airway epithelial

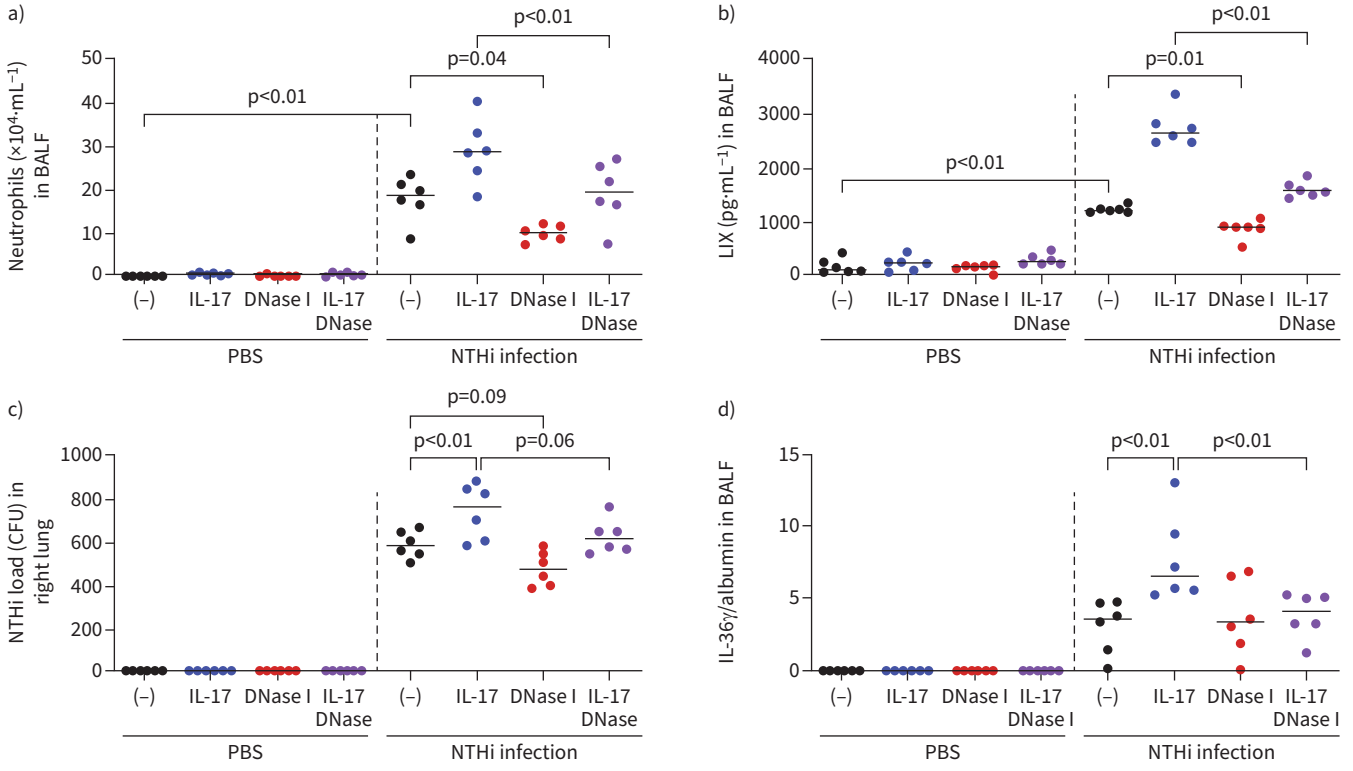

FIGURE 7 Recombinant human DNase I reduces lung neutrophilic inflammation in interleukin (IL)-17-challenged and nontypeable *Haemophilus influenzae* (NTHi)-infected mice. Wild-type C57BL/6 mice were challenged with IL-17 (3 µg/mouse), NTHi (10^7 CFU/mouse) and DNase I (5 µg/mouse) *via* intranasal inoculation. After 24 h, bronchoalveolar lavage fluid (BALF) was collected for analysing total numbers of a) neutrophils, b) neutrophil chemoattractant LIX, c) count of colony forming units (CFU) in the right lung tissue homogenate and d) IL-36γ protein using Western blot. (–) indicates controls. The horizontal bars represent medians.

