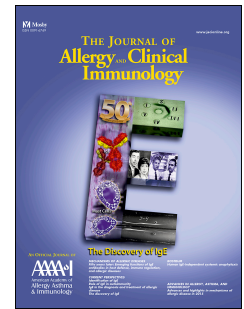


Accepted Manuscript

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

Roua Al-Kufaidy, MSc, Alejandro Vazquez-Tello, PhD, Ahmed S. BaHammam, MD, Qutayba Hamid, MD, PhD, Saleh Al-Muhsen, MD, Rabih Halwani, PhD.



PII: S0091-6749(16)30958-7

DOI: [10.1016/j.jaci.2016.07.037](https://doi.org/10.1016/j.jaci.2016.07.037)

Reference: YMAI 12335

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 23 June 2015

Revised Date: 16 June 2016

Accepted Date: 1 July 2016

Please cite this article as: Al-Kufaidy R, Vazquez-Tello A, BaHammam AS, Hamid Q, Al-Muhsen S, Halwani R, IL-17 enhances the migration of B cells during asthma by inducing CXCL13 chemokine production in structural lung cells, *Journal of Allergy and Clinical Immunology* (2016), doi: 10.1016/j.jaci.2016.07.037.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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

Roua Al-Kufaidy¹, MSc; Alejandro Vazquez-Tello¹, PhD; Ahmed S. BaHamman^{1,2}, MD;
Qutayba Hamid^{3,4}, MD, PhD; Saleh Al-Muhsen¹, MD; and Rabih Halwani^{1*}, PhD.

¹Prince Naif Center for Immunology Research and Asthma Research Chair, department of
pediatrics, College of Medicine, King Saud University, Riyadh, Saudi Arabia. ²Pulmonary
Medicine Department, University Sleep Disorders Center, College of Medicine, King Saud
University, Riyadh, Kingdom of Saudi Arabia. ³Meakins-Christie Laboratories, McGill
University, Montreal, Quebec, Canada. ⁴College of Medicine, University of Sharjah, Sharjah,
UAE.

*** Correspondence and proof:**

Rabih Halwani, Msc. Ph.D

Prince Naif center for immunology Research
Asthma Research Chair, Department of pediatrics,
College of Medicine
King Saud University

P.O.Box 2925, Postal Code 11461
Riyadh, Saudi Arabia
Tel:+966-1-4690146

Fax:+966-1-4679463

Email address: rhalwani@ksu.edu.sa

28 **Summary**

29

30 Here, we report a positive correlation between expression of IL-17 cytokines with CXCL13⁺
31 cells and B cells numbers in asthmatic airways. IL-17A/F stimulated CXCL13 release from
32 fibroblast and endothelial cells which induced B cells chemotaxis.

33 **Keywords**

34 Bronchial asthma, B cells, CXCL13, IL-17, NF- κ B.

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 to the production of immunoglobulin antibodies in response to allergens and infectious pathogens.^{1,2} 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.³ Recent findings have revealed the crucial participation of B-regulatory cells, either as modulators or effectors of the immune response in many chronic autoimmune and allergic disorders.⁴ Despite these advances, little is known about the mechanisms that drive B cell infiltration into the bronchial tissues of asthmatic patients, in particular those of the severe phenotype characterized with a T-helper (Th)-17 cell derived cytokine profile.

Therefore, we evaluated the potential role of Th-17 cytokines in facilitating B cell infiltration in the airways of asthmatic patients. B cells in bronchial tissue sections from 79 asthmatic patients (28 mild, 28 moderate, and 23 severe) and 29 healthy subjects (see Supplementary Table E1) were immunolabeled; the numbers of isolated B cells from mild/moderate and severe asthmatic were significantly higher than those of healthy control subjects ($p=0.046$ and $p=0.007$, respectively) (Figure 1A). 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.^{4,5} Correspondingly, we observed B cell follicles in 13/79 asthmatic patients (16.4%) and 8/29 control subjects (27.6%) (Figure 1B, Supplementary Figure E1A). However, the average number of follicles/mm² is twice as high in

asthmatic patients as in control subjects (2.08 and 1.0 follicles/mm², respectively); and this difference was statistically significant ($p < 0.001$; Figure E1B).

