

T_H17 cytokines induce human airway smooth muscle cell migration

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Background: Migration of airway smooth muscle cells (ASMCs) might contribute to increased airway smooth muscle mass in asthma. T_H17 cells and T_H17-associated cytokines are involved in the pathogenesis of asthma and might also contribute to airway remodeling.

Objective: We sought to explore the possibility that migration of ASMCs might contribute to airway remodeling through the action of T_H17-related cytokines.

Methods: The effect of exogenous T_H17 cytokines on *ex vivo* human ASMC migration was investigated by using a chemotaxis assay. The involvement of signaling pathways, including p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 1/2 MAPK, nuclear factor κ B, and phosphoinositide 3-kinase, was also examined.

Results: We demonstrated that IL-17A, IL-17F, and IL-22 promote migration in a dose-dependent manner. We further demonstrated that ASMCs express receptors for IL-17RA, IL-17RC, and IL-22R1. Using mAbs directed against these receptors, we confirmed that T_H17-associated cytokine-induced migration was dependent on selective receptor activation. Moreover, IL-17A and IL-17F exert their effects through signaling pathways that are distinct from those used by IL-22. The p38 MAPK inhibitor BIRB0796 inhibited the migration induced by IL-17A and IL-17F. PS1145, an inhibitor of nuclear factor κ B, abolished the IL-22-induced migration.

Conclusion: These data raise the possibility that T_H17-associated cytokines promote human ASMC migration *in vivo* and suggest an important new mechanism for the promotion of airway remodeling in asthma. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Asthma, airway remodeling, T_H17, airway smooth muscle cells, migration

Asthma is associated with characteristic changes in the structure of the airway wall, termed airway remodeling, a phenomenon that is increasingly prominent as disease severity increases.¹ Although associated with a variety of changes, the most important features of airway remodeling in asthma are increased airway smooth muscle (ASM) mass and decreased distance between ASM and epithelium.² These changes, which are present in both the large and small airways, are likely to contribute to increased bronchial responsiveness, a hallmark of asthma. However, remodeling might have implications beyond bronchoconstriction. There is increasing evidence that indicates that in addition to their contractile function, airway smooth muscle cells (ASMCs) might be involved in regulating the immune response through the production of inflammatory mediators, including T_H2 cytokines, chemokines, and growth factors.³ Moreover, ASMCs also express receptors for growth factors, activation of which is believed to contribute to airway wall remodeling by promoting hypertrophy and hyperplasia of ASM.⁴ However, the potential mechanisms by which ASMCs might contribute to increased ASM mass in asthmatic patients are not restricted to hypertrophy and hyperplasia. Recently, ASMCs have been demonstrated to have the capacity to move toward the epithelium in asthmatic patients,² suggesting that migration of ASMCs might also play a role in the pathogenesis of this disease.

T_H17 cells, a lineage of CD4⁺ T cells distinct from T_H1 and T_H2 cells, have been recognized as a novel proinflammatory CD4⁺ T effector cell type.⁵ Characterized by predominant production of IL-17A, IL-17F, and IL-22,⁶ T_H17 cells have an important role in host defense against specific pathogens and are potent inducers of autoimmunity and tissue inflammation.⁷ Recent studies also suggest that T_H17 cells and T_H17-associated cytokines are involved in asthma.⁸⁻¹⁰ IL-17A might contribute to asthma through its capacity to promote recruitment of inflammatory cells, including neutrophils, monocytes, and macrophages, to the site of airway inflammation.¹¹⁻¹³ In addition, T_H17 cells and IL-17 might drive airway remodeling through the accumulation of neutrophils or by stimulating the release of matrix metalloproteinases, as well as cytokines. IL-17 could also increase the release of factors from resident cells, including IL-6, IL-8, IL-11, GM-CSF, and vascular endothelial growth factor.¹⁴ To date, however, the evidence implicating IL-17 in the pathogenesis of asthma is largely circumstantial. Although published results support the notion that IL-17 has the potential to contribute to airway remodeling, no direct evidence is yet available.

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Abbreviations used

ASM: Airway smooth muscle
 ASMC: Airway smooth muscle cell
 $\text{IkB}\alpha$: Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α
 MAPK: Mitogen-activated protein kinase
 NF- κ B: Nuclear factor κ B
 PI3K: Phosphoinositide 3-kinase

Recently, Cheng et al¹⁵ reported that IL-17 could promote migration of vascular smooth muscle cells, suggesting the possibility that T_H17 -associated cytokines might contribute to airway remodeling in asthmatic patients through this mechanism. Although important cell-specific differences are likely, the findings for vascular muscle might be relevant to understanding smooth muscle migration in the airways.¹⁶ On the basis of this observation and our recent work that showed that ASMCs can migrate in response to a number of cytokines,^{17,18} we chose to investigate whether T_H17 -associated cytokines would enhance migration of ASMCs. Here, using an *in vitro* cell migration assay, we show for the first time that the T_H17 -associated cytokines IL-17A, IL-17F, and IL-22 promote migration of human ASMCs. These effects are mediated by selective activation of receptors on ASMCs, with IL-17A and IL-17F acting through p38 mitogen-activated protein kinase (MAPK) activation and IL-22 acting through a separate nuclear factor κ B (NF- κ B)-dependent signaling pathway.

METHODS**Isolation and culture of human ASMCs**

Primary cultures of human ASMCs were prepared from transplant specimens, as previously described.¹⁷ Informed consent for ASMC harvesting was obtained from all patients. Cells were cultured in Dulbecco modified Eagle medium/F12 (1:1; GIBCO, Invitrogen, Grand Island, NY) supplemented with 10% FBS and 100 U/mL penicillin/streptomycin (Clonetics, Walkersville, Md) at 37°C with 5% CO₂. Cells from the third to sixth passages were used and were identified as smooth muscle cells by means of positive immunohistochemical staining for smooth muscle-specific α -actin and positive identification of smooth muscle myosin heavy chain and calponin by means of Western blot analysis.

