

Modulation of bronchial epithelial cells by IL-17

Mio Kawaguchi, MD,^{a,b} Fumio Kokubu, MD,^b Hideki Kuga, MD,^b
Satoshi Matsukura, MD,^b Hiroshi Hoshino, MD,^b Koushi Ieki, MD,^b
Toshimichi Imai, MD,^b Mitsuru Adachi, MD,^b and Shau-Ku Huang, PhD^a
Baltimore, Md, and Tokyo, Japan

Background: The induction of epithelial cytokines/chemokines is crucial in the migration of leukocytes, and its regulatory mechanisms remain incompletely defined.

Objective: To determine the role of IL-17, a CD4⁺ T cell-derived cytokine, in modulation of primary bronchial epithelial cells, the expression of IL-6, IL-8, and intercellular adhesion molecule 1 (ICAM-1) and the potential involvement of mitogen-activated protein (MAP) kinases in IL-17-mediated signaling were examined.

Methods: The levels of gene expression and protein production for IL-6 and IL-8 in IL-17-treated cells, in the presence or absence of MAP kinase inhibitors, were analyzed by RT-PCR and ELISA, respectively, and activation of MAP kinases was determined by Western blot analyses.

Results: We showed first that IL-17 induced time-dependent expression of IL-6 and IL-8 but not of the chemokines eotaxin and RANTES. In addition, IL-17 induced activation of extracellular signal-regulated kinase 1/2 but not of p38 or JNK kinases. A selective MAP kinase kinase inhibitor, PD98059, inhibited IL-17-induced IL-6 and IL-8. A combination of IL-17 and each of the cytokines IL-4, IL-13, and IFN- γ further enhanced IL-8 expression. IL-17 alone did not induce ICAM-1 expression and showed no effect on IL-4- or IL-13-induced ICAM-1 expression. In contrast, a combination of IL-17 and IFN- γ augmented IL-6 and ICAM-1 expression.

Conclusion: These findings suggest that IL-17, alone or in combination with other cytokines, modulates airway inflammation via—in part—the expression of epithelial IL-6, IL-8, and ICAM-1. (*J Allergy Clin Immunol* 2001;108:804-9.)

Key words: Adhesion molecule, bronchial epithelial cell, ERK1/2, interleukin-8, interleukin-6, interleukin-17

Pulmonary inflammation is characterized by activation and migration of various inflammatory cells. Airway epithelial cells play an important role in regulation of the inflammatory process through their ability to express a wide range of proinflammatory cytokines and chemokines.¹ Understanding of the regulatory pathway involving modulation of airway epithelium by cytokines will help to delineate the pathogenesis of airway inflammation.

Abbreviations used

DMSO:	Dimethyl sulfoxide
ERK1/2:	Extracellular signal-regulated kinase
ICAM-1:	Intercellular adhesion molecule 1
MAP:	Mitogen-activated protein
MEK:	MAP kinase kinase
PBEC:	Primary bronchial epithelial cell

IL-17 is a novel cytokine derived from CD4⁺ activated memory (CD45⁺RO⁺) T lymphocytes,² and its expression has been found in several disease states, such as multiple sclerosis, contact dermatitis, and rheumatoid arthritis.³⁻⁶ Several in vitro studies have shown that IL-17 is able to induce IL-6, IL-8, granulocyte colony-stimulating factor, nitric oxide, and prostaglandin E₂ from various cell types.⁷⁻¹⁰ It has also been suggested, in a rat model, that IL-17 is able to selectively recruit neutrophils into the airway, at least in part through the release of IL-8.^{11,12} An increased expression of intercellular adhesion molecule 1 (ICAM-1) on human keratinocytes stimulated with both IL-17 and IFN- γ has been demonstrated.^{13,14} However, it is not known whether IL-17 modulates ICAM-1 expression on airway epithelial cells, and the biological function of IL-17 has not been fully elucidated in primary bronchial epithelial cells (PBECs).

