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IL-17 Promotes p38 MAPK-Dependent Endothelial Activation Enhancing Neutrophil Recruitment to Sites of Inflammation

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Neutrophilic inflammation plays an important role in lung tissue destruction occurring in many chronic pulmonary diseases. Neutrophils can be recruited to sites of inflammation via the action of the cytokine IL-17. In this study, we report that IL-17RA and IL-17RC mRNA expression is significantly increased in asthmatic bronchoscopic biopsies and that these receptors are not only expressed on epithelial and inflammatory cells but also on endothelial cells. IL-17 potently stimulates lung microvascular endothelial cells to produce chemoattractants (CXCL8 and derivatives of the 5-lipoxygenase pathway) that selectively drive neutrophil but not lymphocyte chemotaxis. Moreover, IL-17 promotes endothelial activation by inducing the expression of endothelial adhesion markers (E-selectin, VCAM-1, and ICAM-1) in a p38 MAPK-dependent manner. This increased expression of adhesion molecules stimulates the *trans*-endothelial migration of neutrophils, as well as the transmigration of HT-29 colon carcinoma cells, suggesting a further role in promoting lung metastasis. Finally, IL-17 increased neutrophil adhesion to the endothelium *in vivo* as determined by intravital microscopy of mice cremaster muscle. Overall, our results demonstrate that IL-17 is a potent activator of the endothelium *in vivo* leading to neutrophil infiltration. Therefore, preventing neutrophil recruitment by blocking the action of IL-17 on endothelial cells may prove to be highly beneficial in diseases in which neutrophilic inflammation plays a key role. *The Journal of Immunology*, 2010, 184: 4531–4537.

Neutrophilic inflammation of the airways is an important feature in respiratory diseases, including acute respiratory distress syndrome (1), chronic bronchitis (2), chronic obstructive pulmonary disease (2), and cystic fibrosis (CF) (3). Moreover, neutrophilic inflammation has also been reported in patients suffering from severe asthma (4, 5). Furthermore, diseases associated with neutrophilic inflammation are generally found to be poorly responsive to corticosteroids, a common and effective anti-inflammatory (6). Therefore, neutrophilic inflammation may be determinant in chronic lung tissue destruction, and understanding the mechanisms underlying the recruitment of neutrophils to inflammatory sites may shed light on new ways to prevent chronic lung diseases.

Neutrophils can be attracted to the lungs from the circulation via the action of various chemoattractants including the metabolites of

the 5-lipoxygenase (5-LO) pathway, leukotriene B₄ (7) and 5-oxo-6,8, 11,14-eicosatetraenoic acid (8), as well as members of the Cys-Xxx-Cys chemokine family, such as CXCL8 (also known as IL-8) (9). These mediators can be synthesized via the activation of both inflammatory and structural cells of the airways in response to various environmental cues. Dissecting the relationship between these environmental cues and the production of neutrophil chemoattractants will be key to prevent their recruitment in chronic diseases.

Interestingly, it has been recently proposed that an important cue for neutrophil recruitment may be the synthesis of IL-17, also known as IL-17A, a cytokine suggested to play an active role in numerous autoimmune disorders (10). IL-17, CXCL8, and neutrophils have all been found to be elevated in the airways of patients with severe asthma and CF (11, 12). The IL-17 family has six members, including IL-17A and IL-17F homodimers and heterodimers, considered to be the most potent forms recruiting neutrophils in mice (IL-17A/IL-17A > IL-17A/IL-17F >> IL-17F/IL-17F) (13). IL-17A is synthesized by a Th17 lymphocyte subtype, as well as other inflammatory cells including monocytes-macrophages (14). IL-17A and IL-17F bind to the IL-17RA and IL-17RC on target cells (12), which recruit the adaptor protein connection to I κ B kinase and stress-activated protein kinase. Connection to I κ B kinase and stress-activated protein kinase is a U-box E3 ubiquitin ligase that mediates lysine-63 ubiquitination of TNF-R-associated factor 6 (15), which in turn will recruit the protein kinase TGF- β activated kinase that serves as the template for the activation of the transcription factors NF- κ B and CEBP β , as well as the MAPK pathways ERK1/ERK2 and p38 MAPK (16). Human bronchial epithelial cells stimulated with IL-17A and IL-17F show increased secretion of CXCL8 dependent on the MAPK pathways (12). Moreover, airway smooth muscle cells and

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Abbreviations used in this paper: 5-LO, 5-lipoxygenase; CF, cystic fibrosis; FLAP, 5-lipoxygenase activating protein; LMVEC, lung microvascular endothelial cell; QRT-PCR, quantitative real-time PCR.

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fibroblasts can also be a source of CXCL8 in the airways in response to IL-17 via a similar mechanism (17).

In this study, we have investigated further the mechanisms of action of IL-17A (hereafter referred to as IL-17) in recruiting neutrophils to the airways in chronic airway diseases.

Materials and Methods

Materials

SB203580 (p38 MAPK inhibitor) was obtained from Invivogen (San Diego, CA), and MK886 (5-LO inhibitor) was from Cayman Chemical (Ann Arbor, MI). BIRB07896 (p38 MAPK inhibitor) was kindly provided by Professor Sir Philip Cohen (Medical Research Council Protein Phosphorylation Unit, University of Dundee, Dundee, U.K.). Recombinant human IL-17A was purchased from BioVision (#4176-25; Mountain View, CA), TNF- α from Alexis Biochemicals (ALX-520-002; Philadelphia, PA), and IL-4 and mouse IL-17A from R&D Systems (Minneapolis, MN).