Chemotaxis assays

ASMC migration assays were performed as previously described¹⁹ with a 48-well micro Chemotaxis Chamber (Neuro Probe, Inc, Cabin John, Md). Cells that have been growth arrested in media supplemented with 0.1% FBS for 48 hours were harvested with 0.05% trypsin-EDTA solution (Clonetics) and were resuspended (8.0×10^5 cells/mL) in medium containing 0.1% FBS. An 8.0- μ m-pore polycarbonate membrane (Neuro Probe) was treated with 0.01% Collagen type I (rat tail; Nalgene Culture 3D Matrix, Rochester, NY) solution in 0.01N HCl and placed between 2 chambers. Human AMSCs were added to the upper chamber, and different concentrations (0.1, 1, 10, and 100 ng/mL) of cytokines (IL-17A/F and IL-22), 10 ng/mL PDGF-BB (R&D Systems, Minneapolis, Minn), or medium alone were added to the lower chamber. A preliminary experiment was performed to establish the time course of migration (4, 8, 12, and 24 hours). Because the maximal increase in migration was observed at 4 hours (data not shown), all subsequent experiments were performed for 4 hours. After 4 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, Kalamazoo, Mich), according to the manufacturer's instructions. The number of cells was counted in 5 random fields at a magnification of $\times 200$.

Cytokine-specificity examination. Cytokines were pretreated with neutralizing mAbs for 2 hours at 37°C before being added to the ASMCs to investigate the specificity of the cytokines on migration of AMSCs. Specifically, IL-17A was inhibited by addition of 10 μ g/mL mouse anti-human IL-17A mAb (R&D Systems), IL-17F by addition of 10 μ g/mL mouse anti-human IL-17F mAb (R&D Systems), and IL-22 by addition of 10 μ g/mL mouse anti-human IL-22 mAb (R&D Systems). In each case results were compared with the effect of adding the corresponding isotype controls.

Direct effect examination. To investigate whether the effects of exogenous T_H17 cytokines might be mediated indirectly by increasing the release of chemokines from the ASMCs, we added blocking antibodies into both the upper and lower chambers during the migration. We used mouse anti-human CXCL8 (IL-8) mAb to block the potential effects of CXCL8 (R&D Systems). In each case results were compared with the effects of adding the corresponding isotype controls.

Receptor activation. To confirm that the increased migration associated with administration of exogenous T_H17 cytokines was dependent on receptor activation, we pretreated ASMCs with anti-IL-17RA mAb and/or anti-IL-17RC antibody or anti-IL-22R mAb (R&D Systems). Results were compared with the corresponding isotype controls.

Signaling. We used pharmacologic inhibitors directed against various pathways that potentially contribute to increased ASMC migration. Specifically, we used the p38 MAPK inhibitor BIRB0796 (0.1 μ mol/L; Gift of Professor Sir Philip Cohen, MRC PPU, University of Dundee, Dundee, United Kingdom), the extracellular signal-regulated kinase (ERK) 1/2 MAPK inhibitor PD184352 (2 μ mol/L; United States Biological, Inc, Swampscott, Mass), the NF- κ B inhibitor PS1145 (10 μ mol/L; Professor Sir Philip Cohen), and the phosphoinositide 3-kinase (PI3K) inhibitor PI103 (5 μ mol/L; Cayman Chemical, Ann Arbor, Mich). Pertussis toxin (100 ng/mL; Santa Cruz Biotechnology, Santa Cruz, Calif), an inhibitor for G protein-coupled receptors, was also used. In each case the ASMCs were incubated with the inhibitor for 1 hour at 37°C. All results were compared with the corresponding vehicle control dimethyl sulfoxide.

Flow cytometric assay

Cells were prepared for flow cytometry by means of trypsinization of confluent, serum-fed, adherent cultures of human ASMCs or immortalized BEAS-2B cells (see the **Methods** section in this article's Online Repository at www.jacionline.org). Cells were then incubated for 1 hour on ice with the primary antibodies (anti-IL-17RA, anti-IL-17RC, or isotype controls) or anti-IL-22R1 mAb conjugated with phycoerythrin. The cells were incubated in the dark for 30 minutes on ice with either Alexa488-conjugated goat anti-mouse IgG or Alexa647-conjugated rabbit anti-goat IgG (Invitrogen, Camarillo, Calif). The cells were then washed and fixed in 2% paraformaldehyde and analyzed by means of flow cytometry (BD FACSCalibur; BD Biosciences, Franklin Lakes, NJ). The results are presented as a percentage of positive cells by using Cell Quest Pro software (BD Biosciences).

Statistical analysis

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

RESULTS **T_H17 -associated cytokines induced migration of ASMCs**

We first investigated the effect of IL-17A, IL-17F, and IL-22 on migration of ASMCs. We observed that IL-17A (at 100 ng/mL, $149\% \pm 8\%$, $P < .05$), IL-17F (at 100 ng/mL, $160\% \pm 15\%$, $P < .05$), and IL-22 (at 10 ng/mL, $152\% \pm 10\%$, $P < .05$) significantly increased ASMC migration compared with migration seen after use