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 severe asthmatic subjects immunolabeled with anti-CXCL13 antibodies presented abundant positive immunoreactivity in the luminal-side of the epithelium and within the smooth muscle area (Figure 1Ci); and interestingly, close to and within B cells follicle (Figure 1Cii). Immunoreactivity was virtually undetectable in those from healthy subjects (Figure 1Ciii). Average numbers of CXCL13⁺ cells were also significantly higher in mild/moderate and severe asthmatics relative to control subjects ($p < 0.001$ and $p < 0.001$; respectively) (Figure 1D). RT-PCR results confirmed a significant increase in CXCL13 mRNA expression in mild/moderate and severe asthmatics, relative to healthy control subjects ($p < 0.001$) (Figure 1E). Furthermore, in asthmatic bronchial tissues, the number of CXCL13⁺ cells positively correlated with the number of B cells (severe: $r = 0.99$, $p < 0.001$; mild/moderate: $r = 0.99$, $p < 0.001$).

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.⁶ Similarly, accumulation of B cells in ectopic lymphoid follicles during mycobacterium infection is known to be regulated by IL-17-induced CXCL13 release.⁷ Of note, all the recruited patients and subjects had no clinical history of MTB 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⁺ cells in bronchial tissues

81 from mild/moderate and severe asthmatic were significantly higher than control subjects
82 ($p<0.001$ and $p<0.001$, respectively) (Figure E1C). RT-PCR data also consistently showed
83 elevated IL-17A and IL-17F expression in the bronchial tissues from mild/moderate and severe
84 asthmatic relative to control subjects ($p<0.05$) (Figure E1D). Severe asthmatic bronchial tissues
85 had greater IL-17A and IL-17F mRNA expression than mild/moderate tissues ($p=0.001$ and
86 $p=0.001$; respectively). In the same severe asthmatic bronchial tissues, the numbers of IL-17A+,
87 CXCL13+, and CD20+ cells were highly correlated (IL-17A+/CXCL13+, $r=0.93$; IL-
88 17A+/CD20+, $r=0.92$). These data underline the possibility that IL-17 plays a key role in
89 enhancing CXCL13 expression and subsequently leads to increased B cell infiltration in
90 asthmatic airways. To further evaluate the role of IL-17 in CXCL13 expression and B cell
91 infiltration, we (1) stimulated various cell types (i.e. human primary lung fibroblasts, human lung
92 microvascular endothelial cells (HMVEC-L), airway epithelial cells, and airway smooth muscle
93 cells) with or without Th-17 cytokines (i.e. IL-17A, IL-17F or IL-17A+F) and (2) measured
94 CXCL13 mRNA expression levels and CXCL13+ cell numbers. Both the CXCL13 mRNA
95 expression levels and CXCL13+ cell numbers were significantly higher in stimulated than non-
96 stimulated fibroblasts and HMVEC-L cells (Figure 1F and 1G-H). ELISA assays confirmed that
97 fibroblasts and endothelial cells, in response to IL-17A/F stimulations, secreted significantly
98 greater amount of CXCL13 proteins into the culture medium (Figure E1E). In contrast, airway
99 epithelial and smooth muscle cells failed to respond to IL-17A/F stimulations (Figure E1F-I).
100 These findings suggest that fibroblasts and endothelial cells are the principal CXCL13 sources in
101 the airways. These *in vitro* observations contrasted with our *in situ* observation of positive
102 immunoreactivity in the bronchial epithelium of asthmatic subjects (Figure 1Ci). One possible
103 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.⁸ Hence, the observed CXCL13 protein expression in the airway epithelium may be induced by alternative factors/pathways (e.g. 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 down-regulated by various factors (e.g. influenza viruses), consequently hampering chemotaxis and B cell infiltration in the lungs.⁹ Therefore, we tested the possibility that IL-17A/F could modulate CXCR5 expression on B cells. At the basal level (i.e. no stimulation), CXCR5 mRNA expression levels were comparable in the control and asthmatic subjects (Figure E2A). Stimulation with IL-17 increased CXCR5 mRNA expression levels in all subjects compared to their basal levels ($p < 0.05$) (Figure E2A). When compared across stimulated cells, severe asthmatic B cells had the highest levels of CXCR5 mRNA relative to healthy control cells ($p < 0.05$) (Figure E2A). The numbers of CXCR5+ B cells also increased in response to IL-17A+F stimulation in both healthy and asthmatic cells (Figure E2B and C). This ability of IL-17 stimulation to induce higher expression of CXCR5 on asthmatic B cells could be due to upregulation of its receptors on these cells.⁶ Interestingly, stimulation with IL-17 in these B cells also significantly upregulated the expression of lymphocyte function-associated antigen 1 (LFA-1) and very late antigen-4 (VLA-4), ligands for intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) adhesion receptors, respectively ($p < 0.05$) (Figure E2D). Given that ICAM-1 and VCAM-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 towards culture medium from IL-17-stimulated fibroblasts or endothelial cells. B cells from asthmatic and healthy control subjects had significantly greater migration rates towards IL-17-stimulated media than unstimulated media ($p < 0.05$) (Figure 2A 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 (Figure 2A and B).