RNA extraction from human airway biopsies

Adult endobronchial tissue specimens were obtained from the Fonds de recherche en santé du Québec Respiratory Health Network Tissue Bank (Montreal Chest Institute/Meakins-Christie Laboratories Tissue Bank, McGill University, Montreal, Quebec, Canada). Written consent was obtained from all subjects, and the use of tissues was approved by the hospital ethics committee. Clinical diagnosis of asthma was made by a respiratory physician for all patients based on the American Thoracic Workshop for Refractory Asthma (18): mild ($n = 15$); severe asthma; ($n = 15$), and nonasthmatic healthy controls ($n = 12$). Tissue RNA was extracted using the RNeasy micro kit (Qiagen, Ontario, Canada).

Immunohistochemistry

Five-micrometer-thick sections from nasal polyps and CF alveoli biopsies were prepared from adult subjects undergoing septoplasty (nasal polyps) or lung transplantation (CF). All patients gave written informed consent as approved by the hospital ethics committee. Slides were deparaffinated in xylene and rehydrated in decreasing concentrations of alcohol. For staining, heat-induced epitope retrieval was performed using EDTA buffer (pH 8). Sections were then permeabilized with 0.2% Triton X-100, and incubated with 5% hydrogen peroxide. After blocking with universal blocking solution for 30 min (DakoCytomation, Mississauga, Ontario, Canada), slides were incubated overnight with 10 μ g/ml polyclonal IL-17RA Ab (#AF177; R&D Systems) or 10 μ g/ml polyclonal IL-17RC Ab (#69673; Abcam, Cambridge, MA). Isotype controls were prepared by replacing the primary Ab with a nonspecific goat Ig at the same concentration. After rinsing, the slides were incubated with a biotinylated secondary Abs (1:100), followed by an HRP/AB Complex (both from DakoCytomation). Immunoreactivity was developed with diaminobenzidine chromogen (DakoCytomation), and slides were counterstained with hematoxylin and lithium carbonate.

Cell culture

Lung microvascular endothelial cells (LMVECs) were obtained from Cambrex (East Rutherford, NJ) as cryo-preserved primary cultures. The cells were cultured in endothelial basal medium-2 with growth factors (EGM-2-MV BulletKit, Cambrex). LMVECs were cultured from passages 2–6. HUVECs were isolated from freshly derived umbilical cords as described previously (19). Cells were grown in 199 medium, supplemented with 20% heat-inactivated FBS, 60 μ g/ml endothelial cell growth supplement, 2 mM L-glutamine, and 50 μ g/ml heparin. HUVECs were cultured from passages 2–7. Following stimulation of LMVECs or HUVECs, culture supernatants were collected, spun down at 13,000 \times g, and kept for further assays. Neutrophils were freshly prepared from human blood as described previously (8). CD4⁺ cells were prepared from freshly harvested blood by negative selection using CD4⁺ T cell isolation kit 2 (Miltenyi Biotec, Auburn, CA). Cell purity was found to be >84%.

Quantitative real-time PCR

RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), treated with DNase I Amp Grade (Invitrogen), and reverse-transcribed using Superscript II Reverse Transcriptase (Invitrogen). Quantitative real-time PCR (QRT-PCR) was performed in 96-well plate format using SYBR Green-based detection on a Step-One-Plus machine (Applied Biosystems, Foster City, CA) with each 20 μ l reaction containing \sim 50 ng cDNA, 0.3 μ M sense and antisense primers (see Supplemental Table I for sequences), and 1 \times Quantitect SYBR Green supermix (Qiagen). The plate was sealed and cycled under the following conditions: 95°C for 10 min, 50 cycles of 95°C

for 10 s, and 60°C for 45 s. Each reaction was performed in duplicate, mRNA levels of GAPDH were used for normalization, and fold induction was determined from cycle threshold values using the Pfaffl method (20). PCR efficiency was determined from the slope of a standard curve generated using 5-fold dilution series of the DNA template.

ELISA

Human CXCL8/IL-8 (DY208) DuoSet ELISA kits were purchased from R&D Systems. A total of 100 μ l supernatant collected after cell stimulation was directly used for IL-8 quantification.

Neutrophil chemotaxis assay

Neutrophils, CD4⁺ cells, and Jurkat cell migration assays were performed using a modified Boyden chamber (Transwell, Fisher Scientific, Ottawa, Ontario, Canada) with 5- μ m pores. The conditioned media of treated LMVECs was added to the lower chamber, whereas the upper chamber was filled with untreated LMVEC media. To determine the role of CXCL8, supernatants were first incubated for 1 h at room temperature with Ab against CXCL8 (10 μ g/ml, R&D Systems) or with normal goat IgG (R&D Systems) before adding supernatant to the lower chamber. Neutrophils, CD4⁺ cells, and Jurkat cells (0.5×10^6) suspended in DMEM were put into the upper well, and the chamber was placed in a humidified incubator under 5% CO₂ for 4 h at 37°C. Cells located on the upper surface of the membrane were scraped with a cotton swab, and the cells that had crossed the polycarbonate filter were collected from the lower compartment and counted with a hemacytometer.