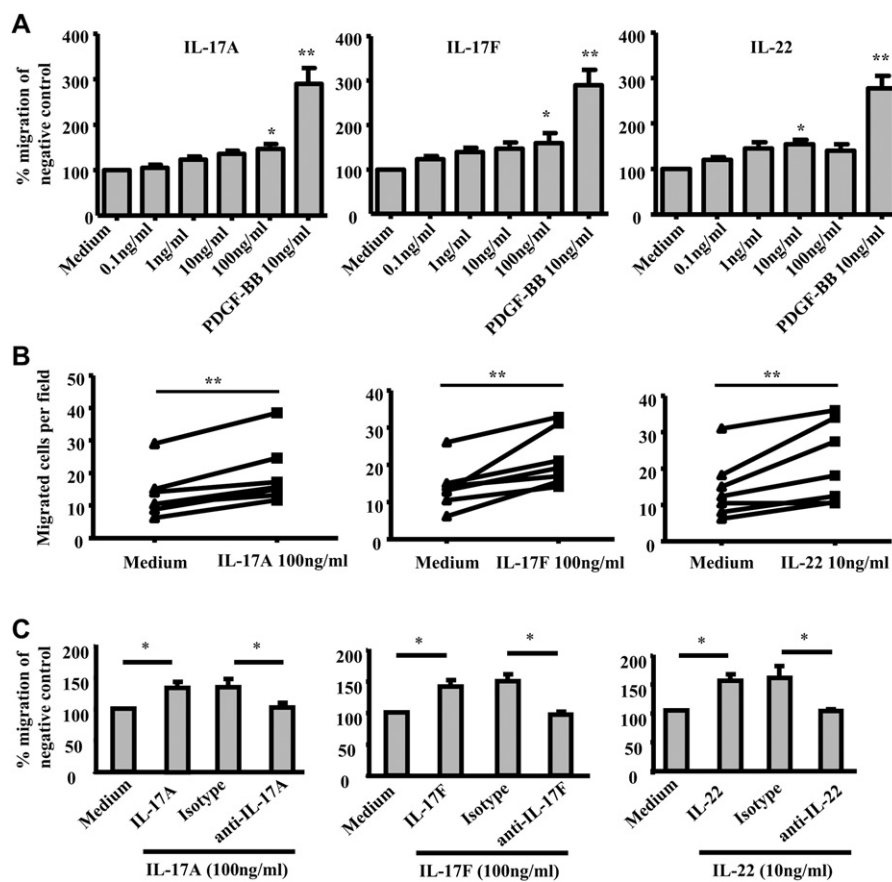


FIG 1. Migration of ASMCs induced by T_H17 cytokines. **A**, T_H17 cytokines induced migration of ASMCs. * $P < .05$ and ** $P < .01$ compared with medium negative control ($n = 11$ experiments including ASMCs from 7 subjects). **B**, Migrated cell number of ASMCs induced by T_H17 cytokines. ** $P < .01$ compared with medium negative control ($n = 7$ subjects). **C**, Cytokine specificity of T_H17 -induced migration of ASMCs. The relative isotype was used as a control. * $P < .05$ ($n = 4$ independent experiments).

of negative controls (Fig 1, A). Migration increased progressively in a dose-dependent manner, although the magnitude of the increase was less than that induced by the positive control PDGF-BB (at 10 ng/mL, $290\% \pm 28\%$, $P < .01$). We found no evidence of synergistic interaction when the cytokines were administered in combination (data not shown). Pretreatment of cytokines with neutralizing antibodies, but not their isotype controls, prevented T_H17 cytokine-induced migration of ASMCs ($P < .05$; Fig 1, B).

A checkerboard analysis was performed to determine whether the effects of T_H17 cytokines were mediated through chemokinesis rather than chemotaxis (see the Methods section in this article's Online Repository). The presence of higher concentrations of T_H17 cytokines in the upper chamber did not enhance migration of ASMCs (see Table E1 in this article's Online Repository at www.jacionline.org). The absence of a concentration gradient did not stimulate the migration of ASMCs (see Table E1). Taken together, our results suggest that T_H17 cytokines mediate ASMC chemotaxis but not enhanced chemokinesis.

T_H17 -associated cytokines directly induced migration of ASMCs

To explore the possible contribution of CXCL8 and CCL11, we first detected the production of CXCL8 and CCL11 in the supernatants after 4 hours of migration. However, we could

only detect increased CXCL8 production in the upper chamber. The production of CXCL8 in the lower chamber and CCL11 in both chambers was not detectable. By adding the neutralizing antibody against CXCL8, the produced CXCL8 was fully neutralized (data not shown), and no evidence of inhibition of T_H17 cytokine-induced ASMC migration was observed (Fig 2).

Receptors for T_H17 -associated cytokines expressed on ASMCs

We examined the expression of receptors for T_H17 cytokines on ASMCs using BEAS-2B cells as a positive control. Using flow cytometry, we detected the expression of the extracellular domains of IL-17RA and IL-17RC (receptors for both IL-17A and IL-17F; Fig 3, A). In ASMCs lower IL-17RC expression was detected compared with that of IL-17RA (percentage of positive cells: $43.6\% \pm 5.8\%$ for IL-17RA and $19.7\% \pm 3.3\%$ for IL-17RC). In contrast, higher expression of IL-17RC was found on the BEAS-2B cell line (percentage of positive cells: $16.7\% \pm 4.5\%$ for IL-17RA and $73.0\% \pm 9.8\%$ for IL-17RC). The expression of IL-22R1, one subunit of the IL-22 receptor, was detected on both ASMCs (percentage of positive cells: $25.3\% \pm 2.6\%$) and the BEAS-2B cell line (percentage of positive cells: $19.3\% \pm 3.6\%$). Additionally, the expression of these 3 receptors on ASMCs and the BEAS-2B cell line was further confirmed by

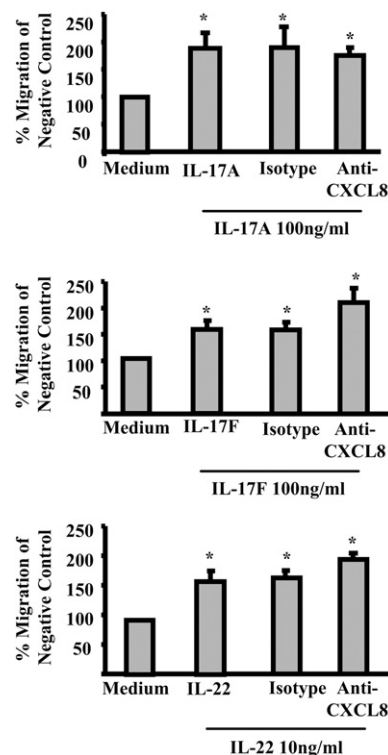


FIG 2. Effect of CXCL8 on the T_H17 cytokine-induced migration of ASMCs. Mouse anti-human CXCL8 (IL-8) mAbs were added into both the upper and lower chambers during the migration. The relative isotype was used as a control. * $P < .05$ compared with medium alone ($n = 3$ independent experiments).

means of Western blotting (Fig 3, B; see the Methods section in this article's Online Repository).