IL-17 cytokines signal through multiple alternative pathways including p38 MAPK, ERK1/2 MAPK, NF- κ B, and PI3K.^{6,10} To identify the IL-17-signaling pathway(s) involved in CXCL13 gene induction, we evaluated four specific pathway inhibitors (i.e. PS1145 (NF- κ B pathway), PD184352 (ERK1/2 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 (Figure 2C and D). These results confirmed that activation of NF- κ 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- κ B/CXCL13 pathway activation. Potentially, therapeutical blockage of IL-17/NF- κ 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.

Roua Al-Kufaidy, MS¹,

Alejandro Vazquez-Tello, PhD¹,

Ahmed S. BaHammam, MD^{1,2}

Qutayba Hamid MD, PhD³

Saleh Al-Muhsen, MD¹

*Rabih Halwani, PhD¹**

¹Prince Naif Center for Immunology Research and Asthma Research Chair, department of pediatrics, College of Medicine, King Saud University, Riyadh, Saudi Arabia. ²Pulmonary Medicine Department, University Sleep Disorders Center, College of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia. ³Meakins-Christie Laboratories, McGill University, Montreal, Quebec, Canada.

*** Corresponding Author.**

Acknowledgments

This project was funded by the National Plan for Science, Technology and Innovation (MAARIFAH), King Abdulaziz City for Science and Technology, Kingdom of Saudi Arabia, Award Number (10-MED1224-02). Airway biopsy sections from asthmatics were obtained from the Strauss Severe asthma program and Meakins-Christie tissue bank, McGill University, Montreal, Canada.

Figure legends

Figure 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/mm². B) Abundant B cells in an airway follicle, stained with anti-CD20 antibodies. C) Representative CXCL13 immunoreactivity in biopsies from severe asthmatic (i, ii) and control subjects (iii). D) Quantification of CXCL13+ cells in biopsies. E) CXCL13 mRNA expression levels in bronchial tissues of asthmatic 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+ fibroblast and endothelial cell numbers * p-values <0.05

Figure 2: IL-17 induced CXCL13 expression is required for B cell migration and regulated by NF- κ B pathway activation. A) Percentage of asthmatic B cells migrated towards 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 (A) with medium from endothelial cells. C) Blockage of NF- κ 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- κ B inhibitor added. * p-values <0.05

