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## IL-17 enhances the migration of B cells during asthma by inducing CXCL13 chemokine production in structural lung cells



### To the Editor:

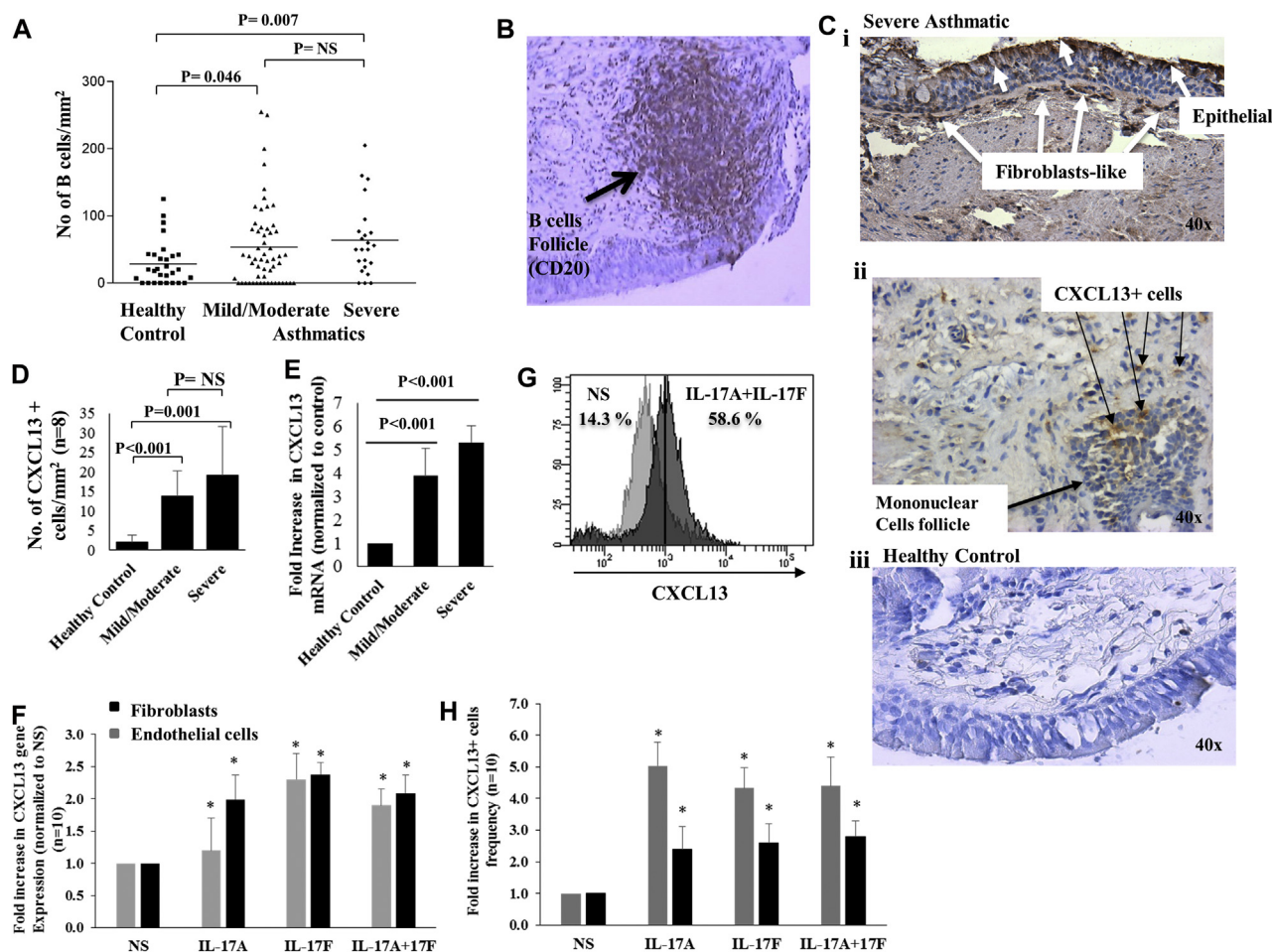
In contrast to T lymphocytes, the critical relevance of B cells in asthma pathogenesis and airway inflammation is less well-recognized. B lymphocytes are principally associated with the production of immunoglobulin antibodies in response to allergens and infectious pathogens.<sup>1,2</sup> Furthermore, B cells are critical determinants for the development of allergen-specific T-cell respiratory tolerance, which prevents reactivity to common airborne particles. However, in allergic asthma, this mechanism is dysregulated and linked potentially to abnormally low numbers of a subset of regulatory B cells with characteristic IL-10 release.<sup>3</sup> Recent findings have revealed the crucial participation of regulatory B cells, either as modulators or as effectors of the

immune response in many chronic autoimmune and allergic disorders.<sup>4</sup> Despite these advances, little is known about the mechanisms that drive B-cell infiltration into the bronchial tissues of patients with asthma, in particular those of the severe phenotype characterized with a T<sub>H</sub>17-cell-derived cytokine profile.