T_H17 cytokine-induced migration is receptor dependent

Having confirmed the expression of T_H17 cytokine receptors on ASMCs, we used neutralizing antibodies to attempt to selectively block these receptors. We demonstrated that blockade of either IL-17RA or IL-17RC alone significantly but incompletely inhibited the migration of ASMCs induced by IL-17A or IL-17F ($P < .05$, Fig 4). Blocking IL-17RA inhibited IL-17A-induced migration more strongly than did IL-17F (inhibition: $64.8\% \pm 20.8\%$ for IL-17A vs $58.3\% \pm 14.3\%$ for IL-17F). Blockade of IL-17RC led to stronger inhibition for IL-17F-induced migration (inhibition: $65.2\% \pm 17.8\%$ for IL-17A vs $86.5\% \pm 9.8\%$ for IL-17F). When both IL-17RA and IL-17RC were blocked, the increased migration induced by IL-17A or IL-17F was totally inhibited (inhibition: $98.5\% \pm 6.6\%$ for IL-17A vs $97.6\% \pm 2.3\%$ for IL-17F). For IL-22-induced migration of ASMCs, blockage of IL-22R1 completely inhibited the migration (inhibition: $96.2\% \pm 5.4\%$, $P < .01$, Fig 4).

T_H17 cytokines induced ASMC migration by differential signaling pathways

Next we explored the signaling pathways involved in the T_H17 cytokine-induced migration of ASMCs. Using pharmacologic inhibitors, we tested for the involvement of p38 MAPK, ERK1/2 MAPK, NF- κ B, and PI3K pathways in the migratory effect. An inhibitor of p38 MAPK, BIRB0796, partially inhibited the migration

of ASMCs induced by IL-17A ($68.5\% \pm 14.0\%$ inhibition, $P < .05$) and IL-17F ($71.8\% \pm 9.0\%$ inhibition, $P < .05$; Fig 5). In contrast, inhibitors for ERK1/2 MAPK, NF- κ B, and PI3K had no effect. These results contrast with those obtained for IL-22-induced migration of ASMCs. Only PS1145, an inhibitor of NF- κ B, significantly prevented the migratory effect of that cytokine ($82.7\% \pm 18.9\%$ inhibition, $P < .05$, Fig 5). For all 3 T_H17 cytokines, no inhibition was observed with 100 ng/mL pertussis toxin.

IL-17A/IL-17F and IL-22 activated p38 MAPK and NF- κ B pathways, respectively

We sought direct evidence of p38 MAPK phosphorylation after administration of exogenous IL-17A and IL-17F (see the Methods section in this article's Online Repository). As shown in Fig 6, stimulation by 100 ng/mL IL-17A or IL-17F resulted in phosphorylation of p38 MAPK starting at 5 minutes, with peak phosphorylation detected at 15 minutes. To confirm the involvement of NF- κ B in IL-22-stimulated ASMC migration, we examined the effect of 10 ng/mL IL-22 on NF- κ B p65 phosphorylation and nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α (I κ B α) degradation. I κ B α degradation was detected as early as 15 minutes and reached its nadir at 45 minutes. Consistent with this finding, phosphorylation of NF- κ B p65 started at 15 minutes and reached its peak at 45 minutes.

DISCUSSION

We used an *in vitro* cell-migration assay system to confirm that T_H17 cytokines might promote ASMC migration. IL-17A and IL-17F, as well as IL-22, led to enhanced migration of human ASMCs in a selective receptor-mediated fashion. Moreover, although IL-17A and IL-17F appeared to share a common signaling pathway, IL-22 promoted migration through a separate signaling system. In the context of growing recognition of the importance of airway remodeling in asthma, as well as the potential for ASMCs to contribute to asthma pathogenesis through their ability to produce various mediators, as well as bronchoconstriction, our findings underscore the potential importance of T_H17-associated cytokines in the pathogenesis of asthma.

There is growing recognition that T_H17 cells are involved in a variety of human diseases, particularly autoimmune diseases.⁷ However, there is increasing evidence to suggest that IL-17-producing cells also contribute to asthma, which has been associated with an increased number of T_H17 cells, and with increased production of cytokines that are characteristically produced by these cells. Interestingly, the expression of T_H17 cytokines has been found to be related to disease severity.^{9,10} Nevertheless, although these results do suggest that T_H17 cells might contribute to asthmatic inflammation, to date, there has been no mechanistic link between T_H17 cytokines and the structural changes that are characteristic of severe asthma.

We found strong evidence that the T_H17 cytokines we tested promoted increased migration of ASMCs (Fig 1, A). Although the magnitude of the increase was less than that of our positive control (PDGF), these effects were reproducible and exhibited a dose-response relationship. Moreover, administration of neutralizing mAbs directed against the cytokines abolished these effects entirely (Fig 1, B). This suggests that the effect of the T_H17 cytokines is direct and selective. However, IL-17A has been reported to release CXCL8 (IL-8) from human ASMCs,²⁰⁻²² and CXCL8 is known to induce migration of human ASMCs.¹⁷ Given the

