Supplemental Material

Methods

Study approval

Animal experiments were performed in accordance with animal guidelines from animal research committee of POSTECH (POSTECH-2017-0109-C1). Informed written consent, including voluntary donation of sputum samples to biobank at Soonchunhyang University Bucheon Hospital, was obtained from all participants. The protocol was approved by the Ethics Committee of the hospital (SCHBC-2017-07-017-002).

Subjects

The diagnosis of asthma was based on the Global Initiative for Asthma (GINA guidelines)\(^1\). All subjects had a clinical diagnosis of asthma that was supported by at least 1 of the following criteria: (1) variability in maximum diurnal peak expiratory flow rate greater than 20% over the course of 14 days, (2) an increase in forced expiratory volume in 1 second (FEV1) greater than 12% and 200 mL after inhalation of albuterol 400 mg, or (3) a 20% decrease in FEV1 in response to a provocative concentration of inhaled methacholine (PC20) of less than 10 mg/mL. Spirometry was performed before and after inhalation of a bronchodilator. At the baseline visit, demographic information, including enrollment age, sex, body mass index (BMI), tobacco consumption, age of asthma onset, and asthma duration, were collected. All patients underwent a standardized assessment that included sputum analysis, complete blood cell counts with differential counts, serum total IgE levels, chest radiography, spirometry, and allergy skin prick tests with 24 common inhalant allergens (Bencard Co., Brentford, UK). Atopy was defined as a mean wheal diameter ≥3 mm over that of the saline control on skin prick tests. Patients with parenchymal lung disease on chest X-ray, including bronchiectasis, pulmonary tuberculosis,
chronic obstructive pulmonary disease (COPD) and interstitial lung diseases, were also excluded.

**Mice**

The following mice were used: 6 to 8 week-old wild-type C57BL/6, IL-17A, and TNF-α knockout mice (Jackson laboratory). IL-17A x TNF-α double KO mice were generated by crossing IL-17A and TNF-α single KO mice. All mice were housed in a specific pathogen-free animal facility at POSTECH. Animal experiments were performed in accordance with animal guidelines from animal research committee of POSTECH.

**In vitro cytokine stimulation**

Mouse lung epithelial cell line (MLE-12) was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM)/Nutrient Mixture F-12 Ham (Sigma) supplemented with 2% fetal bovine serum (FBS, GE Healthcare), Antibiotic-Antimycotic (ThermoFisher scientific), insulin-transferrin-selenium (ITS) solution (Sigma), 10 nM hydrocortisone (Sigma), 10 nM β-estradiol (sigma), and 2 mM L-glutamine (ThermoFisher scientific). Human lung epithelial cell lines A549 and Beas2B were cultured in DMEM and RPIMI medium, respectively, supplemented with 10% FBS. 1 x 10^5 of MLE-12, A549, and Beas2B cells were plated on 48 well cell culture plate and stimulated with 100 ng/ml of rIL-17A (ThermoFisher scientific) and/or 20 ng/ml of rTNF-α (Biolegend) for MLE-12 and human cell lines. Supernatant was harvested 24 hours after stimulation, and level of G-CSF was measured by ELISA.

**Mouse models of asthma and treatments**

For LPS/OVA-induced model, mice were intranasally sensitized with 10 μg of LPS (Sigma) mixed with 75 μg of OVA (Grade V, Sigma) in 20 μl of PBS on days 0, 1, 2, and 7. For
alum/OVA-induced model, mice were intraperitoneally sensitized with 2 mg of alum (Invivogen) with 75 µg of OVA in 200 µl of PBS on days 0 and 7. During challenges, 50 µg of OVA in 20 µl of PBS was intranasally introduced on 2 consecutive days a week for 2 or 4 weeks starting on day 14 for alum/OVA or LPS/OVA models, respectively. For HDM-induced model, mice were intranasally sensitized and challenged with 25 µg of HDM (Greer laboratories) on days 0, 1, 2, 14, 15, 16, and 17. For the experiments of supplemental figure 1, OVA was introduced on 2 consecutive days a week for 2 weeks. All intranasal injection were performed after anesthetization by 120 µl of ketamine (Yuhan)/Rompun (BAYER) solution. For G-CSF neutralizing experiment, mice were injected with 100 µg of α-G-CSF (ThermoFisher scientific) or isotype antibody (BioXcell, BE0089) intraperitoneally the day before intranasal OVA challenges starting on day 28. For neutralization of IL-17A, and TNF-α, 50 µg of α-IL-17A and/or α-TNF-α (BioXcell, BE0173 and BE0244, respectively) or isotype antibodies (BioXcell, BE0083 and BE0091) were injected intraperitoneally 4 hours before the challenge starting on day 28.