Therefore, we evaluated the potential role of T<sub>H</sub>17 cytokines in facilitating B-cell infiltration in the airways of patients with asthma. B cells in bronchial tissue sections from 79 patients with asthma (28 mild, 28 moderate, and 23 severe) and 29 healthy subjects (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) were immunolabeled; the numbers of isolated B cells from those with mild/moderate and severe asthma were significantly higher than those from healthy control subjects ( $P = .046$  and  $P = .007$ , respectively) (Fig 1, A). B cells and accompanying T cells can form organized lymphoid-like structures known as ectopic lymphoid follicles in the lung parenchyma of patients with chronic airway inflammation.<sup>4,5</sup> Correspondingly, we observed B-cell follicles in 13 of 79 patients with asthma (16.4%) and 8 of 29 control subjects (27.6%) (Fig 1, B; see Fig E1, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). However, the average number of follicles/mm<sup>2</sup> is twice as high in patients with asthma as in control subjects (2.08 and 1.0 follicles/mm<sup>2</sup>, respectively) and this difference was statistically significant ( $P < .001$ ; Fig E1, B).

Subsequently, we tested whether the augmented B-cell infiltration in asthmatic airways is correlated with increased CXCL13 expression, a principal B-cell chemoattractant. Bronchial tissue sections from subjects with severe asthma immunolabeled with anti-CXCL13 antibodies presented abundant positive immunoreactivity in the luminal side of the epithelium and within the smooth muscle area (Fig 1, Ci), and interestingly, close to and within B-cell follicles (Fig 1, Cii). Immunoreactivity was virtually undetectable in those from healthy subjects (Fig 1, Ciii). Average numbers of CXCL13<sup>+</sup> cells were also significantly higher in those with mild/moderate and severe asthma relative to control subjects ( $P < .001$  and  $P < .001$ ; respectively) (Fig 1, D). RT-PCR results confirmed a significant increase in CXCL13 mRNA expression in those with mild/moderate and severe asthma, relative to healthy control subjects ( $P < .001$ ) (Fig 1, E). Furthermore, in asthmatic bronchial tissues, the number of CXCL13<sup>+</sup> cells positively correlated with the number of B cells (severe:  $r = 0.99$ ,  $P < .001$ ; mild/moderate:  $r = 0.99$ ,  $P < .001$ ).

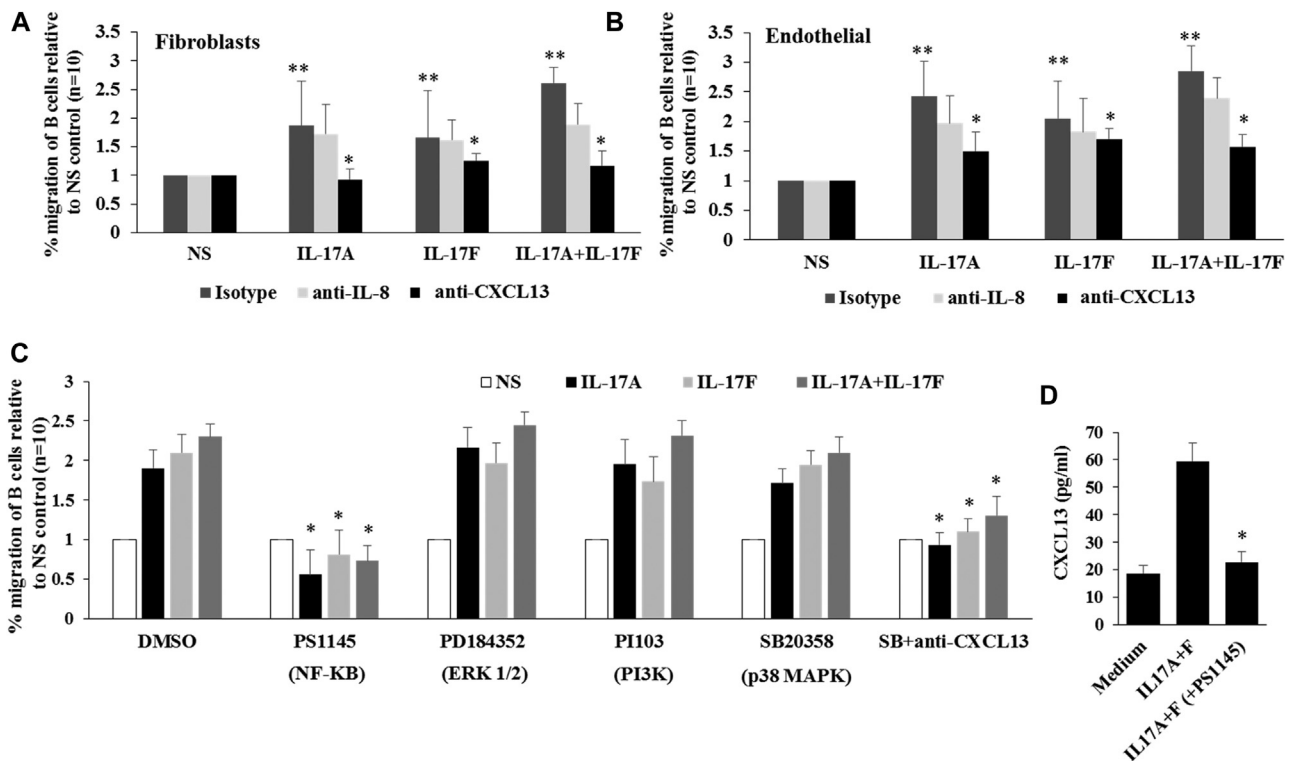
Elevated IL-17 production in lung tissues and bronchial lavage fluid is characteristic of severe asthma and *in vitro* evidence suggested the possibility that B-cell tissue infiltration could be driven by IL-17 stimulation, either directly, or indirectly, via the upregulation of chemoattractant mediators.<sup>6</sup> Similarly, accumulation of B cells in ectopic lymphoid follicles during mycobacterium infection is known to be regulated by IL-17-induced CXCL13 release.<sup>7</sup> Of note, all the recruited patients and subjects had no clinical history of mycobacterium tuberculosis infection. Here, we evaluated the possibility of an IL-17-induced CXCL13 expression in asthmatic airways. As expected, immunohistochemistry revealed that the numbers of IL-17A<sup>+</sup> cells in bronchial tissues from those with mild/moderate and severe asthma were significantly higher than in bronchial tissues from control subjects ( $P < .001$  and  $P < .001$ , respectively) (Fig E1, C). RT-PCR data also consistently showed elevated