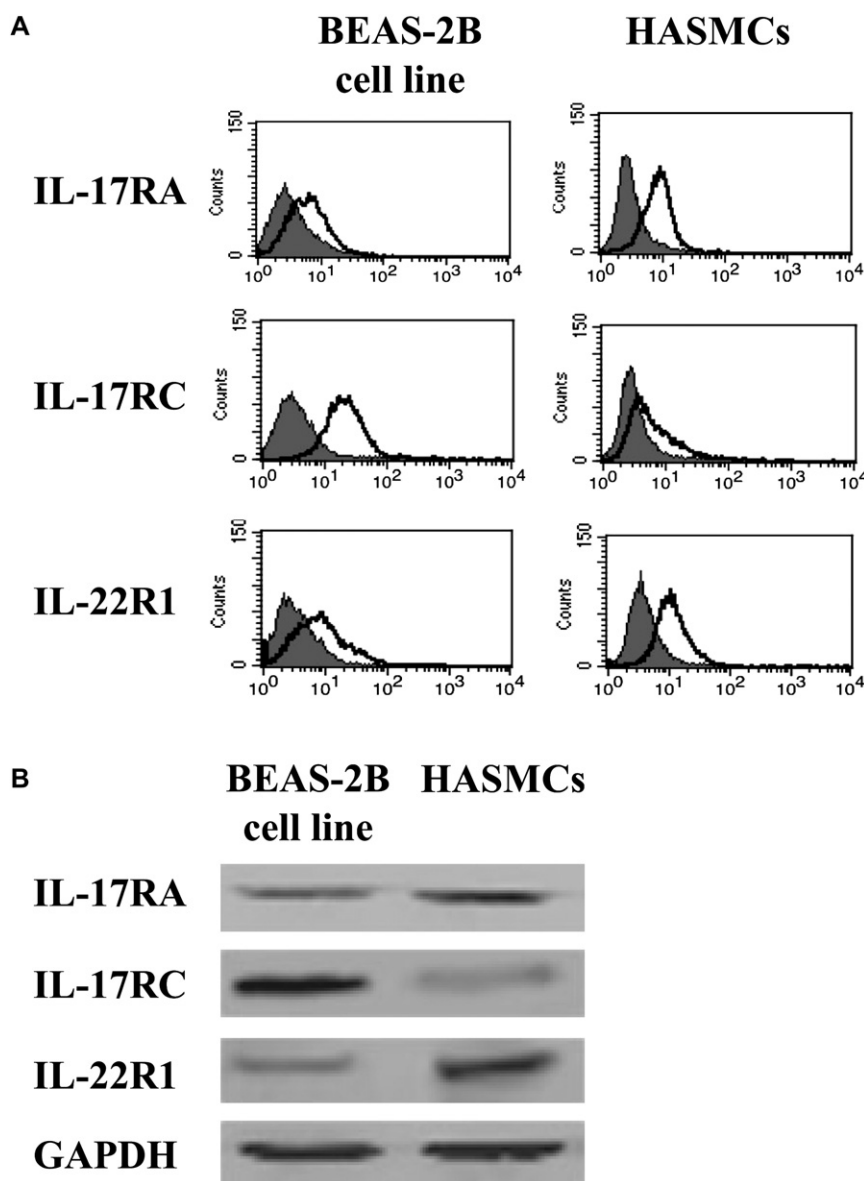


FIG 3. Expression of T_H17 cytokine receptors on human ASMCs (HSMCs). **A**, T_H17 cytokine receptors were examined by using flow cytometry. The shaded sections represent the isotype control staining, and the single line represents the specific antibody staining. A representative result of 4 experiments is shown. **B**, T_H17 receptors were examined by means of Western blot assay. A representative result of 3 experiments is shown. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

potential for this chemokine to confound our results, we selectively used specific neutralizing antibody to block CXCL8 (Fig 2). We found no evidence to suggest that this chemokine accounts for our findings. Although T_H17 cytokines exhibited a relatively modest chemotactic effect on ASMCs compared with CCL11¹⁸ and PDGF, their increased production in asthmatic patients has the potential to amplify their effect on migration of ASMCs and consequently to contribute to increased smooth muscle mass. Of note, recently, IL-17A was shown to mediate severe airway hyperresponsiveness in mice.²³ Together with the findings in this study, this finding suggests that IL-17 deserves further consideration as an important factor in airway remodeling.

We further confirmed the presence of T_H17 cytokine receptors on the human ASMCs (Fig 3). IL-17RA, IL-17RC, and IL-22R1

were present on these ASMCs, with IL-17RA and IL-22R1 detected at a higher level of expression compared with IL-17RC. The lower level of expression of IL-17RC compared with IL-17RA might be a function of variability among tissues. IL-17RA is reported to be highly expressed in the bone marrow, thymus, and spleen but less easily detected in the colon, small intestine, and lung. Conversely, epithelial cells of the prostate, kidney, and joints express high levels of IL-17RC mRNA, whereas low levels of expression are detected in the hematopoietic cell compartments.²⁴ In this regard, although expression of IL-17RA on ASMCs has been previously reported,²⁵ this study appears to be the first demonstration of IL-17RC expression on these cells. As with the IL-17 receptor, the IL-22 receptor consists of 2 subunits, IL-22R1 and IL-10R2. Although IL-10R2 is widely

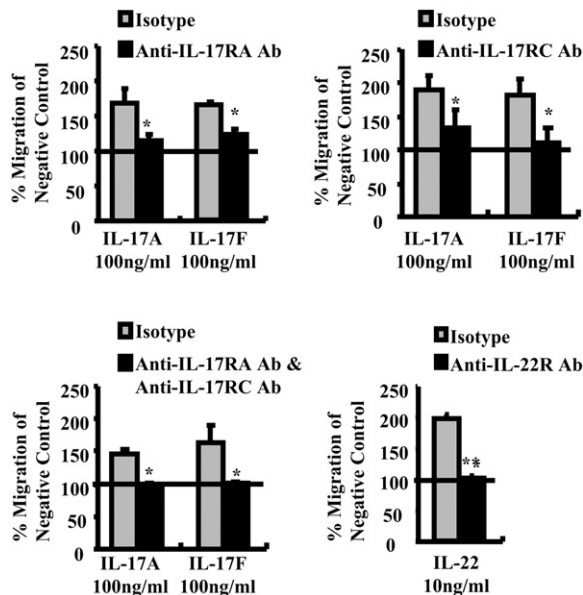


FIG 4. Effect of blockade of T_H17 receptors on migration of ASMCs. ASMCs were pretreated with anti-IL-17RA neutralizing antibody (Ab) and/or anti-IL-17RC neutralizing antibody and anti-IL-22R antibody for 1 hour at 37°C, and isotypes were used as a control (n = 3 experiments).

expressed on immune cells, the expression IL-22R1 is prominent in a variety of nonimmunologic tissues, including the skin, lung, small intestine, liver, colon, kidney, and pancreas.²⁶ In this study we demonstrate for the first time that IL-22R1 is present in human ASMCs.