**Administration of recombinant G-CSF, IL-17A, and TNF-α**

Mice were intraperitoneally injected with 2.5 µg of rG-CSF (Biolegend) for 2 consecutive days and sacrificed after 48 hours to evaluate bone marrow cellularity. 500 ng of rIL-17A and rTNF-α were introduced by intranasal inhalation in 30 µl of PBS for 2 consecutive days a week for 2 weeks.

**Tissue isolation and single cell preparation**

At 6 hours after the last challenge, lungs were collected after perfusion and stored in 4 % paraformaldehyde (PFA) for histology or media for cell preparation. For lung cell preparation,
lung tissues were diced and incubated in mixture of dispase (Gibco), collagenase D (Sigma) DNase I (Sigma) at 37 °C for 40 min with vigorous agitation. After incubation, tissues were passed through a 40 μm strainer to obtain single cell suspensions. For bone marrow cell collection, leg bones (one tibia and one femur per mouse) were collected at 48 hours after the last challenge and flushed with RPMI1640 media (Welgene) supplemented with 2 % of calf serum. Inflammatory aspects in airway were measured at 6 hours after the last challenge. Blood was collected by cardiac puncture at 6, 12, 24, and 48 hours after the last challenge to measure the cytokines. Bronchoalveolar lavage fluid (BALF) was obtained by intubation of catheter into mouse trachea with 1 ml of PBS. Total cell count was determined by Vi-Cell XR counter (Beckman Coulter). For BALF cells, BALF was centrifuged and pellet was re-suspended in 100 μl of 2% FBS-containing PBS and manually counted. BALF cellularity was analyzed by flow cytometry.

For histological analysis, perfused left lung tissues were fixed and paraffin embedded. Tissues were cut in 4 μm section and stained with hematoxylin and eosin. Sections were imaged with microscopy. For histological analysis, perfused left lung tissues were fixed and paraffin embedded. Tissues were cut in 4 μm section and stained with hematoxylin and eosin. Sections were imaged with microscopy. Inflammatory scores that evaluate perivascular and peribronchial inflammation were measured in a blinded fashion. Scores range from 0 to 3.

**Cytokine measurement**

Cytokine levels were measured by using Duoset ELISA kit (R&D systems) for mouse IL-1β (DY401), IL-4 (DY404), IL-6 (DY406), IL-17A (DY421), TNF-α (DY410), IFN-γ (DY485), M-CSF (DY416), G-CSF (DY414), and CXCL1 (DY453), and Ready-SET-Go! (eBioscience) for GM-CSF, according to the manufacturer’s instructions. Cytokine levels of human IL-17A
(DY317), TNF-α (DY210), and G-CSF (DY214) were measured by Duoset ELISA kit (R&D systems) according to the manufacturer’s instructions.

**MPO activity**

10 μl of BALF was incubated with 80 μl of 88 mM H₂O₂ and 110 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (SurModics) at 37 °C for 5 min. The reaction was stopped by 50 μl of 1 N H₂SO₄ and absorption was measured at 450 nm.