To gain further understanding of the role of IL-17 in modulation of airway epithelium, we have investigated the effects of IL-17 on proinflammatory parameters of PBECs—namely, the expression of a cytokine (IL-6), a C-X-C chemokine (IL-8), and an adhesion molecule (ICAM-1). In this study, we demonstrated that IL-17, alone or in combination with either 1 T_H1 cytokine (IFN- γ) or 2 T_H2 cytokines (IL-4 and IL-13), is able to modulate the function of bronchial epithelial cells through—at least in part—the activation of extracellular signal-regulated kinase (ERK1/2) kinase.

MATERIALS AND METHODS

Analysis of IL-6 and IL-8 expression

PBECs were purchased from Clonetics (San Diego, Calif) and cultured in bronchial epithelial basal medium (Clonetics) containing 0.5 ng/mL human recombinant epidermal growth factor, 52 μ g/mL bovine pituitary extract, 0.1 ng/mL retinoic acid, 0.5 μ g/mL hydrocortisone, 5 μ g/mL insulin, 10 μ g/mL transferrin, 0.5 μ g/mL epinephrine, 6.5 ng/mL triiodothyronine, 50 μ g/mL gentamicin, and 50 pg/mL amphotericin-B (Clonetics). PBECs were incubated at 37°C in a humidified 5% CO₂ atmosphere and cultured for no more than 3 passages before the analysis. For analysis of IL-6 and IL-8

From ^athe Asthma and Allergy Center, Johns Hopkins University, and ^bthe First Department of Internal Medicine, Showa University School of Medicine, Tokyo.

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Reprint requests: Shau-Ku Huang, PhD, Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Baltimore, MD 21224-6801.

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expression, PBECs were treated with IL-17 (10 and 100 ng/mL; R&D Systems, Minneapolis, Minn) for various periods. In some cases, the cells were treated with 100 ng/mL of IL-17, 100 ng/mL of IL-4, 100 ng/mL of IL-13, or 100 U/mL of IFN- γ (all purchased from R&D Systems) or with IL-17 (100 ng/mL) in combination with either IFN- γ (100 U/mL), IL-4 (100 ng/mL), or IL-13 (100 ng/mL) for 48 hours.

Total RNA was extracted through use of RNeasy (Qiagen, Chatsworth, Calif) from 1×10^6 cells 4 hours after stimulation. cDNAs were synthesized from 500 ng of total RNA in the presence of MMLV reverse transcriptase (1 U/reaction; Sigma, St Louis, Mo), oligo(dT) primer, and reaction buffer at 42°C for 90 minutes; this was followed by PCR. The sequences of PCR primers were as follows:

IL-8:

forward, 5'-TCTGCAGCTCTGTGTGAAG-3';
reverse, 5'-TAATTTCTGTGTTGGCGCA-3'

IL-6:

forward, 5'-ATGAACTCCTTCTCCACAAGCGC-3';
reverse, 5'-GAAGAGCCCTCAGGCTGGACTG-3'

G3PDH:

forward, 5'-ACCACAGTCCATGCCATCAC-3';
reverse, 5'-TCCACCACCTGTTGCTGTA-3'.

In addition, gene expression for 2 chemokines, eotaxin and RANTES, was also analyzed. The sequences of primers for eotaxin and RANTES were as follows:

eotaxin:

forward, 5'-ACAGGAGAATCACCAGTGGC-3';
reverse, 5'-ACTTCATGGAATCCTGCACC-3'

RANTES:

forward, 5'-CGCTGTCATCCTCATTGCTA-3';
reverse, 5'-TGATGTACTCCCGAACCCAT-3'.