We used neutralizing antibodies to confirm the importance of specific receptor binding. As shown in Fig 4, each receptor contributed to the increased migration of the cells. Of interest, blockade of either IL-17RA or IL-17RC only partially prevented the increase in migration induced by exogenous administration of IL-17A or IL-17F. However, when blocking antibodies directed against both receptors were administered, the ability of either IL-17A or IL-17F to promote increased cell migration was abolished. Both IL-17A and IL-17F have been shown to bind to IL-17RA and IL-17RC. However, whereas IL-17A and IL-17F bind IL-17RC with similar affinities, the affinity of IL-17F for IL-17RA is considerably lower.²⁷ Based on results in other systems, it has been argued that the binding of the IL-17RC receptor might not be essential for the activity of IL-17A and IL-17F.^{24,27-30} However, our results support an important role of IL-17RC for the functions of IL-17A and IL-17F in human ASMCs. Nevertheless, activation of each of the 2 receptor subtypes leads to different effects. IL-17A-induced migration of ASMCs appears to be more dependent on IL-17RA, whereas IL-17F-induced migration of ASMCs is more closely related to activation of IL-17RC.

Several cytokines and chemokines, including IL-1 β ,³¹ TGF- β 1,³¹ CCL11,¹⁸ CCL19,³² and CXCL8,¹⁷ have been reported to function as chemoattractants for ASMCs. However, the signaling pathways involved have not been fully elucidated, except for the observation that the p38 MAPK pathway is involved in IL-1 β - and TGF- β 1-induced migration.³¹ To explore intracellular signaling in response to T_H17 cytokines, we administered BIRB0796, which inhibits p38 α and p38 β MAPK isoforms at 0.1 μ mol/L.³³ BIRB0796 significantly inhibited the increased

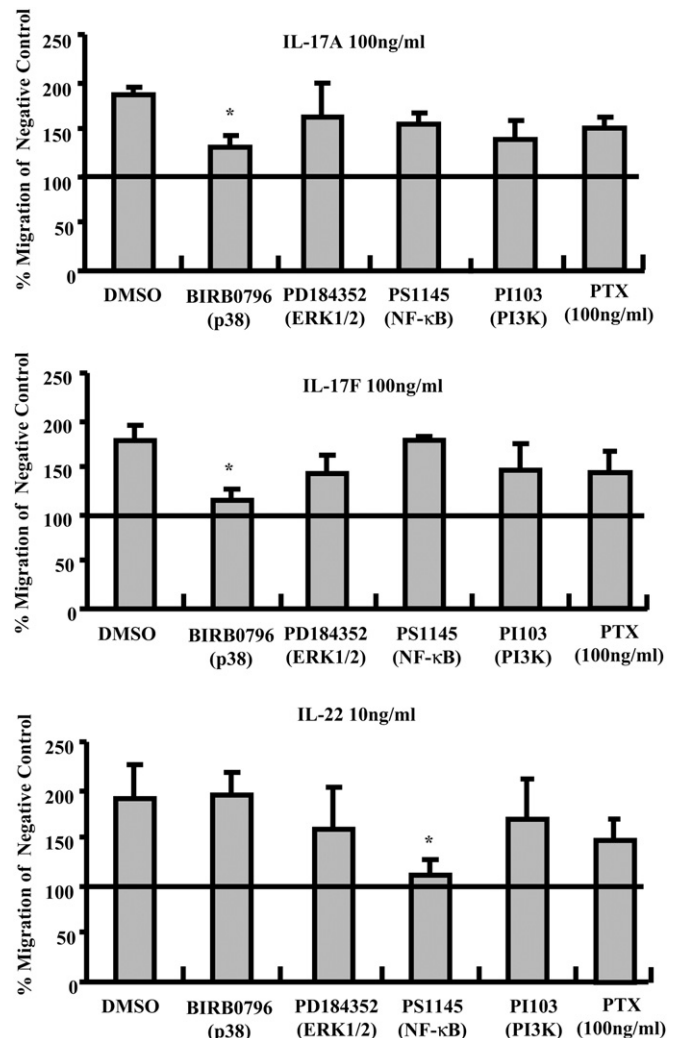


FIG 5. Signaling pathways involved in T_H17 cytokine-induced migration of ASMCs. ASMCs were pretreated with inhibitors for certain signaling pathways, and the solvent dimethyl sulfoxide (DMSO) was used as a control. * $P < .05$ compared with the control (N = 4 experiments). PTX, Pertussis toxin.

migration, although not completely. Nevertheless, our results indicate that any effect of IL-17A or IL-17F on NF- κ B is likely to be weak, as previously reported.²⁴ The activation of p38 MAPK by IL-17A/F was further confirmed by the assessment of p38 MAPK phosphorylation. Interestingly, IL-17A and IL-17F exhibited similar kinetics for the activation of p38 MAPK, suggesting that they fulfill similar biological functions using the same signaling pathways. To our knowledge, this is the first demonstration of p38 MAPK activation by IL-17F in ASMCs. Our results differ from studies of IL-17A-induced CXCL8 production by ASMCs, which suggest that both the p38 MAPK and ERK1/2 MAPK pathways are involved.²⁰

We inhibited IL-22-induced ASMC migration using PS1145, a highly specific small-molecule selective inhibitor of I κ B kinase known to efficiently inhibit both basal and induced NF- κ B activity.³⁴ Additionally, we explored p65 phosphorylation and I κ B α degradation. NF- κ B is maintained as a latent form in the cytoplasm of cells, where it is complexed to I κ B inhibitor proteins. NF- κ B activation involves the degradation of the inhibitory