Transendothelial cell migration assay

HUVECs were grown to confluence for 72 h on 5.0- μ m pore size gelatinized polycarbonate membranes of modified Boyden chambers (Transwell, Fisher Scientific). Neutrophils were left in suspension for 1 h before being labeled for 30 min with Calcein AM (0.5 μ M) and washed with M199 migration buffer (10 mM HEPES [pH 7.4], 1 mM MgCl₂, and 0.5% BSA). Thereafter, HUVECs were treated as indicated and then washed with M199 migration buffer. The culture media was changed for fresh M199 migration buffer. Neutrophils or HT-29 (150,000) were added for 5 h in the upper chamber. Then, cells on the upper face of the membrane were scraped using a cotton swab, and the number of Calcein-stained neutrophils or HT-29 that had migrated to the lower face of the filter was counted with a fluorescence microscope.

Flow cytometry

Following treatments, HUVECs were detached by incubation for 10 min at 37°C in 10 mM EDTA in sterile PBS and then washed with PBS. Mouse IgG1 anti-human VCAM-1, clone 1G11B1 (Abcam), was added at a concentration of 1 μ g Ab per 5×10^5 cells. After washing with staining buffer, the cells were incubated with rat anti-mouse IgG1-FITC (BD Biosciences, San Jose, CA) and immediately analyzed with an FACSCalibur flow cytometer system using the CellQuest Software (BD Biosciences). Background staining was determined by staining the cells with the secondary Ab alone or preceded by an isotype control (normal mouse IgG1, Santa Cruz Biotechnology, Santa Cruz, CA). Differential VCAM-1 surface expression on the cells was evaluated by comparing the mean fluorescence intensity.

Intravital microscopy

Six-week-old CD1 male (Charles River Laboratories, St. Constant, Quebec, Canada) of \sim 25 g were maintained in the Royal Victoria Hospital Animal Facility, and all experiments were conducted in accordance with the McGill University Animal Care Committee guidelines. The method was performed as previously described (21). Two hours after intrascrotal injection of cytokines, the mice were anesthetized with a mixture of 200 mg/kg ketamine (Wyeth-Ayerst, Guelph, Ontario, Canada) and 10 mg/kg xylazine (Bayer, Etobicoke, Ontario, Canada) injected i.p. and maintained throughout experiments with additional doses administered i.v. via a jugular vein cannula. Cremaster muscle, prepared on a Plexiglas microscope stage with a five-point fixation technique as described (21), was viewed with an inverted microscope (Nikon TE300, Nikon, Montreal, Quebec, Canada) equipped with a \times 40 objective. The image was captured with a video camera (Panasonic Digital KR222, Panasonic, Toronto, Ontario, Canada) and recorded on an iMac G5 (Apple Computer, Cupertino, CA) equipped with video acquisition software. Postcapillary venules of 2.0–6.0 mm/s and a diameter of 25–40 μ m were chosen for online video recording of 10 min. Recorded vessels were analyzed offline by an investigator blinded to the intervention and were assessed for neutrophil adherence defined as the number of leukocytes remaining stationary for 30 s over a 100- μ m length of endothelium for a 5-min time period.

Statistical analysis

ANOVA followed by a multiple comparison test (Bonferroni) was used to test differences in mean between groups; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

IL-17RA and IL-17RC expression is increased in asthma

Asthma affects ~8% of the adult population and 20% of children worldwide (22). We first sought to determine the expression of the IL-17A receptors in bronchial tissue of subjects with asthma, as it was reported previously that Th17 cells and IL-17 are increased in the airways of patients with asthma (11, 23). Accordingly, we found a significant increase of IL-17RA and IL-17RC mRNA by QRT-PCR in bronchoscopic biopsy tissues of patients with asthma when compared with nonasthmatic individuals (Fig. 1A, 1C). IL-17RA expression was greater in mild compared with severe asthmatics (Fig. 1B). Clinical data for the three groups illustrated can be found in Supplemental Table II.

IL-17RA is expressed at the surface of lung endothelial cells

To identify which cells express the IL-17RA receptor in the airways, we performed immunohistochemistry on human nasal polyp biopsies, where neutrophilic airway inflammation is occurring (2). As expected IL-17RA expression was detected on airway epithelial cells as well as inflammatory cells in the nasal polyps (Fig. 1D). Interestingly, we also found a strong labeling of the endothelium in the nasal polyps (Fig. 1E), suggesting that these cells may also respond to IL-17 as they were shown to express IL-17RC previously (24). A similar staining pattern for IL-17RA was found in alveolar tissue from a patient with CF (Fig. 1F). Immunohistochemistry staining for IL-17RC confirms the coexpression with IL-17RA in endothelial cells of CF alveoli (Fig. 1G). These findings suggest that endothelial cells respond to IL-17 and led us to hypothesize that IL-17 may act through the endothelium to promote neutrophil recruitment.

IL-17 increases CXCL8 production by LMVECs via the p38 MAPK pathway

CXCL8 is a potent neutrophil chemoattractant present in the inflamed lungs of patients with asthma, chronic obstructive pulmonary disease, and CF. Therefore, we checked whether endothelial cells synthesize CXCL8 upon IL-17 stimulation similar to what was seen with other structural cells of the airways (25). In primary human LMVECs, IL-17 stimulation led to an increase in CXCL8 mRNA (2-fold) and protein (5-fold) as determined by QRT-PCR and ELISA, respectively (Fig. 2A, 2B). Moreover, when the cells were incubated with two structurally unrelated p38 MAPK inhibitors prestimulation with IL-17, namely SB203580 and BIRB0796 (26, 27), this upregulation was essentially abolished (Fig. 2A, 2B). It is worth noting that the induction and inhibition are more pronounced at the protein rather than mRNA level, supporting the notion that the regulation of CXCL8 mainly occurs at the posttranscriptional level, as previously reported in other cell types (28). These results show that lung endothelial cells contribute to the secretion of the neutrophil chemoattractant CXCL8 in a p38 MAPK-dependent manner.