**FIG 1.** B-cell infiltration in bronchial tissue increased in asthma and correlated with IL-17 cytokines stimulation and elevated CXCL13 expression. **A**, B cells in bronchial tissues were immunolabeled with anti-CD20 antibodies and expressed as number/mm<sup>2</sup>. **B**, Abundant B cells in an airway follicle, stained with anti-CD20 antibodies. **C**, Representative CXCL13 immunoreactivity in biopsies from those with severe asthma (i, ii) and control subjects (iii). **D**, Quantification of CXCL13<sup>+</sup> cells in biopsies. **E**, CXCL13 mRNA expression levels in bronchial tissues of subjects with asthma and healthy control subjects. **F**, Fold increase in IL-17-stimulated CXCL13 mRNA expression levels in fibroblasts and endothelial cells by RT-PCR. **G**, Flow cytometric analysis of fibroblasts stimulated with or without IL-17A+17F and immunostained with anti-CXCL13 antibodies. **H**, Fold increase in IL-17-stimulated CXCL13<sup>+</sup> fibroblast and endothelial cell numbers. NS, Nonsignificant. \**P* < .05.

IL-17A and IL-17F expression in the bronchial tissues from those with mild/moderate and severe asthma relative to control subjects (*P* < .05) (Fig E1, D). Severe asthmatic bronchial tissues had greater IL-17A and IL-17F mRNA expression than mild/moderate tissues (*P* = .001 and *P* = .001; respectively). In the same severe asthmatic bronchial tissues, the numbers of IL-17A<sup>+</sup>, CXCL13<sup>+</sup>, and CD20<sup>+</sup> cells were highly correlated (IL-17A<sup>+</sup>/CXCL13<sup>+</sup>, *r* = 0.93; IL-17A<sup>+</sup>/CD20<sup>+</sup>, *r* = 0.92). These data underline the possibility that IL-17 plays a key role in enhancing CXCL13 expression and subsequently leads to increased B-cell infiltration in asthmatic airways. To further evaluate the role of IL-17 in CXCL13 expression and B-cell infiltration, we (1) stimulated various cell types (ie, human primary lung fibroblasts, human lung microvascular endothelial

cells, airway epithelial cells, and airway smooth muscle cells) with or without T<sub>H</sub>17 cytokines (ie, IL-17A, IL-17F, or IL-17A+F) and (2) measured CXCL13 mRNA expression levels and CXCL13<sup>+</sup> cell numbers. Both the CXCL13 mRNA expression levels and CXCL13<sup>+</sup> cell numbers were significantly higher in stimulated than in nonstimulated fibroblasts and human lung microvascular endothelial cells (Fig 1, F and G and H). ELISA assays confirmed that fibroblasts and endothelial cells, in response to IL-17A/F stimulations, secreted a significantly greater amount of CXCL13 proteins into the culture medium (Fig E1, E). In contrast, airway epithelial and smooth muscle cells failed to respond to IL-17A/F stimulations (Fig E1, F-I). These findings suggest that fibroblasts and endothelial cells are the principal CXCL13 sources in the airways. These *in vitro*



**FIG 2.** IL-17-induced CXCL13 expression is required for B-cell migration and regulated by NF- $\kappa$ B pathway activation. **A**, Percentage of asthmatic B cells migrated toward culture medium from fibroblasts stimulated with IL-17 cytokines, in the presence/absence of either anti-IL-8 or anti-CXCL13 antibodies. **B**, Similar as (Fig 2, A) with medium from endothelial cells. **C**, Blockage of the NF- $\kappa$ B pathway in fibroblasts stimulated with IL-17 cytokines abolished B-cell migration. **D**, ELISA determination of CXCL13 proteins released into the medium by fibroblasts, stimulated with or without IL-17 cytokines and NF- $\kappa$ B inhibitor added. *DMSO*, Dimethyl sulfoxide; *ERK*, extracellular signal-regulated kinase; *MAPK*, mitogen-activated protein kinase; *NS*, nonstimulated. \* $P < .05$ , \*\* $P < .01$ .

observations contrasted with our *in situ* observation of positive immunoreactivity in the bronchial epithelium of subjects with asthma (Fig 1, C). One possible explanation is that although there are no reports of CXCL13 expression in bronchial epithelial cells, CXCL13 expression has been detected in the salivary epithelial cells from patients with Sjögren syndrome, evidently induced by oral and gastrointestinal infections.<sup>8</sup> Hence, the observed CXCL13 protein expression in the airway epithelium may be induced by alternative factors/pathways (eg, Toll-like receptors) acting either independently or in association with IL-17 signaling pathways.