References

1. Baena-Cagnani CE, Teijeiro A, Canonica GW. Four-year follow-up in children with moderate/severe uncontrolled asthma after withdrawal of a 1-year omalizumab treatment. *Curr Opin Allergy Clin Immunol*. 2015;15(3):267-71.
2. Arnaboldi PM, Behr MJ, Metzger DW. Mucosal B cell deficiency in IgA-/- mice abrogates the development of allergic lung inflammation. *J Immunol*. 2005; 175(2):1276-85.
3. van der Vlugt LE, Mlejnek E, Ozir-Fazalalikhan A, Janssen Bonas M, Dijksman TR, Labuda LA, et al. CD24(hi)CD27(+) B cells from patients with allergic asthma have impaired regulatory activity in response to lipopolysaccharide. *Clin Exp Allergy*. 2014;44(4):517-28.
4. Floudas A, Amu S, Fallon PG. New Insights into IL-10 Dependent and IL-10 Independent Mechanisms of Regulatory B Cell Immune Suppression. *J Clin Immunol*. 2016 Mar 23. DOI: 10.1007/s10875-016-0263-8.
5. Yadava K, Marsland BJ. Lymphoid follicles in chronic lung diseases. *Thorax*. 2013;68(6):597-8.
6. Halwani R, Al-Kufaidy R, Vazquez-Tello A, Pureza MA, BaHammam AS, Al-Jahdali H, et al. IL-17 Enhances Chemotaxis of Primary Human B Cells during Asthma. *PLoS One*. 2014;9(12):e114604.
7. Gopal R, Rangel-Moreno J, Slight S, Lin Y, Nawar HF, Fallert Junecko BA, et al. Interleukin-17-dependent CXCL13 mediates mucosal vaccine-induced immunity against tuberculosis. *Mucosal Immunol*. 2013;6(5):972-84.
8. Salomonsson S, Larsson P, Tengner P, Mellquist E, Hjelmstrom P, Wahren-Herlenius M. Expression of the B cell-attracting chemokine CXCL13 in the target organ and autoantibody production in ectopic lymphoid tissue in the chronic inflammatory disease Sjogren's syndrome. *Scandinavian journal of immunology*. 2002;55(4):336-42.

9. Wang X, Chan CC, Yang M, Deng J, Poon VK, Leung VH, et al. A critical role of IL-17 in modulating the B-cell response during H5N1 influenza virus infection. *Cell Mol Immunol.* 2011;8(6):462-8.
10. Chang Y, Al-Alwan L, Risse PA, Roussel L, Rousseau S, Halayko AJ, et al. TH17 cytokines induce human airway smooth muscle cell migration. *J Allergy Clin Immunol.* 2011;127(4):1046-53 e1-2.

Supplementary figure Legends

Figure E1: (A) Representative figure of cell follicles (H&E staining) formed in an asthmatic bronchial tissue. (B) Number of B cell follicles/mm² in bronchial tissue of asthmatic and healthy subjects. (C) IL-17A⁺ cells/mm² in bronchial tissues of mild/moderate, severe asthmatic, and healthy control subjects. (D) IL-17 mRNA expression levels in bronchial biopsies from mild/moderate or severe asthmatic 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 (F) except in asthmatic epithelial cells. (H) Same as (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. (D-H, n=10). Data is expressed as means $\pm$ SE. * $P < 0.05$.

Figure E2: CXCR5, VLA-4, LFA-1 receptors are upregulated on B cells following stimulation with IL-17. (A) Relative expression of CXCR5 mRNA in B cells from asthmatic 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.

238 (C) % of CXCR5⁺ cells following stimulation with IL-17 from healthy and severe asthmatic B
239 cells. (D) Fold increase in VLA-4 and LFA-1 mRNA expression levels in asthmatic B cells
240 following stimulation with IL-17 cytokines normalized to baseline. Data is expressed as means $\pm$
241 SE. * $P < 0.05$ compared to the Non stimulated (NS) condition. ** $P < 0.05$ compared to the
242 control condition.

243

Methods and materials

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 asthmatic patients (28 mild, 28 moderate, and 23 severe) and 29 healthy subjects. Asthma status was classified according to the ATS criteria (1). Forced expiratory volume in 1 second (FEV_1) is expressed as a percentage relative to the lower limit values obtained from 'normal' (e.g. non-asthmatic) subjects and it is calculated based on the age, gender, height, and ethnicity of the patient. Mild asthmatic subjects had a baseline FEV_1 $>75\%$ predicted and were treated with a short-acting β_2 -agonist as needed (≤ 3 times/week). Moderate asthmatic subjects had a baseline FEV_1 of 60-80% predicted and were treated with a short-acting β_2 -agonist as needed (≥ 4 times/week). Severe asthmatic patients needed high-dose inhaled corticosteroid or daily anti-leukotriene for $>50\%$ and at least 1 other add-on therapy on daily for the previous 12 months. They were also required to have two of the following criteria: (i) daily short-acting β -agonist, (ii) persistent FEV_1 $<60\%$ and FEV_1 /forced vital capacity (FVC) $<75\%$ predicted, (iii) 1 urgent visit or at least 3 steroid bursts in the previous year, (iv) prompt deterioration with $<25\%$ steroid dose reduction, or (v) previous near-fatal asthma within the last 3 years.