IL-17 promotes selective migration of neutrophils

We next tested whether IL-17–treated LMVEC culture media could promote neutrophil migration. When fresh human neutrophils isolated from peripheral blood were exposed to IL-17–treated LMVEC conditioned medium, their migration was significantly upregulated (7-fold; Fig. 2C), supporting the notion that IL-17 promotes neutrophil recruitment. We then checked whether this was also true for T lymphocytes using the human immortalized Jurkat cells and CD4⁺ cells freshly isolated from human blood. In striking contrast to neutrophils, Jurkat and CD4⁺ cells were completely unresponsive to IL-17–treated LMVEC conditioned medium, whereas they were apt to migrate in response to serum (Fig. 2D, 2E). These results suggest that IL-17 selectively promotes the migration of neutrophils.

As shown in the previous section, IL-17 increases CXCL8 production, a potent neutrophil chemoattractant. We then went on

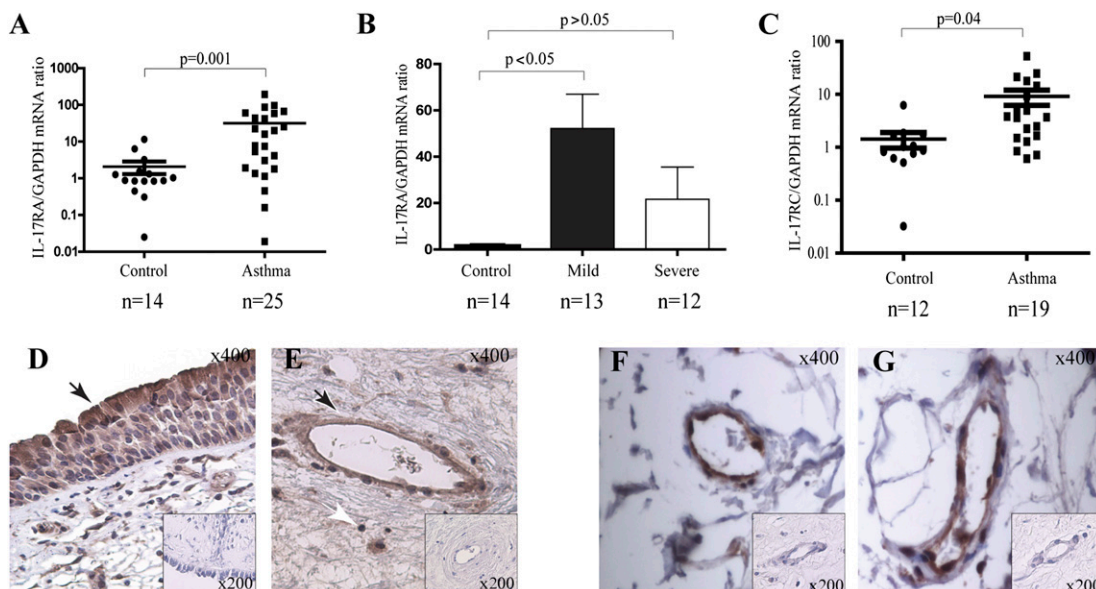
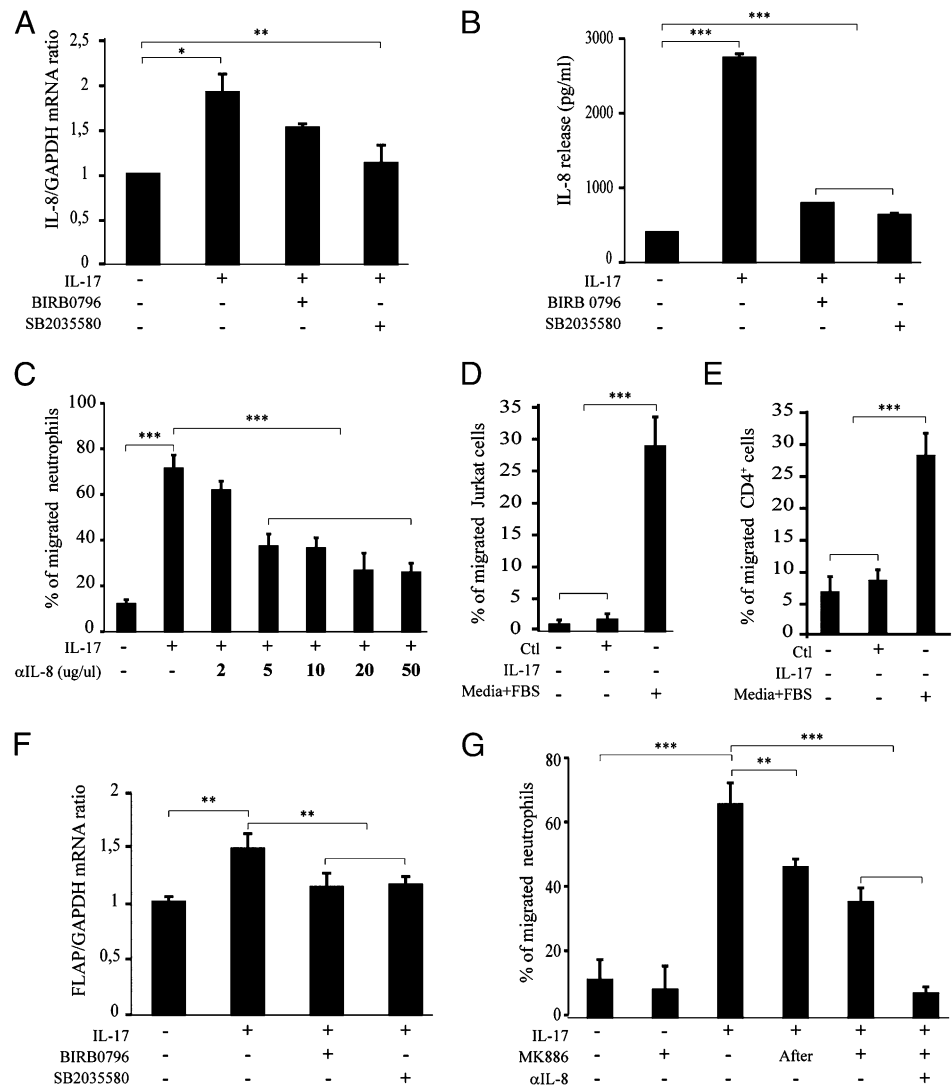