The amplification reaction was performed for 23 cycles with denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, and extension at 72°C for 45 seconds. The expected size was 154 bp for IL-8, 628 bp for IL-6, 114 bp for eotaxin, and 233 bp for RANTES. PCR products were detected by ethidium bromide staining and quantified by video densitometry through use of a gel documentation system with Image 1.61 software (NIH Public Software; National Institutes of Health, Bethesda, Md). The level of gene expression was quantified by calculating the ratio of densitometric readings of the band intensity for IL-6/IL-8 and G3PDH from the same cDNA sample. IL-6/IL-8 protein levels in the supernatants of IL-17-stimulated cells were determined with a commercially available ELISA kit (Biosource, Camarillo, Calif), used according to the manufacturer's instruction.

Detection of MAP kinases

For analysis of activation of MAP kinases, PBECs were treated with IL-17 (100 ng/mL) for various periods, and in some cases with or without treatment with one of the following for 1 hour: a MAP kinase kinase (MEK) 1/2 inhibitor, PD98059 (1–50 μ M/L; Calbiochem, La Jolla, Calif)¹⁵; the p38 inhibitor SB202190 (0.5–10 μ M/L)¹⁶; or a vehicle control, dimethyl sulfoxide (DMSO). The final concentration of DMSO did not exceed 0.1% (v/v). After the treatment, the cells were washed with ice-cold PBS and the cell pellets were immediately lysed in cold lysis buffer (20 mmol/L Tris [pH 7.4], 4 mmol/L EDTA, 1 mmol/L PMSF, 100 mg/mL aprotinin, 200 mg/mL leupeptin, 50 mmol/L NaF, 5 mmol/L Na₄P₂O₇, 1 mmol/L Na₃VO₄, and 1% Nonidet P-40 [all purchased from Sigma]). Extracts (1×10^6 cell equivalents per lane) were suspended with an equal volume of 2x loading buffer (0.1 mol/L Tris-HCl [pH 6.8], 4% SDS, 0.005% bromophenol blue, and 20% glycerol) containing 2-ME (0.7 mol/L) and subjected to 4% to 20% Tris-

glycine gel electrophoresis (NOVEX, San Diego, Calif). Gels were then transferred to PVDF membranes (BIO-RAD, Hercules, Calif) with a Trans Blot apparatus (NOVEX). The membranes were immersed overnight in Tris-buffered saline/Tween 20 containing 5% nonfat dry skim milk (Carnation, Los Angeles, Calif).

Immunoreactive proteins were detected through use of antibodies against various kinases and phosphorylated kinases. The antibodies used were rabbit anti-ERK1/2 Ab, anti-phospho-ERK1/2 Ab, anti-p38 Ab, anti-JNK Ab, and anti-phospho-JNK Ab (New England Biolabs, Beverly, Mass), as well as rabbit anti-phospho-p38 Ab (Santa Cruz Biotechnology, Santa Cruz, Calif). All antibodies were suspended in Tris-buffered saline/Tween 20 containing 5% skim milk for 1 hour. After washing, the membranes were incubated with peroxidase-linked donkey antirabbit Ig Ab (Amersham, Arlington Heights, Ill) for 1 hour. After washing, membrane-bound antirabbit Ig Ab was visualized with enhanced chemiluminescence. Western blot detection reagents (Amersham) and Hyper ECL luminescence detection film (Amersham) were used. In some cases, protein standards (New England Biolabs) were run in parallel and served as positive controls.

Flow cytometry analysis for ICAM-1 surface expression

When PBECs were grown to near confluence, the cells were treated either with 100 ng/mL of IL-17, 100 ng/mL of IL-4, 100 ng/mL of IL-13, or 100 U/mL of IFN- γ or with IL-17 (100 ng/mL) in combination with either IFN- γ (100 U/mL), IL-4 (100 ng/mL), or IL-13 (100 ng/mL) for 48 hours. The cells were harvested after treatment with 0.025% trypsin-EDTA and suspended in PBS containing 2% FBS and 0.02% sodium azide; 10^6 cells were then incubated with an anti-ICAM-1 mAb (R&D Systems) on ice for 30 minutes. After 3 washes with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated goat antimouse IgG (Gam-FITC, Biosource) on ice for 30 minutes. After 3 additional washes with PBS, the cells were resuspended in PBS and analyzed by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ). Mean fluorescence intensity was determined by subtracting the mean background fluorescence (negative control, Gam-FITC) from that measured with the mAb to ICAM-1. Values are expressed as means $\pm$ SDs.