Exclusion criteria included smoking history or any other pulmonary diseases or co-existing medical conditions such as cardiac and renal diseases and uncontrolled hypertension. All normal control subjects were non-smokers with normal lung function, no history or symptoms of allergy and respiratory diseases, and were not taking any medications for the preceding four

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 (IRB) of the college of Medicine, King Saud University (Protocol number E-11-555). All patients and control subjects in this study signed an informed consent approved by the IRB. 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 asthmatic and control subjects, as described elsewhere (4). Slides were incubated overnight at 4°C with either diluted goat anti-human CD20 (R&D Systems) 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 min at room temperature. After washing in PBS, the complex Streptavidin/Horse Radish Peroxidase (Vector) was applied for 30 min at room temperature. The reaction result was visualized with DAB/hydrogen peroxide (DAB Kit, Dako). 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 sub-epithelial area were counted at a magnification of 400x; the area analyzed was measured using the software Image Pro 6.2 (MediaCybernetics, Bethesda, USA). The final result

was expressed as number of positive cells/mm².

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 (20 ml) was drawn from each subject. Blood was layered over Ficoll gradient (Axis shield, Norway) 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 >98% as evaluated by fluorescence-activated cell sorting (FACS) analysis and their viability was >99% as evaluated by trypan blue dye exclusion.

Cell culture

Human primary lung fibroblasts isolated from 5 healthy subjects and primary airway smooth muscle (ASM) cells isolated from 3 asthmatic subjects were cultured in DMEM/F12 (1:1) supplemented with 10% FCS and antibiotics. Primary human bronchial epithelial cells from 2 healthy control subjects and 2 asthmatic patients (Lonza, Walkersville, MD) were maintained in Bronchial Epithelial Cell Growth Medium (BEGM, BulletKit, Lonza) according to the supplier's recommendations. Human lung microvascular endothelial cells (HMVEC-L) (Lonza, Walkersville, MD) were cultured in microvascular endothelial cell growth medium-2 (EGM2-MV BulletKit; Lonza). All experiments were performed using second- or third-passage cells in 75–85% 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 (i.e. IL-17A, IL-17F, or both) (50ng/ml) for 12 hours prior to cell harvest. Cells (5×10^5 cells) were then harvested and total RNA was extracted (RNeasy Mini kit, Qiagen). mRNA expression levels of CXCL13, CXCR5, VLA-4, and LFA-1 were then determined using semi-quantitative RT-PCR (7900 Fast system from Applied Biosystems). Gene expressions were normalized with GAPDH and relative expressions were determined by the delta-delta Ct method (2).

Flow cytometry assay

Human primary lung fibroblasts and HMVEC-L cells (5×10^5 cells) were stimulated with IL-17A and IL-17F (50ng/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 (5×10^5 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). Gates were set based on non-stained cells (using Fluorescein Minus One (FMOs) 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 E2B).

Chemotaxis assays

B cell migration assays were performed as previously described (3) using a 48-well micro Chemotaxis Chamber (Neuro Probe). B cells isolated from severe asthmatic patients 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 0.01N HCl and placed between two chambers. B cells were added to the upper chamber, and supernatants from non-stimulated cells or IL-17 stimulated (50ng/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), according to the manufacturer's instructions. The number of cells was counted in 5 random fields at a magnification of 3200. Since B cells are non-adherent cells, migrating cells were also counted in the lower chamber and total number of cells both at the membrane and in media was obtained.

To determine whether the increased migration of B cells towards 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) 1 hour prior to the start of the migration assay. Results were compared with the corresponding isotype controls.