The CXCL13 receptor, C-X-C motif chemokine receptor 5 (CXCR5), is expressed on B cells and can be downregulated by various factors (eg, influenza viruses), consequently hampering chemotaxis and B-cell infiltration in the lungs.<sup>9</sup> Therefore, we tested the possibility that IL-17A/F could modulate CXCR5 expression on B cells. At the basal level (ie, no stimulation), CXCR5 mRNA expression levels were comparable in control subjects and subjects with asthma (see Fig E2, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Stimulation with IL-17 increased CXCR5 mRNA expression levels in all subjects compared with their basal levels ( $P < .05$ ) (Fig E2, A). When compared across stimulated cells, severe asthmatic B cells had the highest levels of CXCR5

mRNA relative to healthy control cells ( $P < .05$ ) (Fig E2, A). The number of CXCR5+ B cells also increased in response to IL-17A+F stimulation in both healthy and asthmatic cells (Fig E2, B and C). This ability of IL-17 stimulation to induce higher expression of CXCR5 on asthmatic B cells could be due to the upregulation of its receptors on these cells.<sup>6</sup> Interestingly, stimulation with IL-17 in these B cells also significantly upregulated the expression of lymphocyte function-associated antigen 1 and very late antigen-4, ligands for intercellular adhesion molecule 1 and vascular cell adhesion molecule-1 adhesion receptors, respectively ( $P < .05$ ) (Fig E2, D). Given that intercellular adhesion molecule 1 and vascular cell adhesion molecule-1 are expressed in lung endothelial cells, upregulation of their ligands by IL-17 on B cells implies mechanistically the role of IL-17 in transvasation and tissue infiltration.

To further investigate the mechanism of IL-17-induced CXCL13-mediated B-cell infiltration in asthmatic airways, we assessed B-cell chemotaxis toward culture medium from IL-17-stimulated fibroblasts or endothelial cells. B cells from asthmatic and healthy control subjects had significantly greater migration rates toward IL-17-stimulated media than toward unstimulated media ( $P < .05$ ) (Fig 2, A and B). The specificity of CXCL13-mediated chemotaxis was confirmed when the

addition of anti-CXCL13 but not anti-IL-8 antibodies to the medium hindered migration (Fig 2, A and B).

IL-17 cytokines signal through multiple alternative pathways including p38 mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase, nuclear factor kappa B (NF- $\kappa$ B), and PI3K.<sup>6,10</sup> To identify the IL-17 signaling pathway(s) involved in CXCL13 gene induction, we evaluated 4 specific pathway inhibitors (ie, PS1145 [NF- $\kappa$ B pathway], PD184352 [extracellular signal-regulated kinase 1/2 pathway], PI103 [PI3K pathway], and SB20358 [p38 MAK pathway]) in blocking CXCL13 release from fibroblasts and subsequent inhibition of B-cell chemotaxis. Of the 4 pathway inhibitors tested, only PS1145 substantially decreased CXCL13 release into the medium and inhibited B-cell chemotaxis (Fig 2, C and D). These results confirmed that activation of the NF- $\kappa$ B pathway is required for IL-17-induced CXCL13 production and subsequent B-cell migration, further illustrating the potential mechanism of an IL-17-induced CXCL13-mediated B-cell infiltration in inflamed tissues in asthma.

To our knowledge, this is the first report that illustrates the possible role of IL-17 in B-cell infiltration in human asthmatic airways, via a mechanism involving NF- $\kappa$ B/CXCL13 pathway activation. Potentially, therapeutic blockage of IL-17/NF- $\kappa$ B/CXCL-13 signaling could help reduce B-cell infiltration in the airways, as an alternative approach to control bronchial inflammation in asthma and other chronic inflammatory diseases.

Airway biopsy sections from patients with asthma were obtained from the Strauss Severe Asthma Program and Meakins-Christie Tissue Bank, McGill University, Montreal, Canada.

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## Heterogeneous inflammatory patterns in chronic rhinosinusitis without nasal polyps in Chicago, Illinois



### To the Editor:

Although it is accepted that chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP) is characterized by type 2 inflammation with pronounced eosinophilia and the presence of high levels of IL-5 and IL-13 in Western countries, the mechanism of inflammation in patients with chronic rhinosinusitis without nasal polyps (CRSSNP) is poorly understood.<sup>1,2</sup> Initial studies by Van Zele et al<sup>3</sup> in Belgium suggested that CRSSNP is characterized by type 1 inflammation on the basis of elevation in IFN- $\gamma$  levels. Although several articles from the same group have confirmed these findings,<sup>4</sup> other groups, including our own, have been unable to find elevation in IFN- $\gamma$  levels in CRSSNP.<sup>5,6</sup> We therefore examined potential differences in experimental design between these studies. Studies that found type 1 inflammation in CRSSNP compared inferior turbinate (IT) tissue from controls, ethmoid tissue (ET) from patients with CRSSNP, and nasal polyp (NP) tissue from patients with CRSwNP.<sup>3,4,7</sup> In contrast, those that did not find type 1 inflammation compared uncinatate tissue (UT) from controls, patients with CRSSNP and CRSwNP, and patients with NPs.<sup>5,6</sup> It was thus unclear whether reported IFN- $\gamma$  elevations were due to differences in sampled anatomy or countries.