Data analysis

The statistical significance of differences was determined by ANOVA. Any difference with a *P* value of less than .05 was considered to be statistically significant. When ANOVA indicated a significant difference, the Scheffé *F* test was used to determine the difference between groups.

RESULTS

IL-17 induces IL-6 and IL-8 production and MAP kinase activation in PBECs

As shown in Fig 1, A, stimulation of PBECs with IL-17 elicited a time-dependent increase in both IL-6 and IL-8 production. IL-17 significantly induced IL-6 and IL-8 production in comparison with control at 2 different doses at the 24-hour and 48-hour time points. However, though detectable expression of eotaxin gene was seen in unstimulated cells, IL-17 did not induce the gene expression for 2 CC chemokines, eotaxin and RANTES, known to be expressed in bronchial epithelial cells (Fig 1, B).

To determine the signaling pathway involved in IL-17-induced IL-6 and IL-8 expression, untreated or IL-17-stimulated cells were lysed at various time points,

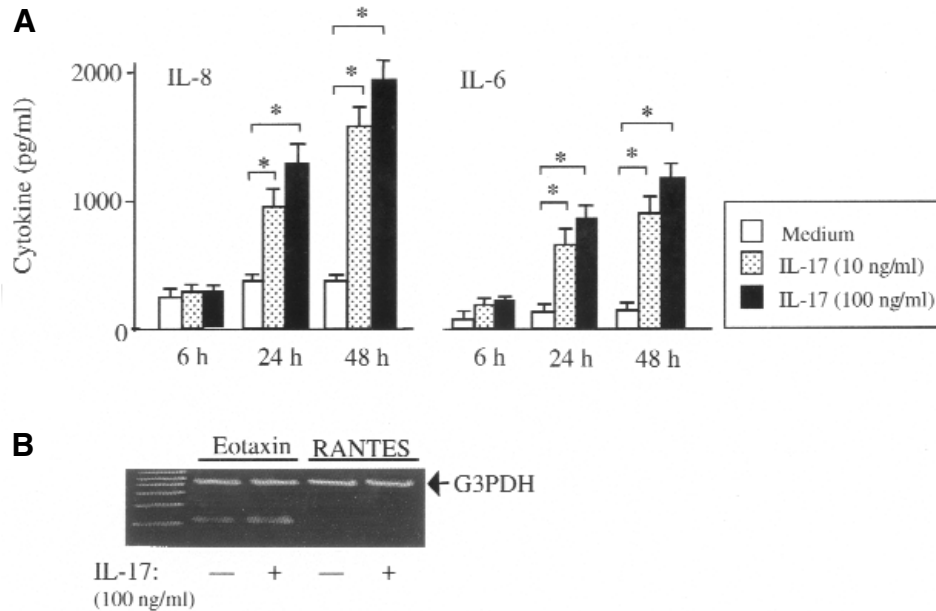


FIG 1. Analysis of IL-6, IL-8, eotaxin, and RANTES expression in PBECs stimulated by IL-17. **A**, The IL-6 and IL-8 protein release in the supernatant was determined by ELISA. The results are expressed as means $\pm$ SDs ($n = 6$). * $P < .05$ was considered significant vs control. **B**, Gene expression of eotaxin and RANTES. RT-PCRs were performed as described in the Materials and Methods section.