FIGURE 1. IL-17RA and IL-17RC in bronchial asthma: evidence for expression by endothelial cells of the airways. A–C, IL-17RA and IL-17RC transcript levels in tissues obtained from a cohort of subjects with asthma and healthy controls were analyzed by QRT-PCR. The data are presented for all subjects with diagnosed asthma compared with control subjects (A, C), or in subjects with mild and severe asthma compared with controls (B). D–G, The localization of IL-17RA (D–F) and IL-17RC (G) was determined in nasal polyps (D, E) or CF alveolar (F, G) tissue sections by immunohistochemistry (see *Materials and Methods*). Representative staining of IL-17RA in epithelial cells (D, black arrows), inflammatory cells (E, white arrows), and endothelial cell (E, black arrows) is shown from two independent experiments. Representative staining of IL-17RC in endothelial cells (G) is shown from two independent experiments. Isotype controls (normal goat serum for the IL-17RA and normal mouse serum for IL-17RC) are illustrated as insets (D–G). All magnifications are $\times 400$ except insets, which are $\times 200$.

FIGURE 2. IL-17 promotes selective neutrophil migration mainly through CXCL8. *A* and *B*, LMVECs were preincubated for 1 h with vehicle (DMSO) (–), 0.1 μ M BIRB0796, or 5 μ M SB2035580 as indicated. The cells were then left untreated (–) or exposed to 20 ng/ml IL-17 for 2 h. Post-stimulation, the media was collected and total RNA extracted. The amount of CXCL8 mRNA (*A*) and protein in the media (*B*) were determined by QRT-PCR and ELISA, respectively. Neutrophil (*C*), Jurkat (*D*), and CD4⁺ (*E*) cell chemotaxis was assayed in the absence (–) or presence of IL-17–treated (20 ng/ml, 4 h) LMVEC-conditioned media (+) or media containing 20% of FBS. The contribution of CXCL8 was determined by the addition of a neutralizing Ab at the indicated concentrations (*C*). *F*, LMVECs were treated as in *A* and FLAP mRNA levels determined as above. *G*, LMVECs were preincubated with the 5-LO inhibitor MK886 (7 μ M) for 24 h, followed by the addition of the CXCL8 neutralizing Ab (α CXCL8). Alternatively, MK886 was added to the IL-17–treated LMVEC-conditioned media (MK886 After). All experiments were repeated three times except *B*, *E*, and *G*, which were repeated twice.



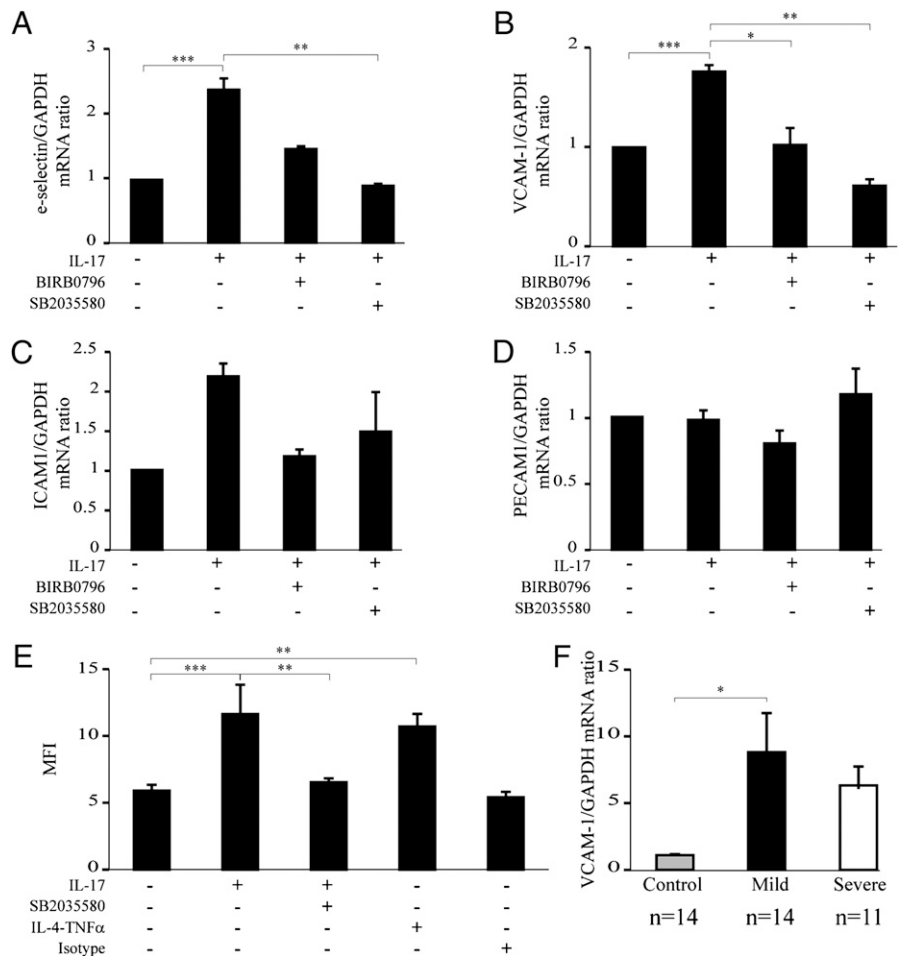
to determine if this increased migration of neutrophils was entirely dependent on CXCL8 using a neutralizing Ab. Our results show that CXCL8 plays a major role to stimulate neutrophil migration in IL-17–conditioned LMVEC medium, as >50% of the neutrophil migration was inhibited in presence of saturating doses of the CXCL8–neutralizing Ab (Fig. 2C).