CXCL13 ELISA assay

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

Inhibition of IL-17 induced CXCL13 mediated B cell migration

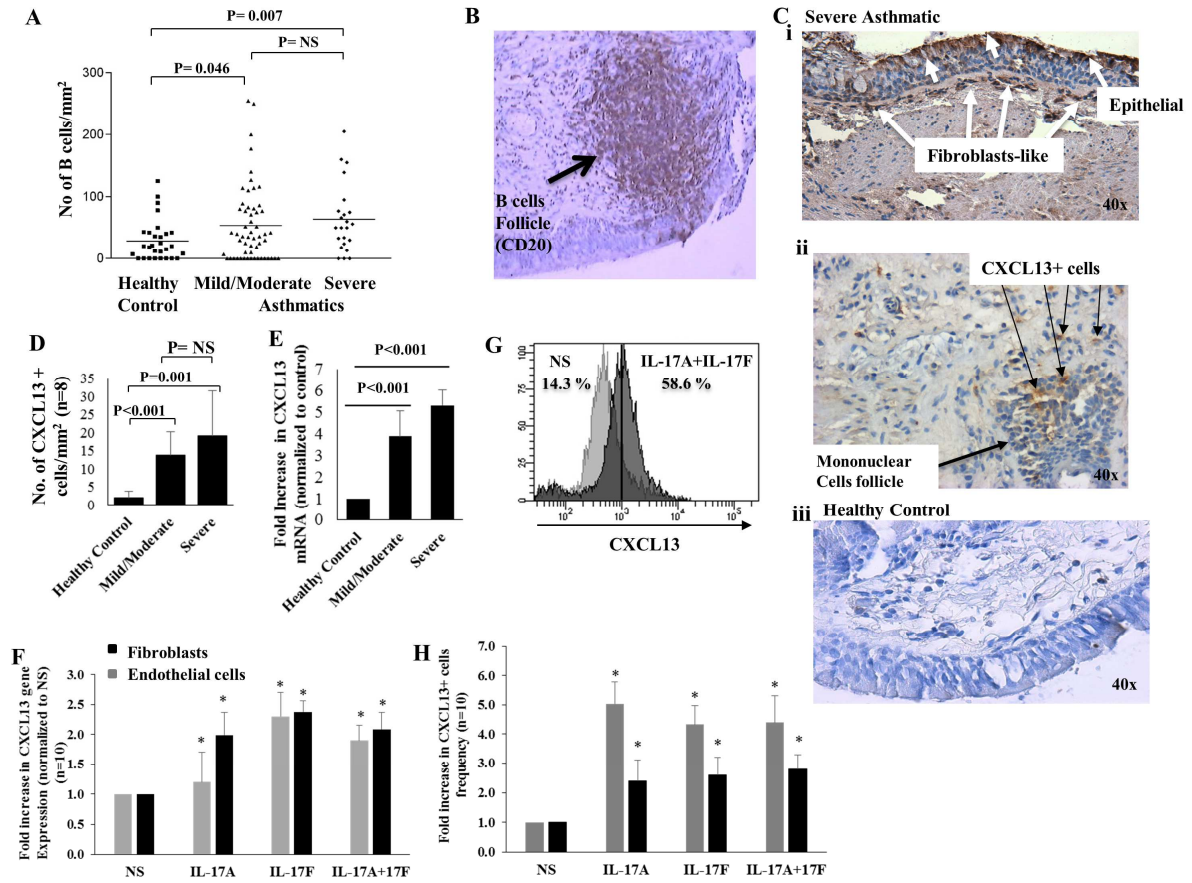
Pharmacologic inhibitors directed against various pathways potentially activated by IL-17 cytokines were used to determine the pathway required for CXCL13 production by lung structural cells. Human primary lung fibroblasts and HMVEC-L cells were incubated with various inhibitors (p38 MAPK inhibitor SB203580 (0.1 mmol/L; Axon Medchem), the extracellular signal-regulated kinase (ERK) 1/2 MAPK inhibitor PD184352 (2 mmol/L; United States Biological, Inc), the NF- κ 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 prior to IL-17 stimulation. A dose response experiment for each inhibitor has been performed to determine the concentration 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 (3). Cells were then stimulated with IL-17A and IL-17F (50ng/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 (SE). 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 <0.05 was considered statistically significant. Statistical analyses were performed with GraphPad InStat 3 software (GraphPad Software).

1. Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. Am J Respir Crit Care Med. 2000;162(6):2341-51.
2. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
3. Chang Y, Al-Alwan L, Risse PA, Roussel L, Rousseau S, Halayko AJ, et al. TH17 cytokines induce human airway smooth muscle cell migration. J Allergy Clin Immunol. 2011;127(4):1046-53 e1-2.
4. Laberge S, Pinsonneault S, Ernst P, Olivenstein R, Ghaffar O, Center DM, et al. Phenotype of IL-16-producing cells in bronchial mucosa: evidence for the human eosinophil and mast cell as cellular sources of IL-16 in asthma. Int Arch Allergy Immunol. 1999;119(2):120-5.



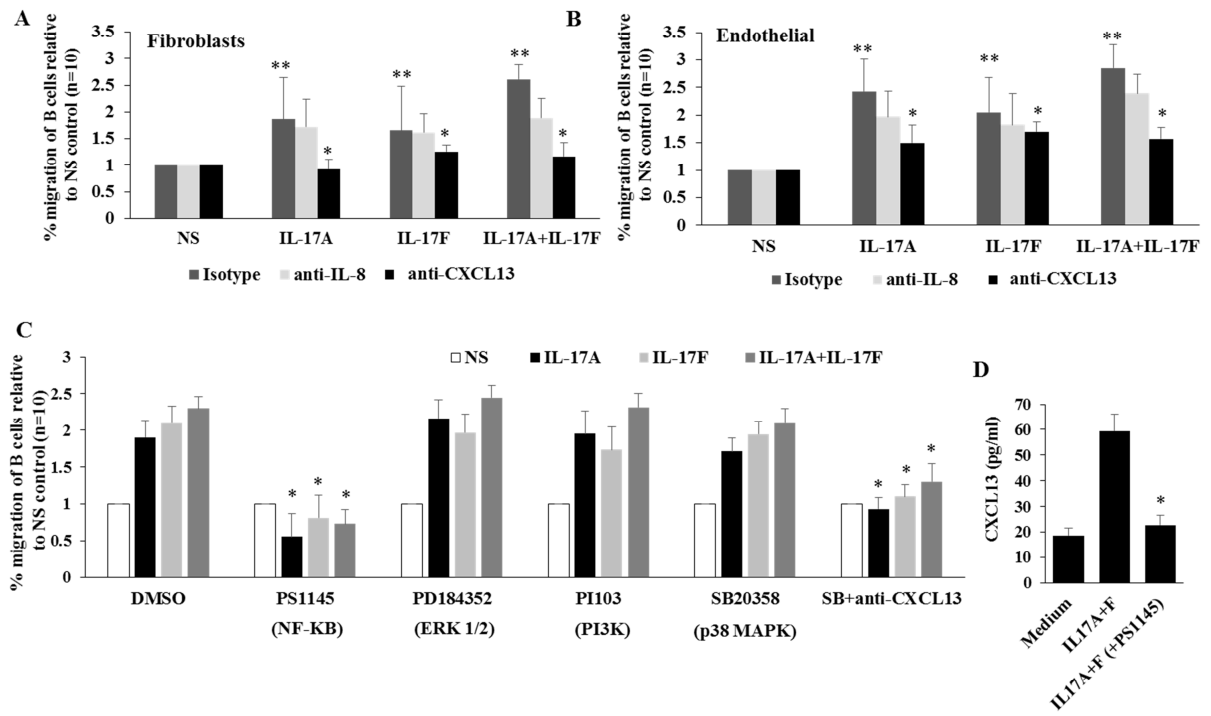
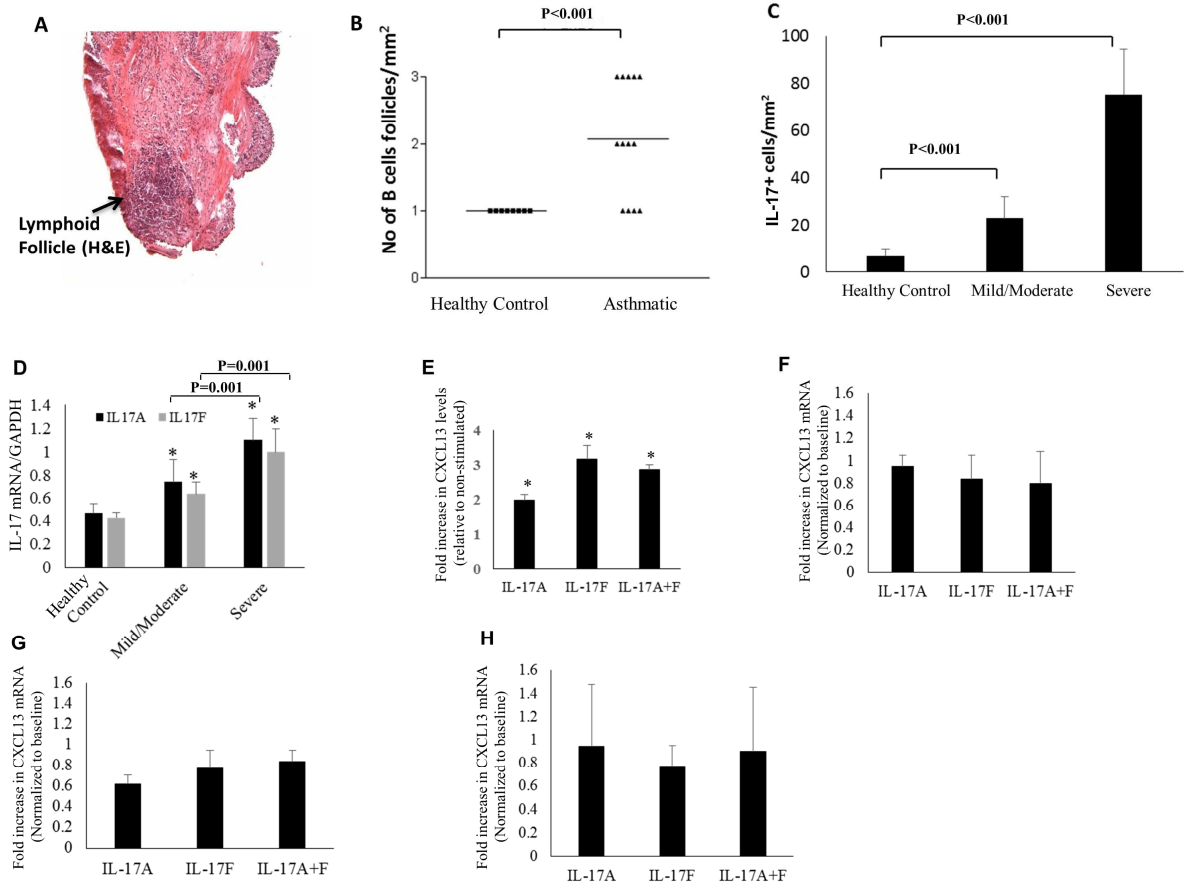