**Flow cytometry**

To analyze hematopoietic cells in the bone marrow, lung, and BALF, following monoclonal antibodies and phenotypic markers were used for flow cytometry. For LSK cell analysis, mature lineage cells were excluded by lineage markers such as TER119 (clone TER-119), CD11b (clone M1/70), Gr-1 (clone RB6-8C5), CD3ε (clone 145-2C11), NK1.1 (clone PK136), MHC class II (I-A/I-E, clone M5/114.15.2), and B220 (clone RA3-6B2). Cells were then positively identified with c-Kit (clone 2B8) and Sca-1 (clone D7). Progenitor cells were identified with lineage−, c-Kit, Sca-1, CD34 (clone RAM34), and FcγRII/III (CD16/32, clone 93) for CMPs (LIN−Sca-1−c-Kit+CD34+FcyRlo), GMPs (LIN−Sca-1−c-Kit+CD34+FcyRhi), and MEPs (LIN−Sca-1−c-Kit+CD34+FcyRlo). Mature immune cells in the BM, lung, and BALF were identified with TER119, CD11b, B220, SiglecF (E50-2440), and Gr-1 (RB6-8C5) for neutrophils (TER119−CD11b+Gr-1+), eosinophils (TER119−CD11b−SiglecF+Gr-1low), B cells (TER119−Gr-1−CD11b−B220+), and alveolar macrophage (FSChighSSChighTER119−CD11blowSiglecFhighGr-1−). CD62L expression in lung neutrophils was measured with CD62L (clone MEL-14) antibody. For identification of non-hematopoietic cells in the lung, cells were stained with TER119, CD45 (clone 30-F11), EpCAM (clone G8.8), and CD31 (clone 390 and MEC13.3) for epithelial cells
(TER119^CD45^-CD31^-EpCAM^+) and endothelial cells (TER119^-CD45^-CD31^+EpCAM^-). Neutrophil apoptosis was measured by the expression levels of Annexin V and 7-AAD (BD Biosciences). Flow cytometric analysis was performed with LSR Fortessa, LSR Fortessa 5 lasers, and FACSCanto II (BD Biosciences). Data were analyzed with FlowJo software (Tree Star Inc.).

**AHR measurement**

Airway hyper-responsiveness was measured by using flexiVent (SCIREQ). Mice were anesthetized with pentothal sodium (JW pharmaceutical), and then trachea was intubated with the catheter. Respiratory system resistance (Rrs) was measured after methacholine treatment ranging from 0 to 50 mg/ml.

**Cell sorting**

For lung epithelial cell sorting, hematopoietic cells were excluded by lineage cell depletion kit (Miltenyi Biotec, 130-090-858). Remaining cells were stained with CD45, TER119, CD31, and EpCAM and then hematopoietic cells (CD45^+), epithelial cells (TER119^-CD45^-CD31^-EpCAM^+), and endothelial cells (TER119^-CD45^-CD31^+EpCAM^-) were sorted by using MoFlo-XDP (Beckman Coulter).

**RNA extraction and real-time PCR**

RNA was extracted from sorted lung cells by using a TRIzol (Invitrogen) according to manufacturer’s instructions. 1 µg of RNA was used for cDNA synthesis by using QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer’s instructions. Real-time PCR was performed with ViiA 7 Real-Time PCR system (ThermoFisher scientific) using Power SYBR Green PCR master mix (ThermoFisher scientific). Gene expression was normalized to the level of L32. The primer used were as follows; L32: forward: 5´-GAA ACT GGC GGA AAC
CCA-3' and reverse: 5'-GGA TCT GGC CCT TGA ACC TT-3'; G-CSF: forward: 5'- TGA CAC AGC TTG TAG GTG GC-3' and reverse: 5'-TCC TGC TTA AGT CCC TGG AG -3'.

**Sputum induction and preparation**

Sputum was induced by using isotonic saline containing short-acting bronchodilator2. The samples were processed as previously described3. Briefly, all samples with visibly greater solidity were carefully selected and placed in a pre-weighed 1.5 ml tube to which eight volumes of 0.05% dithiothreitol (Sputolysin; Calbiochem Corp) in Dulbecco’s phosphate-buffered saline. Protease inhibitor (0.1 M methylene-diamine-tetra-acetic acid and 2 mg phenyl-methyl-sulfonyl-fluoride/mL) was then added to the homogenized sputum at a ratio (v/v) of 1:100+6. Total cell count was determined using a hemocytometer. Sputum cells were collected by cytocentrifugation, and 500 cells were examined by Diff-Quick staining (American Scientific Products). Samples that contained >10% squamous epithelial cells were excluded from the study. Total of 355 sputum samples were examined for differential cell counts, and 114 cases had more than 10% of squamous cells. The 241 remainders of the homogenized sputum sample were centrifuged at 1,000 g for 5 min; the supernatant was collected and stored at −80 °C for subsequent protein analysis. Patients were categorized according to the inflammatory subtype of their sputum sample7-9: neutrophilic (neutrophils ≥ 60%, eosinophils < 3%, n = 95), eosinophilic (neutrophils < 60%, eosinophils ≥ 3%, n = 56), and mixed (neutrophils ≥ 60%, eosinophils ≥ 3%, n = 64).