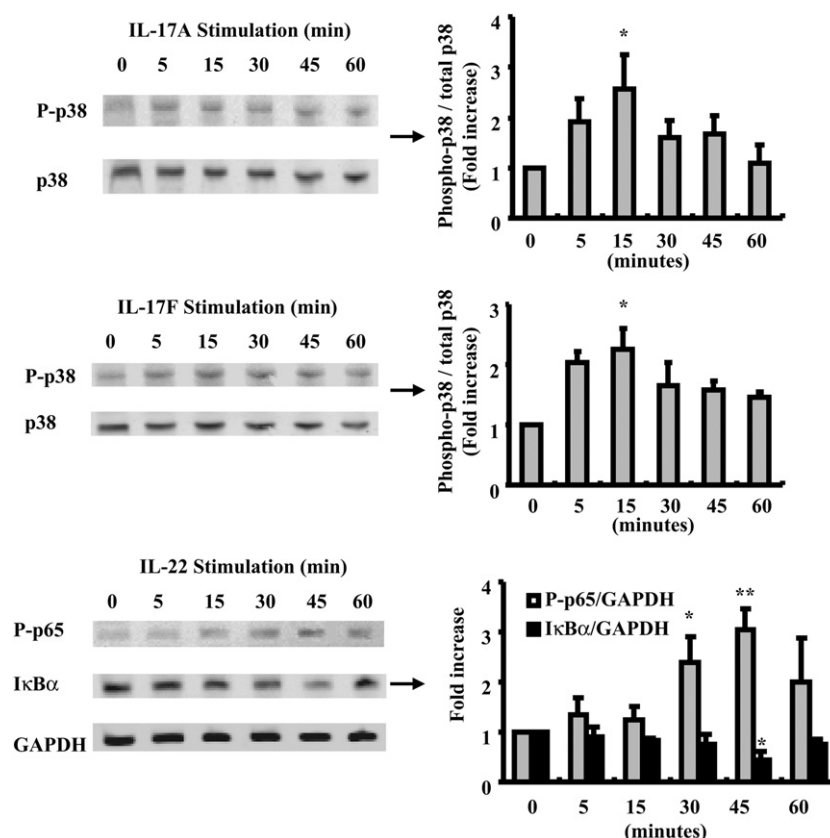


FIG 6. Induction of p38 MAPK phosphorylation by IL-17A and IL-17F and activation of NF- κ B by IL-22 in human ASMCs. * $P < .05$ compared with the control. The results represent one of similar results from 4 independent experiments. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

protein I κ B α , as well as the phosphorylation of p65 protein.³⁵ Our observations of p65 phosphorylation and I κ B α degradation reinforce the findings with PS1145 and suggest that, in contrast to IL-17A and IL-17F, the effect of IL-22 on ASMC migration is dependent on NF- κ B activity. This is consistent with a previous report of IL-22 signaling through NF- κ B activation in colonic subepithelial myofibroblasts.³⁶ Thus the intracellular signaling mechanisms by which promotion of ASMC migration is effected are different for IL-22 compared with IL-17A and IL-17F. Pertussis toxin is mainly used as an inhibitor of G protein-coupled receptors and has an inhibitory effect on PDGF-induced migration of ASMCs.³⁷ In contrast, we found no evidence that pertussis toxin inhibited increased migration after administration of any of the 3 T_H17 cytokines, suggesting that G protein-coupled receptor activation is not involved in this phenomenon.

Although our results do not, on their own, confirm a role for T_H17-associated cytokines in the pathogenesis of airway remodeling, they provide for the first time at least one mechanism by which T_H17 cytokines might contribute to this phenomenon. Furthermore, these data provide a strong rationale for subsequent studies in animal models to assess the role of T_H17 cytokines in smooth muscle remodeling. However, additional work needs to be done to put these findings in context. In addition to more information about the expression and localization of T_H17 cytokines in airway wall structures in asthmatic tissue, we need better models to help understand the relative importance of cell migration, as opposed to hypertrophy or hyperplasia, in the development of

increased ASM mass. It is likely that progress in this area will require the careful study of biopsy specimens from asthmatic subjects, possibly using *ex vivo* techniques, such as explants.

In summary, we investigated the possibility that T_H17-associated cytokines induce ASMC migration. We demonstrated that IL-17A, IL-17F, and IL-22 all have the capability of inducing increased ASMC migration in a selective receptor-mediated manner. Moreover, IL-17A and IL-17F exert their effects through signaling pathways that are distinct from those used by IL-22, suggesting that there are multiple mechanisms by which ASMC migration can be increased in the context of T_H17 inflammation. These observations provide clear evidence of an important new mechanism for the promotion of airway remodeling in this disease.

Key message

- In the face of growing recognition of the importance of T_H17 cytokines in the pathogenesis of asthma, these observations provide clear evidence of an important new mechanism for the promotion of airway remodeling in this disease.

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METHODS

Culture of human BEAS-2B cell line

The BEAS-2B cell line was obtained from the American Type Culture Collection (Manassas, Va). Cells were cultured in Dulbecco modified Eagle medium (GIBCO, Invitrogen) supplemented with 10% FBS and 100 U/mL penicillin/streptomycin (Clonetics) at 37°C with 5% CO₂.

Checkerboard analysis

Checkerboard analysis was performed to determine whether T_H17 cytokines are chemotactic or chemokinetic for ASMCs, as previously described.^{E1} Various concentrations of IL-17A, IL-17F, or IL-22 were added into upper wells of a chemotaxis chamber together with cells and added into lower wells without cells. After the incubation, the number of migrated cells was counted, as described above.