To clarify patterns of inflammatory cytokines in CRSSNP in our study population, we collected IT, UT, and ET from control patients and patients with CRSSNP and CRSwNP (see Fig E1 and Tables E1 and E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), and determined the presence of IFN- $\gamma$  by real-time RT-PCR. Detailed methods are given in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). We found that the IFN- $\gamma$  level was not significantly elevated in CRSSNP when compared with controls or patients with CRSwNP within the same tissue type (Fig 1, A). We also compared CRSSNP ET to control IT or CRSwNP NP as previously reported.<sup>3,4</sup> However, the IFN- $\gamma$  level was not elevated in ET from CRSSNP (Fig 1, B). We also analyzed a marker of eosinophilia, Charcot-Leyden crystal galectin (CLC, also known as eosinophil lysophospholipase), type 2 cytokines, IL-5 and IL-13, and the type 3 cytokine IL-17A. Because we found significant differences in the levels

## METHODS

### Subjects' characteristics

The bronchial biopsies were provided by the severe asthma program at the Meakins-Christie Laboratories with the support of the Richard and Edith Strauss Foundation. Bronchial biopsies were obtained from 79 patients with asthma (28 mild, 28 moderate, and 23 severe) and 29 healthy subjects. Asthma status was classified according to the ATS criteria.<sup>E1</sup> FEV1 is expressed as a percentage relative to the lower limit values obtained from "normal" (eg, non-asthmatic) subjects and it is calculated on the basis of age, sex, height, and ethnicity of the patient. Subjects with mild asthma had a baseline FEV1 of more than 75% predicted and were treated with a short-acting  $\beta$ 2-agonist as needed ( $\leq 3$  times/wk). Subjects with moderate asthma had a baseline FEV1 of 60% to 80% predicted and were treated with a short-acting  $\beta$ 2-agonist as needed ( $\geq 4$  times/wk). Patients with severe asthma needed high-dose inhaled corticosteroid or daily antileukotriene for more than 50% and at least 1 other add-on therapy daily for the previous 12 months. They were also required to have 2 of the following criteria: (1) daily short-acting  $\beta$ -agonist, (2) persistent FEV1 of less than 60% and FEV1/forced vital capacity of less than 75% predicted, (3) 1 urgent visit or at least 3 steroid bursts in the previous year, (4) prompt deterioration with less than 25% steroid dose reduction, or (5) previous near-fatal asthma within the last 3 years.

Exclusion criteria included smoking history or any other pulmonary diseases or coexisting medical conditions such as cardiac and renal diseases and uncontrolled hypertension. All normal control subjects were nonsmokers with normal lung function, no history or symptoms of allergy and respiratory diseases, and were not taking any medications for the preceding 4 weeks. All patients and control subjects have been examined for evidence of active tuberculosis using chest X-ray and PCR assay; they had no clinical history of mycobacterium tuberculosis.

The study protocol was approved by the Institutional Review Board of the College of Medicine, King Saud University (Protocol no. E-11-555). All patients and control subjects in this study signed an informed consent approved by the Institutional Review Board. General characteristics of subjects and patients are summarized in Table E1.

### Immunohistochemistry assay

To determine whether airway structural cells have the capacity to express CXCL13 protein, immunohistochemistry was performed on endobronchial biopsies thin sections from subjects with asthma and control subjects, as described elsewhere.<sup>E2</sup> Slides were incubated overnight at 4°C with either diluted goat antihuman CD20 (R&D Systems, Minneapolis, Minn) or CXCL13 (R&D systems) polyclonal antibodies or relevant isotype controls (AB-108-C, R&D Systems). Stained slides were rinsed and incubated with a biotinylated secondary antibody for 30 minutes at room temperature. After washing in PBS, the complex streptavidin/horse radish peroxidase (Vector Laboratories, Burlingame, Calif) was applied for 30 minutes at room temperature. The reaction result was visualized with diaminobenzidine/hydrogen peroxide (DAB Kit, Dako, Carpinteria, Calif). The sections were finally rinsed in distilled water, lightly stained with hematoxylin, dehydrated, cleared, and cover slipped. The specificity of the CXCL13 immune staining was confirmed by preabsorption of the antibody with excess purified CXCL13 antigen, which resulted in a loss of reactivity (data not shown). Immunostained cells in the airway subepithelial area were counted at a magnification of 400 $\times$ ; the area analyzed was measured using the software Image Pro 6.2 (MediaCybernetics, Bethesda, Md). The final result was expressed as number of positive cells/mm<sup>2</sup>.

### Isolation of peripheral blood B cells

Forty study participants (mild asthma [N = 10], moderate [N = 10], severe [N = 10], and healthy control [N = 10]) were randomly selected and 20 mL of peripheral blood was drawn from each subject. Blood was layered over Ficoll gradient (Axis Shield, Dundee, United Kingdom) and centrifuged at 1000g for 30 minutes. The mononuclear cells layer (PBMC) was then collected and B cells were isolated by negative selection using EasySep Human B cell

enrichment kit (StemCell, cat# 19054). B cells' purity was consistently more than 98% as evaluated by fluorescence-activated cell sorting analysis and their viability was more than 99% as evaluated by trypan blue dye exclusion.

### Cell culture

Human primary lung fibroblasts isolated from 5 healthy subjects and primary airway smooth muscle cells isolated from 3 subjects with asthma were cultured in Dulbecco modified Eagle medium/F12 (1:1) supplemented with 10% FCS and antibiotics. Primary human bronchial epithelial cells from 2 healthy control subjects and 2 patients with asthma (Lonza, Walkersville, Md) were maintained in Bronchial Epithelial Cell Growth Medium (BulletKit, Lonza) according to the supplier's recommendations. Human lung microvascular endothelial cells (HMVEC-L) (Lonza) were cultured in microvascular endothelial cell growth medium-2 (EGM2-MV BulletKit; Lonza). All experiments were performed using second- or third-passage cells in 75% to 85% confluent monolayers.