This observation prompted us to look at other neutrophil chemoattractants, in particular those for which synthesis is dependent on 5-LO. IL-17 increased the expression of 5-LO activating protein (FLAP) at the mRNA levels in a p38 MAPK-dependent fashion in LMVEC (Fig. 2F). Similar results were obtained when LMVECs were treated with TNF- α (Supplemental Fig. 1). Moreover, an inhibitor of 5-LO, MK886 (29), attenuated the IL-17–conditioned LMVEC medium-driven neutrophil migration when added to LMVECs prior to their stimulation with IL-17 (Fig. 2G). However, MK886 also affected neutrophil chemotaxis directly, as shown when MK886 was added to IL-17 conditioned medium poststimulation, which decreased neutrophils migration, albeit to a lesser extent than when it is added pretreatment (Fig. 2G). A combination of MK886 and the CXCL8 neutralizing Ab completely abrogated neutrophil chemotaxis induced by IL-17–conditioned LMVEC medium (Fig. 2G). These results suggest that IL-17 enhances the synthesis of 5-LO–derived neutrophil chemoattractants, further supporting its key function in promoting neutrophil chemotaxis.

IL-17 promotes endothelial activation

For neutrophils to be recruited to sites of inflammation, they must cross the endothelium. The neutrophil–endothelium interaction is crucial for both physiological and pathological roles of neutrophil-mediated inflammation. The molecular interactions required for neutrophils to cross the endothelium have been extensively studied (reviewed in Ref. 30). Very briefly, the first step in the capture of neutrophils involves selectin molecules, which enable neutrophil rolling on endothelial cells and permit firmer adhesion via integrins, followed by diapedesis and transvasation across the endothelium. This process is regulated via the expression of adhesion molecules on the endothelium in response to specific environmental stimuli, which is termed endothelial activation. We wanted to check whether IL-17 could lead to activation of the endothelium via expression of various adhesion molecules necessary for neutrophil transvasation. We found that IL-17 stimulation of LMVECs could increase the mRNA expression of E-selectin (Fig. 3A), VCAM-1 (Fig. 3B), and ICAM-1 (Fig. 3C) in a p38 MAPK-dependent manner, as both SB203580 and BIRB0796 were able to reverse this effect (Fig. 3A–C). However, we did not see any change in expression of PECAM-1 (Fig. 3D). The IL-17–driven VCAM-1 and E-selectin gene expression was similar to the increase observed when LMVECs were stimulated with TNF- α (Supplemental Fig. 1). Similar results were also obtained in IL-17

FIGURE 3. IL-17 induces p38 MAPK-dependent expression of adhesion molecules by endothelial cells. *A–D*, LMVECs were preincubated for 1 h with vehicle (DMSO) (–), 0.1 μ M BIRB0796, or 5 μ M SB2035580 as indicated. The cells were then left untreated (–) or exposed to 20 ng/ml IL-17 for 2 h. Poststimulation, transcript levels of E-selectin (*A*), VCAM-1 (*B*), ICAM-1 (*C*), and PECAM-1 (*D*) were determined by QRT-PCR. *E*, Flow cytometric analysis of VCAM-1 surface expression on untreated and IL-17 (20 ng/ml) or IL-4 (1 ng/ml) plus TNF- α -treated (10 ng/ml) HUVECs for 12 h. The HUVEC population (86%) was gated, and VCAM-1 expression was quantified as the mean fluorescence intensity values and illustrated as bar graphs. Refer to Supplemental Fig. 3 for more details. *F*, VCAM-1 transcript levels are presented for subjects with mild ($n = 14$) and severe ($n = 11$) asthma compared with controls ($n = 14$) as in Fig. 1*A*. All experiments were repeated at least three times.



stimulated HUVECs (Supplemental Fig. 2). We also verified that VCAM-1 cell surface expression was indeed increased in a p38 MAPK-dependent manner on IL-17-stimulated LMVECs by flow cytometry analysis, which was comparable to levels induced by the combination of IL-4 and TNF- α (Fig. 3*E*, Supplemental Fig. 3) (31). E-selectin is important for rolling, whereas VCAM-1 and ICAM-1 are key adhesion molecules involved in leukocyte recruitment (30). PECAM-1 is a molecule associated with the later stages of transvasation (30). Interestingly, we found a significant increase of VCAM-1 mRNA by QRT-PCR in bronchoscopies of patients with asthma when compared with nonasthmatic individuals (Fig. 3*F*). A number of other genes (S100A9, IL-1R1, IRF2, COL1A2, CASP1) found to be upregulated by IL-17 in a p38 MAPK-dependent manner are shown in Supplemental Fig. 4. Taken together, these results suggest that IL-17 promotes endothelial activation, which may increase neutrophil recruitment in the airways.