**Statistical analyses**

For mouse experiments, statistical analysis was performed using Prism 5 (GraphPad Software), and data were presented as mean ± S.E.M or median as indicated in the figure legends. Unpaired two-tailed t-test for the comparison of two groups and one-way ANOVA with Bonferroni’s post hoc test for multiple comparisons were used for comparison of parametric
samples. Mann-Whitney U-test and Kruskal–Wallis test with post hoc analysis were used for comparison of categorical or non-parametric samples. Normality was tested by D'Agostino and Pearson omnibus normality test. Correlations between G-CSF level and cellularity in BALF were analyzed by Spearman’s correlation coefficient analysis. $P$ values of less than 0.05 were considered to be significant.

For human studies, data analysis was performed using the SPSS Statistics 20 (IBM SPSS). Comparisons were performed using the Kruskal–Wallis test with post hoc analysis to evaluate levels of sputum cytokines. $P$ values of less than 0.05 were considered to be significant.
Supplemental figures and tables

Supplemental Figure 1. The inflammatory phenotypes of asthma are determined by types of sensitizing adjuvant, not by different time courses

(A-C) Experimental schemes of mouse models of asthma. Total and immune cell counts in BALF. Total-total cell counts, AM-alveolar macrophage, Neut-neutrophils, and Eos-eosinophilis (A) Alum/OVA-induced mixed type asthma. (B) LPS/OVA-induced neutrophilic asthma. (C) HDM-induced eosinophilic asthma. (D) Concentration of G-CSF in BALF. (E and F) (n = 4 to 7 for each group). Correlations between BALF G-CSF with airway (E) neutrophil and (F) eosinophil frequency. Two or three independent experiments were performed and represented as median for (A-D). P values were determined by Mann-Whitney U-test for (A-C), Kruskal–Wallis test with post hoc analysis for (D), and nonparametric Spearman’s correlation test for (E and F). * P < 0.05; ** P < 0.01; *** P < 0.001.

Supplemental Figure 2. Concurrent stimulation of IL-17A and TNF-α induces G-CSF production from lung epithelial cells

(A) Cytokine profiles were measured in the lung homogenates of control and inflamed mice with 6 hours after last OVA challenge (n = 6 in all groups). (B) Level of G-CSF in the supernatant of mouse lung epithelial cell line (MLE-12) with 100 ng/ml of recombinant mouse (rm)-IL-17A and/or 20 ng/ml of rm-TNF-α stimulation for 24 hours (n = 9 in all groups). (C and D) Levels of G-CSF in the supernatant of human lung epithelial cell line cultures (C) A549 and (D) Beas2B. Cells were stimulated with 100 ng/ml of recombinant human (rh)-IL-17A and/or 20 ng/ml of rh-TNF-α for 24 hours (n = 9 in all groups). Two or three independent experiments were performed
and represented as median for (A-D). * P values were determined by Mann-Whintey U-test for (A) and Kruskal–Wallis test with post hoc analysis for (B-D). * P < 0.05; ** P < 0.01; *** P < 0.001.