### RNA extraction and real-time RT-PCR

Human primary lung fibroblasts, HMVEC-L cells, and peripheral blood B cells were stimulated with IL-17 cytokines (ie, IL-17A, IL-17F, or both) (50 ng/mL) for 12 hours before cell harvest. Cells ( $5 \times 10^5$  cells) were then harvested and total RNA was extracted (RNeasy Mini kit, Qiagen, Valencia, Calif). mRNA expression levels of CXCL13, CXCR5, very late antigen-4, and lymphocyte function-associated antigen 1 were then determined using semi-quantitative RT-PCR (7900 Fast system from Applied Biosystems, Foster City, Calif). Gene expressions were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and relative expressions were determined by the delta-delta Ct method.<sup>E3</sup>

### Flow cytometry assay

Human primary lung fibroblasts and HMVEC-L cells ( $5 \times 10^5$  cells) were stimulated with IL-17A and IL-17F (50 ng/mL), either alone or combined, for 18 hours and treated with Brefeldin A for the last hour of incubation. Cells were permeabilized using 0.25% saponin and stained with anti-CXCL13 APC antibodies (R&D Systems).

CXCR5 receptor expression on B cells was determined following IL-17A and IL-17F stimulation. B cells ( $5 \times 10^5$  cells) were stimulated with these cytokines for 18 hours and then immunostained with anti-CXCR5 antibody (R&D Systems). Cells were then washed and fixed in 2% paraformaldehyde and the percentage of positive cells was analyzed by flow cytometry and DIVA software (BD LSRII; from BD Biosciences, San Jose, Calif). Gates were set on the basis of nonstained cells (using Fluorescein Minus One technique) and the isotype signal. A small background peak was detected using the isotype control and it was necessary to adjust the gate and subtract the isotype background signal (7.3%) from the reported CXCR5-positive signals (Fig E2, B).

### Chemotaxis assays

B-cell migration assays were performed as previously described<sup>E4</sup> using a 48-well micro Chemotaxis Chamber (Neuro Probe, Gaithersburg, Md). B cells isolated from patients with severe asthma were growth arrested in RPMI media supplemented with 0.1% FBS for 4 hours. An 8.0-mm-pore polycarbonate membrane (Neuro Probe) was treated with 0.01% Collagen type I (rat tail; Nalgene Culture 3D Matrix) solution in 0.01 N HCl and placed between 2 chambers. B cells were added to the upper chamber, and supernatants from nonstimulated cells or IL-17-stimulated (50 ng/mL) fibroblasts or endothelial cells were added to the lower chamber. Neutralizing anti-IL-17A and anti-IL-17F antibodies were added to the IL-17-treated fibroblast and endothelial supernatants before using them for B-cell migration assay. A preliminary experiment was performed to establish the time course of migration (4, 6, 18, and 24 hours). Because the maximal increase in migration was observed

at 18 hours (data not shown), all subsequent experiments were performed for 18 hours. After 18 hours of incubation at 37°C, the membrane was removed and its upper face was scraped clear of cells. Cells that migrated to the lower side of the membrane were fixed and stained with the Hema 3 kit (Fisher, Pittsburgh, Pa), according to the manufacturer's instructions. The number of cells was counted in 5 random fields at a magnification of 3200. Because B cells are nonadherent cells, migrating cells were also counted in the lower chamber and the total number of cells both at the membrane and in media was obtained.

To determine whether the increased migration of B cells toward the supernatant of IL-17 cytokines-stimulated cells is due to the production of B-cell chemoattractants CXCL13 or IL-8, we pretreated the supernatants of stimulated fibroblasts and HMVEC-L cells with anti-CXCL13 and anti-IL-8 neutralizing antibodies (R&D Systems) for 1 hour before the start of the migration assay. Results were compared with the corresponding isotype controls.

### CXCL13 ELISA

To analyze the production of CXCL13, fibroblasts and HMVEC-L cells were stimulated with IL-17 cytokines (ie, IL-17A [50 ng/mL], IL-17F [50 ng/mL], or combined) and in the presence or absence of PS1145 (10 mmol/L), an NF- $\kappa$ B inhibitor. The secreted levels of CXCL13 in the supernatants were determined using the Human CXCL13/BLC PicoKine ELISA Kit (EK0739, Boster Bio, Pleasanton, Calif) following the manufacturer's instructions.

### Inhibition of IL-17–induced CXCL13-mediated B-cell migration

Pharmacologic inhibitors directed against various pathways potentially activated by IL-17 cytokines were used to determine the pathway required for CXCL13 production by lung structural cells. Human primary lung fibroblasts and HMVEC-L cells were incubated with various inhibitors (p38 mitogen-activated protein kinase inhibitor SB203580 [0.1 mmol/L; Axon Medchem, Reston, Va], the extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase inhibitor PD184352 [2 mmol/L; United States Biological, Inc, Salem, Mass], the NF- $\kappa$ B inhibitor PS1145 [10 mmol/L; Professor Sir Philip