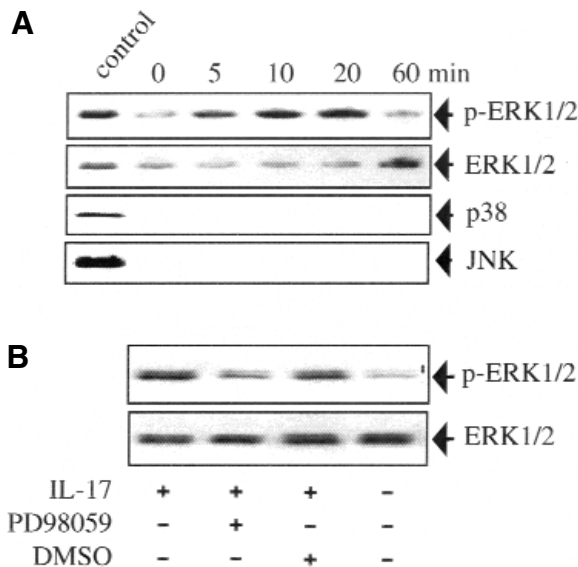


FIG 2. **A**, Kinetic activation of ERK1/2 by IL-17 in PBECs. PBECs were incubated with or without IL-17 (100 ng/mL) for different time points, as indicated. **B**, Effect of PD98059 on IL-17-induced phosphorylation of ERK1/2. PBECs were preincubated with PD98059 (10 μ mol/L) or DMSO vehicle control for 1 hour; this was followed by stimulation of the cells with medium or IL-17 for 20 minutes. The results shown are representative of 3 separate experiments.

and Western blot analyses were performed through use of various antibodies against various MAP kinases.

MAP kinases constitute 3 major groups of serine/threonine kinases: ERK1/2, p38, and JNK. The results showed that though there were no detectable p38 and

JNK kinases in the whole-cell lysates at any time points (Fig 2, **A**), Western blot analysis revealed the phosphorylation of ERK1/2 to reach a maximum at approximately 20 minutes and to return to baseline levels by 60 minutes (Fig 2, **A**). In addition, preincubation of the cells with an MEK inhibitor, PD98059 (10 μ mol/L), diminished the activation of ERK1/2 in PBECs, and preincubation with 0.1% DMSO did not affect the phosphorylation of ERK1/2 (Fig 2, **B**).

We next asked whether the activation of ERK1/2 was necessary for the stimulation of IL-6 and IL-8 production in PBECs. PBECs were stimulated with IL-17 in the presence or the absence of PD98059. As demonstrated in Fig 3, **A**, PD98059 inhibited, in a dose-dependent manner, IL-6 and IL-8 production, whereas treatment of the cells with DMSO did not affect the protein release. Decreased levels of IL-6 and IL-8 gene expressions were also noted in PD98059-treated cells but not in vehicle-treated cells, suggesting that the inhibitory effect was at the level of transcription (Fig 3, **B**). In addition, the protein levels of both IL-6 and IL-8 were unchanged in IL-17-treated cells in the presence of a p38 kinase inhibitor, SB202190, even at a dose of 10 μ mol/L (Fig 3, **A**).

IL-17 augments the expression of IL-6, IL-8, and ICAM-1 in cytokine-treated PBECs

To examine whether IL-17 modulates the expression of proinflammatory parameters in cells stimulated with either 1 T_H1 cytokine (IFN- γ) or 2 T_H2 cytokines (IL-4 and IL-13), we first conducted a comparative study of IL-6, IL-8, and ICAM-1 expression in PBECs after stimulation with individual cytokines. Stimulation of the cells with each of the 3 cytokines IL-17, IL-4, and IL-13