cells exposed to bacterial DNA. However, we cannot exclude the involvement of STING signalling 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 enhances the IL-36γ signalling pathway. In our preliminary bulk RNA sequencing studies in HSAECs, we observed an increase (about five-fold, n=3) of IL-36γ mRNA expression by IL-17, but not by DNA treatment (supplementary figure S4). Our data suggest that IL-17 increased IL-36γ protein production especially in the presence of bacterial DNA, leading to a significant increase of IL-8. The role of IL-36γ in asthma was further supported by the fact that IL-36γ protein levels were significantly higher in airways of patients with NA. Importantly, for the first time, we demonstrated a synergistic effect of bacterial DNA on IL-17-mediated IL-36γ protein production, which may contribute to IL-8 production in airway epithelial cells. Previous studies showed that IL-36γ facilitates TLR9 translocation from ER to lysosomes and potentiates TLR9 activation [39]. Interestingly, both IL-36γ and TLR9 signalling pathways are mediated by MyD88/IRAK signalling to activate NF-κB signalling. Thus, TLR9 and IL-36γ 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-36γ 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.

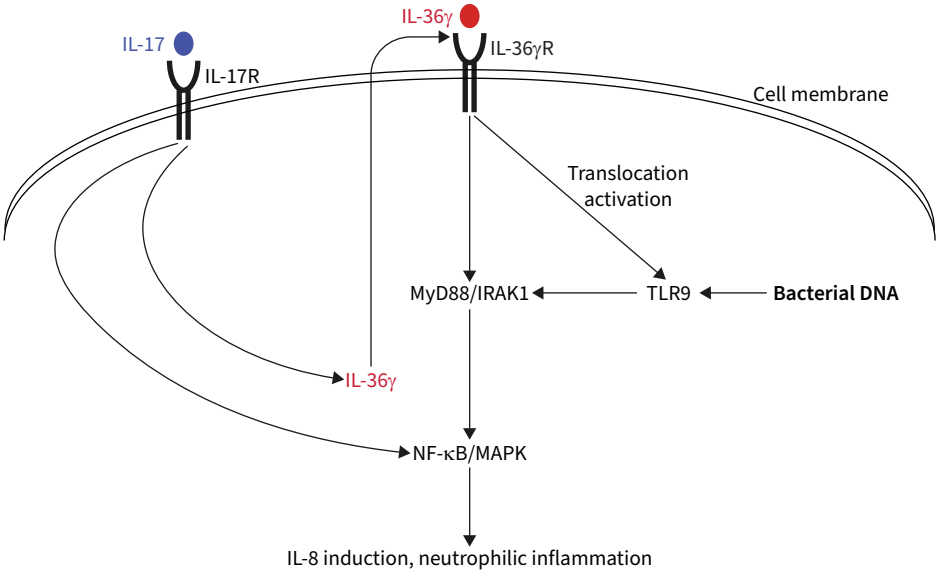

FIGURE 8 Proposed mechanisms by which bacterial DNA and interleukin (IL)-17 cooperate to amplify airway neutrophilic inflammation. IL-17 binds to its receptor and induces IL-36γ expression. IL-36γ binds to its heterodimeric receptor complex, subsequently recruits MyD88, activates MAPK and NF-κB signalling cascades, and induces IL-8 and neutrophilic inflammation. Bacterial DNA binds to TLR9, leading to activation of MAPK and NF-κB signalling pathways. Activation of IL-36γ signalling also results in TLR9 translocation, leading to further activation of TLR9 signalling 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-κB: nuclear transcription factor kappa B; TLR9: Toll-like receptor 9.

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 NA [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-36γ as IL-36γ 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 utilise 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 an IL-17-high environment. We found more airway eosinophils in patients with NA compared to healthy controls, but not patients with NNA. 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 cGAS-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 NA patients may also utilise the cGAS-STING pathway to induce the recruitment of eosinophils into the airway. The NA group in our current study appeared to have more patients on high-dose inhaled corticosteroids (ICS) compared to the NNA 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 NA 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 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 whether DNase reduces 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 need 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-36γ signalling may serve as one of the mechanisms by which airway neutrophilic inflammation is exaggerated (figure 8). We anticipate that our findings may have implications in developing several potential therapeutic approaches, including TLR9 antagonists, IL-36γ neutralising antibodies and DNase I, to attenuate asthma exacerbations in patients who are insensitive to corticosteroid therapy.

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