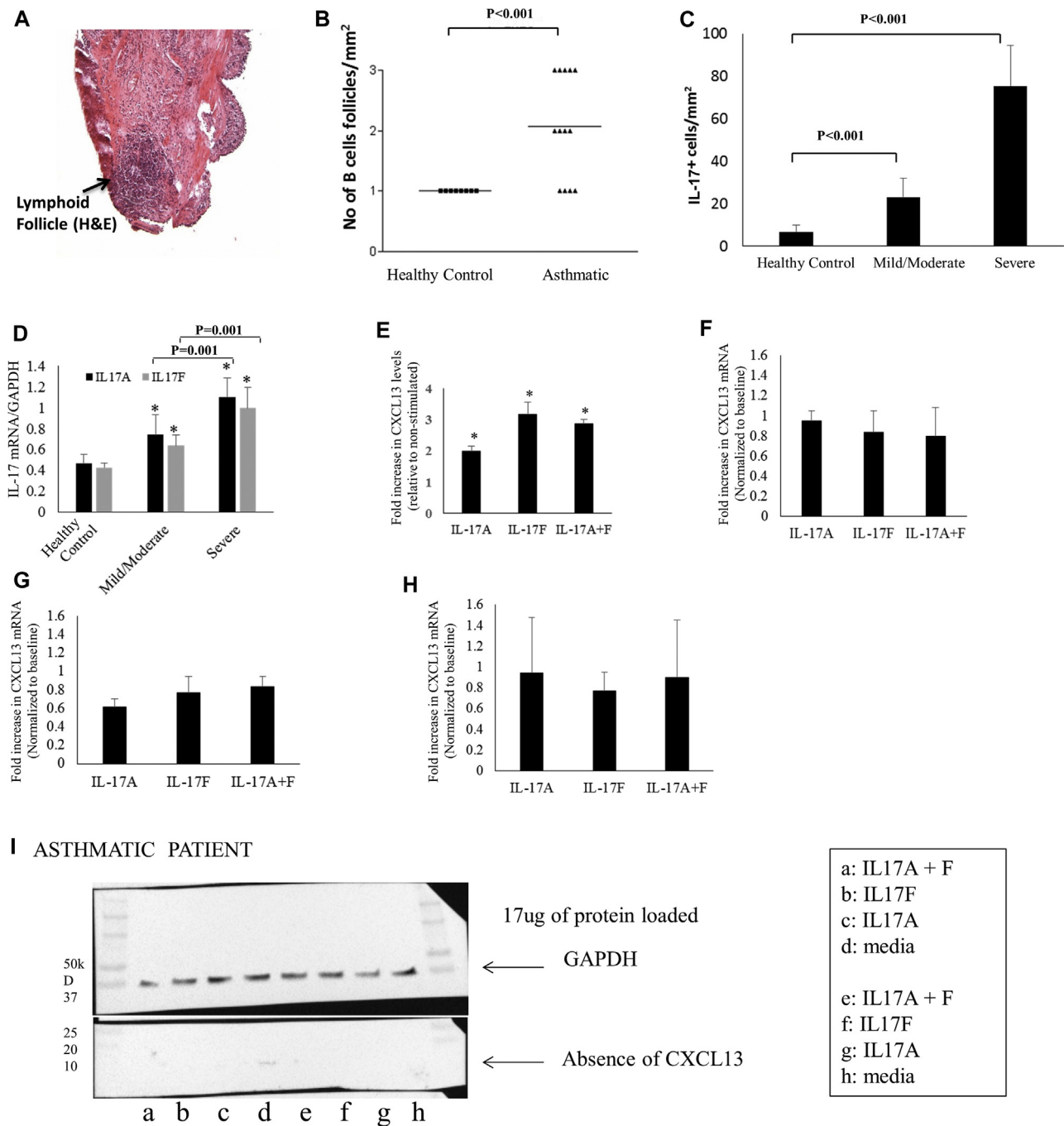
Cohen], and the phosphoinositide 3-kinase [PI3K] inhibitor PI103 [5 mmol/L; Cayman Chemical, Ann Arbor, Mich]) for 1 hour at 37°C before IL-17 stimulation. A dose-response experiment for each inhibitor has been performed to determine the concentration that resulted in the highest effect (above which no increase in inhibition effect was observed). The dose concentrations were consistent with those previously reported for structural cells.<sup>E4</sup> Cells were then stimulated with IL-17A and IL-17F (50 ng/mL) for 16 hours and supernatants were then treated with neutralizing anti-IL-17A and IL-17F antibodies to remove remnants of IL-17 cytokines. Supernatants were then used to examine the inhibiting effect on CXCL13 production and B-cell migration. All results were compared with the corresponding vehicle control dimethyl sulfoxide.