Supplemental Figure 3. Both IL-17A and TNF- α are required for airway neutrophilia

(A-C) Airway neutrophilia in PBS or cytokine treated groups. (A) Total and immune cell counts in the BALF. Total-total cell counts, AM-alveolar macrophage, Neut-neutrophils, and Eos-eosinophils. (B) Neutrophil numbers in the lung tissues. (C) Concentration of CXCL1 in the serum (n = 4 in all groups). (D-F) Airway neutrophilia in PBS or LPS/OVA treated WT, IL-17A KO, TNF-α KO, and DKO mice. (D) Total and immune cell counts in the BALF. Total-total cell counts, AM-alveolar macrophage, Neut-neutrophils, and Eos-eosinophils (n = 10 to 15 for each group). (E) Neutrophil numbers in the lung tissues. (F) Concentration of CXCL1 in the serum (n = 6 to 10 for each group). Empty and filled circle indicate PBS and LPS/OVA groups, respectively. Two or three independent experiments were performed and represented as median for (A-C, E, and F) and mean + S.E.M. for (D). * P values were determined by Kruskal–Wallis test with post hoc analysis for (A-C, E, and F) and one-way ANOVA with Bonferroni’s multiple comparison test for (D). * P < 0.05; ** P < 0.01; *** P < 0.001.

Supplemental Figure 4. G-CSF-mediated hematopoietic dysregulation contributes to neutrophilic inflammation in the airway

(A and B) Frequency of LSK cells, committed progenitors, and lineage cells in the BM. (C) Total and immune cell counts in BALF. Total-total cell counts, AM-alveolar macrophage, Neut-
neutrophils, and Eos-eosinophils. (D) Neutrophil numbers in the lung tissues. Groups consist of PBS-, LPS/OVA-, LPS/OVA + α-IL-17A/α-TNF-α-, LPS/OVA + α-IL-17A/α-TNF-α + rG-CSF-treated mice (n = 4 to 6 for each group). Two or three independent experiments were performed and represented as median for (A-D). P values were determined by Kruskal–Wallis test with post hoc analysis for (A-D). * P < 0.05; ** P < 0.01; *** P < 0.001.

Supplemental Figure 5. Simultaneous blockade of IL-17A and TNF-α is necessary for achieving the optimal therapeutic efficacy against neutrophilic asthma

(A) An experimental scheme of cytokine blockades for asthma therapy. (B) Total and immune cell counts in BALF. Total-total cell counts, AM-alveolar macrophage, Neut-neutrophils, and Eos-eosinophils. (C) Neutrophil numbers in the lung tissues. (D) Representative H&E-stained lung tissue from PBS, LPS/OVA + isotype, LPS/OVA + α-IL-17A, LPS/OVA + α-TNF-α, and LPS/OVA + α-IL-17A + α-TNF-α mice. Scale bars, 100 µm. (E) Perivascular and peribronchial inflammation were scored. (F and G) Levels of G-CSF in the (F) BALF and (G) serum. (H) Concentration of CXCL1 in the serum (n = 4 to 5 for each group). Two or three independent experiments were performed and represented as median for (B-H). P values were determined by Kruskal–Wallis test with post hoc analysis for (B-H). * P < 0.05; ** P < 0.01; *** P < 0.001.

Supplemental Figure 6. A schematic abstract

During neutrophilic asthma progression, G-CSF is increased in airway of mice and humans. Th17-associated cytokines, i.e. IL-17A and TNF-α provoke the lung epithelium to induce two distinct types of inflammatory mediators such as CXCL1 and G-CSF. CXCL1 acts as a local regulatory factor for recruiting neutrophils from the circulation into the inflamed airway,
whereas G-CSF, as a long-range regulatory factor, alters hematopoiesis in the BM enhancing neutrophil supply in the circulation. Therefore, inhibition of G-CSF can attenuate neutrophilic airway inflammation by targeting the lung-BM axis, while the dual blockade of IL-17A and TNF-α can abolish asthma progression by targeting the lung epithelium responses.

**Supplemental Table 1. Clinical parameters of asthmatics stratified by sputum cellularity**

Abbreviations: BMI, body mass index; N/Y/ND, No/Yes/not done; NS, non-smoker; CS, cigarettes smokers; FEV1, forced expiratory volume for 1 second; FCV, forced vital capacity; PC20, provocative concentration causing a 20% fall in FEV1. Data are median (inter-quartile range) unless indicated otherwise. *P < 0.01 compared with mixed asthma. ¥P < 0.01 compared with neutrophilic asthma. §P < 0.01 compared with eosinophilic asthma.
References