IL-17 promotes neutrophil trans-endothelial migration

To test this latest hypothesis, we used an endothelial transmigration assay (19). We found that IL-17 increased the transmigration of neutrophils through the endothelium and that this could be reversed by a p38 MAPK inhibitor (Fig. 4*A*). We further found that IL-17-mediated endothelial activation was as strong as that of TNF- α and the β form of pro-IL-1, two very potent proinflammatory cytokines (Fig. 4*A*, 4*B*). IL-17 stimulation of endothelial cells also promoted the transmigration of HT-29 colon carcinoma cells (Fig. 4*B*). Interestingly, like neutrophils, HT-29 migration was partially dependent on CXCL8 (Fig. 4*C*). However, in contrast to neutrophils, the 5-LO pathway does not play a role in their migration (Fig. 4*D*).

Therefore, other chemotactic molecules must be involved. These findings suggest that IL-17 is a key cytokine regulating the infiltration of neutrophils into tissues and that it may also contribute to lung metastasis of colon cancer cells.

IL-17 promotes neutrophil adhesion to the endothelium of the mice cremaster muscle

Finally, it was important to determine whether IL-17 promoted neutrophil adhesion to the endothelium *in vivo*. We therefore checked if IL-17 increased neutrophil adhesion in the mouse cremaster muscle by intravital microscopy (32). Intrascrotal TNF- α has been previously shown to increase leukocyte-endothelial interactions within the cremaster muscle (21, 32, 33). In this assay, murine IL-17 (4.5-fold) was even more potent than TNF- α (3-fold) in promoting adhesion of neutrophils to the endothelial wall of the cremaster muscle (Fig. 5; Supplemental Videos).

Discussion

Overall, our results demonstrate that IL-17 is a potent activator of the endothelium *in vivo*, leading to neutrophil recruitment at the site of local inflammation. The rise of IL-17 as a key cytokine in a number of autoimmune diseases has prompted great interest in a better understanding of its functions. One of its main roles appears to be the recruitment of neutrophils (10). Our results demonstrate that endothelial cells are additional sites for the action of IL-17, as they express its receptors and respond potently to its stimulation by producing neutrophil chemoattractants and expressing adhesion molecules involved in leukocyte transvasation. This latter role may make endothelial cells a prime site of IL-17 function and an attractive therapeutic target.

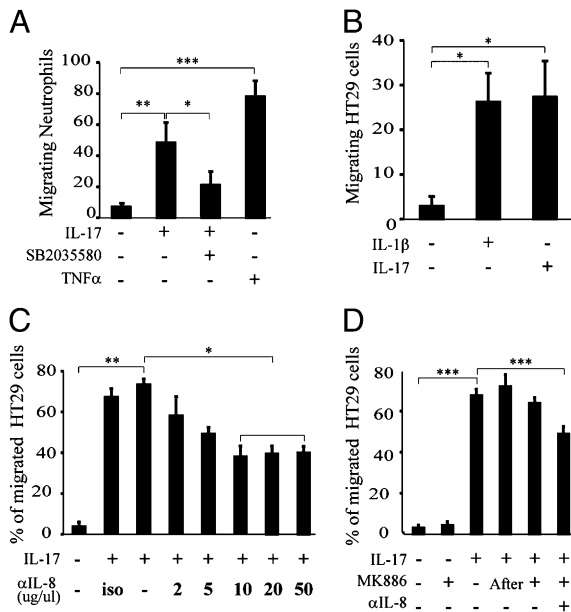


FIGURE 4. IL-17 promotes neutrophils and colon carcinoma cells migration across an endothelial cell monolayer in a p38 MAPK-dependent fashion. *A* and *B*, HUVECs grown to confluence were preincubated for 1 h with vehicle (DMSO) (–) or 5 μ M SB2035580. Cells were then exposed to 20 ng/ml IL-17 or 10 ng/ml TNF- α for 2 h or 20 ng/ml β form of pro-IL-1 for 4 h. Thereafter, culture media was changed for fresh media and cells incubated for an additional 2.5 h. Calcein-stained neutrophils were then added on the endothelial layer and left to migrate for 5 h at 37°C. Results show the number of neutrophils (viability >85%) that have crossed the activated endothelial layer. Data points represent the means from triplicates, and results are representative of two independent experiments. *B*, As in *A*, except that HT-29 tumor cells were used instead of neutrophils. *C* and *D*, HT-29 chemotaxis was assayed in the absence (–) or presence of IL-17-treated (20 ng/ml, 4 h) LMVEC-conditioned media (+) or media containing 20% of FBS. *C*, The contribution of CXCL8 was determined by the addition of a neutralizing Ab at the indicated concentrations or an isotype control (iso). *D*, LMVECs were preincubated with the 5-LO inhibitor MK886 (7 μ M) for 24 h, followed by the addition of the CXCL8 neutralizing Ab (α CXCL8). Alternatively, MK886 was added to the IL-17-treated LMVEC-conditioned media (MK886 After). Representative results from four (*A*, *B*) or two (*C*, *D*) distinct experiments are shown.