### Statistical analysis

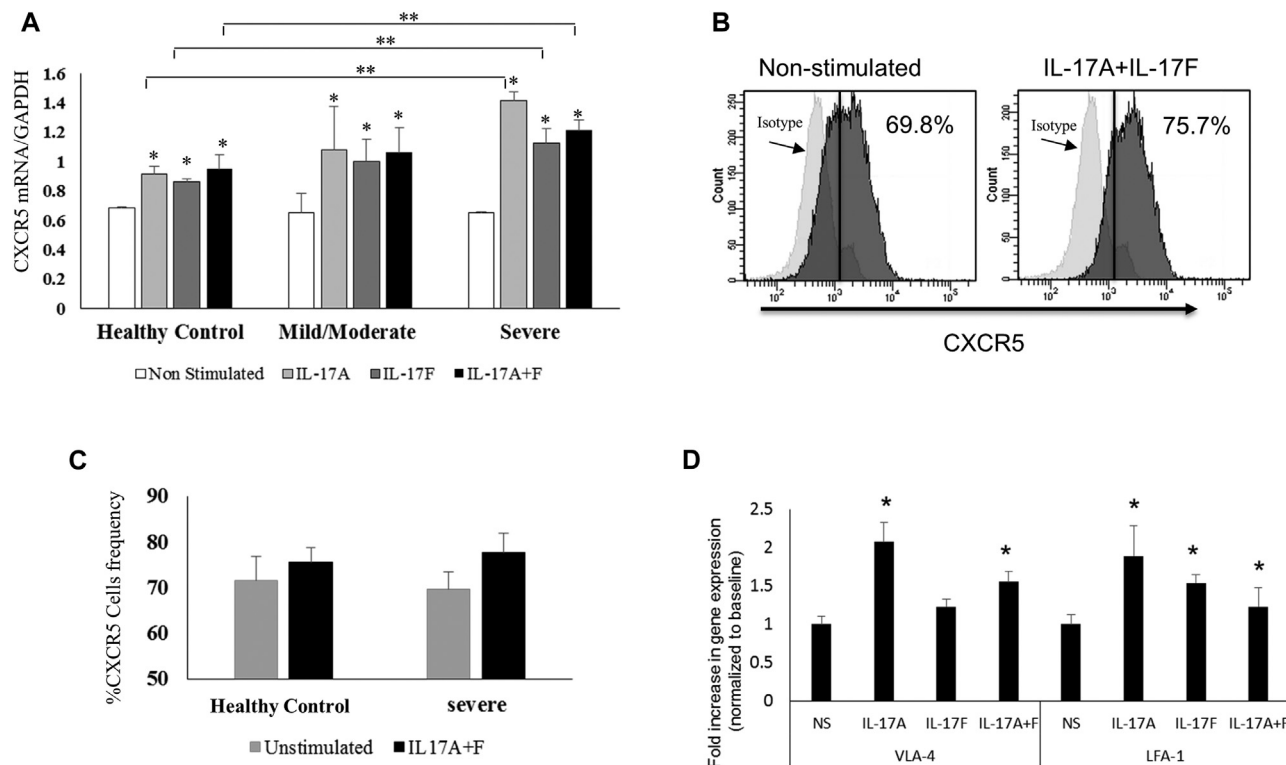
All data are presented as means  $\pm$  standard error. Statistical analysis for cytokine-induced migration was first performed within the subgroups by using ANOVA, followed by the post hoc Bonferroni test if the ANOVA result reached statistical significance. The paired *t* test was performed for the other assays. *P* values of less than .05 were considered statistically significant. Statistical analyses were performed with GraphPad Instat 3 software (GraphPad Software, Inc, La Jolla, Calif).

### REFERENCES

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**FIG E1.** **A**, Representative figure of cell follicles (H&E staining) formed in an asthmatic bronchial tissue. **B**, Number of B-cell follicles/mm<sup>2</sup> in bronchial tissue of subjects with asthma and healthy subjects. **C**, IL-17A<sup>+</sup> cells/mm<sup>2</sup> in bronchial tissues of subjects with mild/moderate and severe asthma and healthy control subjects. **D**, IL-17 mRNA expression levels in bronchial biopsies from subjects with mild/moderate or severe asthma and healthy control subjects. **E**, Fold increase in secreted CXCL13 protein levels from fibroblasts stimulated with or without IL-17. **F**, Fold increase in CXCL13 mRNA in epithelial cells (healthy) stimulated with or without IL-17. **G**, Same as Fig E1, **F**, except in asthmatic epithelial cells. **H**, Same as Fig E1, **F**, except in asthmatic ASM cells. **I**, Western analysis indicating the absence of CXCL13 protein expression following stimulation of asthmatic primary epithelial cells with IL-17 cytokines. (Fig E1, **D-H**, *n* = 10). *ASM*, Airway smooth muscle; *H&E*, hematoxylin and eosin; *SE*, standard error. Data are expressed as means ± SE. \**P* < .05.



**FIG E2.** CXCR5, VLA-4, and LFA-1 receptors are upregulated on B cells following stimulation with IL-17. **A**, Relative expression of CXCR5 mRNA in B cells from subjects with asthma and healthy control subjects following stimulation with IL-17. **B**, Representative flow cytometric analysis of asthmatic B cells immunostained with anti-CXCR5 antibodies, stimulated with or without IL-17. **C**, % of CXCR5<sup>+</sup> cells following stimulation with IL-17 from healthy and severe asthmatic B cells. **D**, Fold increase in VLA-4 and LFA-1 mRNA expression levels in asthmatic B cells following stimulation with IL-17 cytokines normalized to baseline. *LFA-1*, Lymphocyte function-associated antigen 1; *SE*, standard error; *VLA-4*, very late antigen-4. Data are expressed as means  $\pm$  SE. \**P* < .05 compared with the nonstimulated (NS) condition. \*\**P* < .05 compared with the control condition.



**TABLE E1.** General characteristics of subjects and patients

<b>Group</b>	<b>No.</b>	<b>Age (y)</b>	<b>Sex (M/F)</b>	<b>Atopy (yes/no)</b>	<b>FEV<sub>1</sub> (mean ± SD)</b>
Normal controls	29	33.4 ± 3.7	18/11	4/25	110.7 ± 5.3
Mild/moderate	56	33.8 ± 2.7	35/21	44/12	81.9 ± 3.0
Severe asthmatic	23	37.3 ± 4.2	15/8	20/3	57.65 ± 3.4

F, Female; M, male.