Western blotting assay for T_H17 cytokine receptor expression

Western blotting was carried out on cells obtained by means of trypsinization of confluent serum-fed adherent BEAS-2B epithelial cells and ASMCs, which were resuspended in lysis buffer. Protein was extracted from these cells and loaded (20 µg) on a 10% acrylamide SDS-PAGE NEXT GEL (Amresco, Solon, Ohio) for separation before transfer to a nitrocellulose membrane (Bio-Rad, Hercules, Calif). The membrane was incubated at room temperature for 1 hour in Odyssey blocking buffer (LI-COR Biosciences, Inc, Lincoln, Neb) to block nonspecific protein binding. The membrane was then incubated overnight at 4°C with mouse anti-human glyceraldehyde-3-phosphate dehydrogenase mAb (Millipore, Temecula, Calif), mouse anti-human IL-17RA mAb (R&D Systems), goat anti-human IL-17RC Ab (R&D Systems), or goat anti-human IL-22R Ab (R&D Systems) in 1:1 blocking buffer/PBS to detect the expression of T_H17 cytokine receptors on ASMCs. The membrane was washed 4 times with 0.1% Tween 20 in PBS and then incubated with a 1:15,000

dilution of IRDye 800 goat anti-mouse IgG or IRDye 680 donkey anti-goat IgG (Rockland, Philadelphia, Pa) in blocking buffer. Membranes were scanned and analyzed with an Odyssey IR scanner using Odyssey imaging software 3.0 (LI-COR Biosciences, Inc).

Assessment of p38 MAPK, p65 NF-κB, and IκBα phosphorylation

Nearly confluent ASMCs were growth arrested by medium with 0.1% FBS for 48 hours. Cells were then stimulated with 100 ng/mL IL-17A, 100 ng/mL IL-17F, or 10 ng/mL IL-22 for 0, 5, 15, 30, 45, and 60 minutes. The cells were washed twice with cold PBS, and total proteins were extracted with lysis buffer (50 mmol/L Tris-HCl, 1 mmol/L ethyleneglycol-bis-[β-aminoethylether]-N,N,N',N'-tetraacetic acid, 1 mmol/L EDTA, 1% Triton X-100, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate decahydrate, 0.27 mol/L sucrose, and protease inhibitor mixture [Roche, Mannheim, Germany]). Protein lysate (10 µg) was loaded on 10% acrylamide SDS-PAGE NEXT GEL (Amresco), followed by transfer to nitrocellulose membranes (Bio-Rad). The blots were then blocked with Odyssey blocking buffer (LI-COR Biosciences, Inc) for 1 hour at room temperature and then incubated overnight at 4°C with antibodies specific for phosphorylated p38 MAPK (Millipore), p38 MAPK (Millipore), phosphorylated NF-κB p65 (GenScript, Piscataway, NY), IκBα (Millipore), and glyceraldehyde-3-phosphate dehydrogenase (Millipore). After washing with 0.1% Tween 20 in PBS, the membranes were incubated with a 1:15,000 dilution of IRDye 680 goat anti-mouse IgG and IRDye 680 goat anti-rabbit IgG (Rockland) in blocking buffer. Membranes were scanned and analyzed with an Odyssey IR scanner using Odyssey imaging software 3.0 (LI-COR Biosciences, Inc).

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TABLE E1. Checkerboard analysis of ASMC migration in response to T_H17 cytokines

Upper compartment (IL-17A [μg/mL])					
	0	0.1	1	10	100
Lower compartment (IL-17A [μg/mL])					
0	100	101.0 ± 2.1	102.3 ± 5.1	98.3 ± 16.1	101.3 ± 10.8
0.1	114.0 ± 3.1	87.6 ± 6.7	100.3 ± 6.7	103 ± 12.5	106.6 ± 9.5
1	120.7 ± 6.1	104.3 ± 12.7	89.1 ± 7.6	109.6 ± 3.2	102.3 ± 12.7
10	158.7 ± 12.9*	121.3 ± 4.5	100 ± 10.8	95.6 ± 9.5	103.3 ± 7.5
100	178.3 ± 14.3*	133.3 ± 4.5*	122.3 ± 12.8	112.3 ± 9.8	97.8 ± 7.9
Upper compartment (IL-17F [μg/mL])					
	0	0.1	1	10	100
Lower compartment (IL-17F [μg/mL])					
0	100	94.9 ± 3.8	94.7 ± 8.7	92.2 ± 9.5	105.4 ± 5.5
0.1	114.6 ± 4.9	91.5 ± 8.3	95.0 ± 7.8	97.9 ± 8.2	106.7 ± 11.4
1	115.3 ± 9.3	126.9 ± 3.9	91.0 ± 9.6	97.5 ± 6.0	110.4 ± 10.4
10	140.9 ± 15.1*	147.6 ± 20.9*	125.9 ± 8.4	114.0 ± 4.7	117.1 ± 4.5
100	181.1 ± 22.1*	143.9 ± 17.8*	131.0 ± 9.5*	124.4 ± 7.7	103.1 ± 8.9
Upper compartment (IL-22 [μg/mL])					
	0	0.1	1	10	100
Lower compartment (IL-22 [μg/mL])					
0	100	103.5 ± 3.7	106.5 ± 6.8	104.9 ± 8.1	96.6 ± 3.6
0.1	110.2 ± 9.3	93.1 ± 13.9	103.1 ± 6	103.3 ± 3.1	101.5 ± 12.3
1	131.7 ± 5.6*	113.2 ± 5.9	104.3 ± 10.4	105.6 ± 3.3	100.6 ± 6.7
10	152.5 ± 8.3*	113.9 ± 14.2	120.8 ± 12.2	91.4 ± 17.8	102.4 ± 2.1
100	120.3 ± 10.9	110.2 ± 4.6	123.4 ± 9.7	111.2 ± 4.3	92.4 ± 10.4

Various concentrations of T_H17 cytokines were added into the upper wells of a chemotaxis chamber together with cells and added into the lower wells without cells. Results are presented as means of migration percentages ± SEMs (n = 3 independent experiments).

**P* < .05 compared with the medium control.