IL-17, CXCL8, and neutrophils have all been found to be elevated in the airways of patients with severe asthma and CF (11, 12). Increased responsiveness of tissues to IL-17 may play a key role in further promoting neutrophilic inflammation. In accordance, we found that IL-17RA and IL-17RC mRNA was increased in asth-

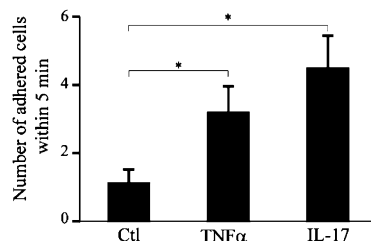


FIGURE 5. Neutrophil adhesion is potently increased by the intracranial injection of IL-17. Murine rTNF- α (0.5 μ g), rIL-17 (1.0 μ g), or vehicle (bicarbonate-buffered saline) was administered intracranially. Two hours postinjection, mice were prepared for intravital microscopy as described in *Materials and Methods*. Adherence was defined as the number of leukocytes newly adherent on a 100- μ m length of postcapillary venule over a 5-min period. Each group represents three mice.

matic subjects. The increased expression of IL-17RA was more pronounced in mild when compared with severe asthmatics, which could be explained by extensive tissue remodeling that occurs in severe asthma, whereas in bronchoscopies the proportional representation of various cell types may be skewed, favoring smooth muscle cells over other types in patients with severe asthma (34). We also found that the mRNA for the adhesion molecule VCAM-1 was increased in a similar fashion as that of IL-17RA in asthmatics. These results suggest that some of the observed tissue increase in IL-17RA may be due to increased blood vessel numbers in asthmatic airways. Increased angiogenesis and blood vessels in asthma have been reported, which is further supported by our data (35, 36). Interestingly, uncommon hyperactive VCAM-1 polymorphisms in the African American population have been associated with asthma (37). Therefore, endothelial coexpression of IL-17RA and IL-17RC may contribute significantly to the biological function of IL-17 in inflamed tissues.

It is well established that an important function of IL-17 is to stimulate the release of CXCL8, a potent neutrophil chemoattractant, by airway epithelial cells, fibroblasts, and smooth muscle cells (12, 17). Accordingly, endothelial cells also synthesize CXCL8 upon IL-17 challenge, which accounts for approximately half of the neutrophil chemotactic activity found in the conditioned media. Murine lung endothelial cells have also been shown to express both 5-LO and FLAP (38), two key enzymes involved in the synthesis pathway of alternate neutrophil chemoattractants (leukotriene B₄ and 5-oxo-6,8, 11,14-eicosatetraenoic acid). We found that blocking this pathway led to a reduction in the chemotactic potential of LMVEC-conditioned media toward neutrophils, but interestingly not HT-29 cells. This suggests that IL-17 drives the activation of this pathway in part through increase expression of FLAP (Fig. 2) and by the activation of 5-LO through its phosphorylation by the p38 MAPK-activated protein kinase MAPKAP-K2 (39). Interestingly, FLAP inhibitors have demonstrated benefits in allergic asthma clinical trials (29), whereas naturally occurring mutations in the promoter of region of 5-LO may be associated with asthma (40).

Endothelial cells sit at the frontier between circulating leukocytes and inflamed tissues and, as such, play an important role as a gateway for neutrophil infiltration in addition to their role in chemoattractant synthesis. Our study has revealed that IL-17 is a very potent activator of the endothelium, comparable to TNF- α , promoting expression of adhesion molecules (E-selectin, VCAM-1, and ICAM-1), leading to enhanced neutrophil transmigration both in cell culture and in vivo. Moreover, we have shown that these activities of IL-17 are dependent on the p38 MAPK pathway. As was suggested previously, p38 MAPK makes an attractive target in corticosteroid-unresponsive diseases (6), which is further supported by the data reported in this study. However, as we pointed out in a previous review (41), p38 MAPK itself may not be the ideal drug target, but either upstream activators or downstream effectors may represent more suitable candidates. Therefore, the results presented in this study prompt a closer investigation of interfering with IL-17RC-initiated cell signaling to prevent neutrophil infiltration and metastasis formation.

The contradictory roles of IL-17 in either promoting tumor growth (42) or preventing it (43) may be explained by its different functions on distinct cells, as suggested by our investigation. At the endothelium, IL-17 may promote metastasis through increased cancer cell adhesion, whereas its absence in draining lymph nodes leads to a reduction of immune cells (for example, NK cells) targeted at restraining tumors (43). Therefore, therapies aimed at IL-17 should take into consideration its various roles on distinct tissues to achieve the maximum therapeutic benefits.

Neutrophilic inflammation leads to tissue destruction in multiple chronic diseases. Therefore, there is great interest in strategies aimed at blocking its progression. As neutrophils must cross the endothelium to contribute to inflammation, preventing their recruitment by blocking the action of IL-17 on endothelial cells may prove to be highly beneficial for disorders dependent on IL-17 driven inflammation and resistant to the action of corticosteroids. These therapies could also be beneficial in reducing metastasis of cancer cells to the lung if their actions are restricted to the endothelium. In that regard, blocking IL-17 activation in the blood flow may be easier to achieve than delivering neutralizing reagents deep into tissues.

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Disclosures

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