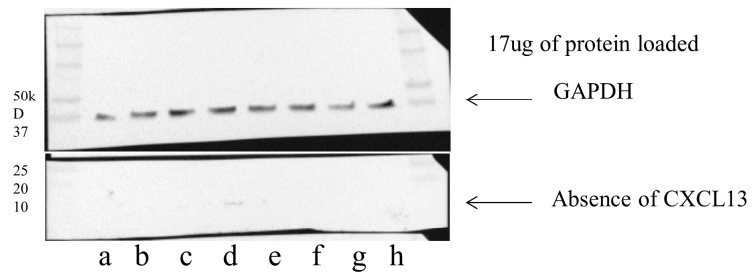
Table E1

	number	Age (years)	Sex (M/F)	Atopy (yes/No)	FEV1 (mean $\pm$ SD)
Normal Controls	29	33.4 $\pm$ 3.7	18/11	4/25	110.7 $\pm$ 5.3
Mild /Moderate	56	33.8 $\pm$ 2.7	35/21	44/12	81.9 $\pm$ 3.0
Severe Asthmatic	23	37.3 $\pm$ 4.2	15/8	20/3	57.65 $\pm$ 3.4



I

ASTHMATIC PATIENT



a: IL17A + F
b: IL17F
c: IL17A
d: media

e: IL17A + F
f: IL17F
g: IL17A
h: media