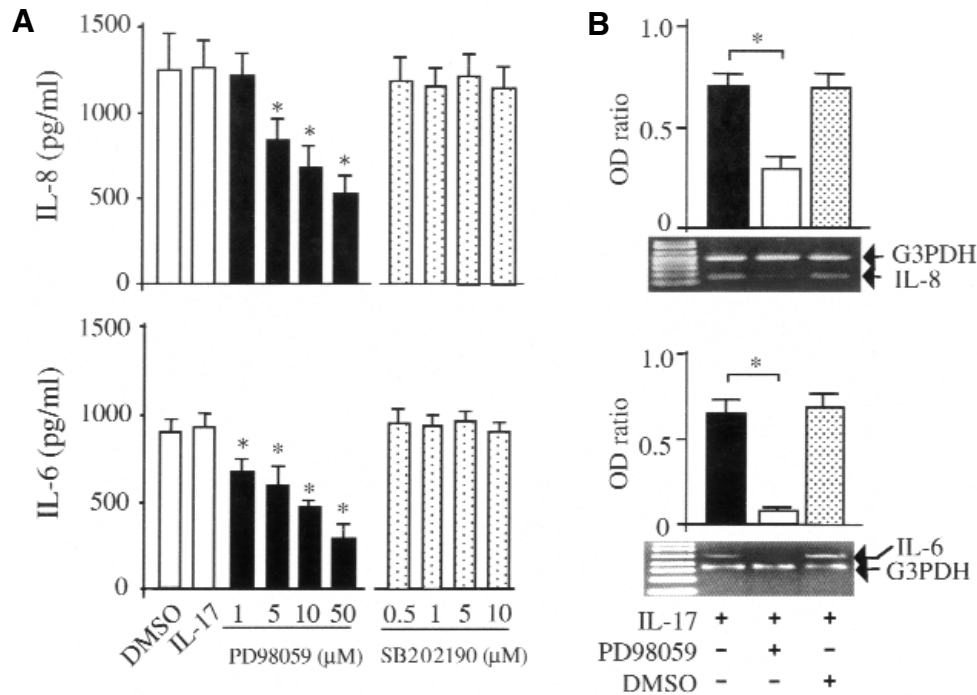


FIG 3. A, Effect of PD98059 on IL-6 and IL-8 expression. PBECs were preincubated with varying concentrations of PD98059, SB202190, or DMSO vehicle control for 1 hour; this was followed by stimulation with IL-17 for 24 hours. The results are expressed as means $\pm$ SDs ($n = 6$). * $P < .05$ in comparison with cultures without the addition of PD98059. **B**, Semiquantitative analysis of IL-6 and IL-8 gene expression by RT-PCR.

induces the expression of both IL-6 and IL-8 (Fig 4), and among all 4 cytokines IL-17 is the most potent inducer. However, IL-17 alone did not induce ICAM-1 expression, which is in contrast to the finding of a significant induction of ICAM-1 expression by 3 other cytokines (Fig 4). The combination of IL-17 and each of these 3 cytokines (IFN- γ , IL-4, and IL-13) significantly augmented the expression of IL-8 in comparison with that seen in cells stimulated with individual cytokines (Fig 4). Of interest is the finding that the expression of IL-6 and ICAM-1 is significantly augmented only when the cells are stimulated with a combination of IL-17 and IFN- γ ; this is in contrast to cells stimulated with either IL-17 or IFN- γ alone (Fig 4). At lower doses (0.1 and 1 ng/mL), IL-17 did not show any augmenting effect with IFN- γ (data not shown). The augmenting effect was not due to increased expression of IL-17R, because the expression of IL-17R mRNA was not upregulated by the treatment of the cells with each of the cytokines analyzed (data not shown).

DISCUSSION

IL-17 is a new cytokine produced by activated CD4⁺ T lymphocytes.² The biological function of IL-17 in the airway is not completely understood. We demonstrate, in this report, that IL-17 is able to induce IL-6 and IL-8 production through the activation of ERK1/2 kinase and to augment cytokine (IL-4, IL-13, and IFN- γ)-induced IL-8 production. As a corollary, a recent study of a rat model provided evidence that IL-17-induced neutrophil recruit-

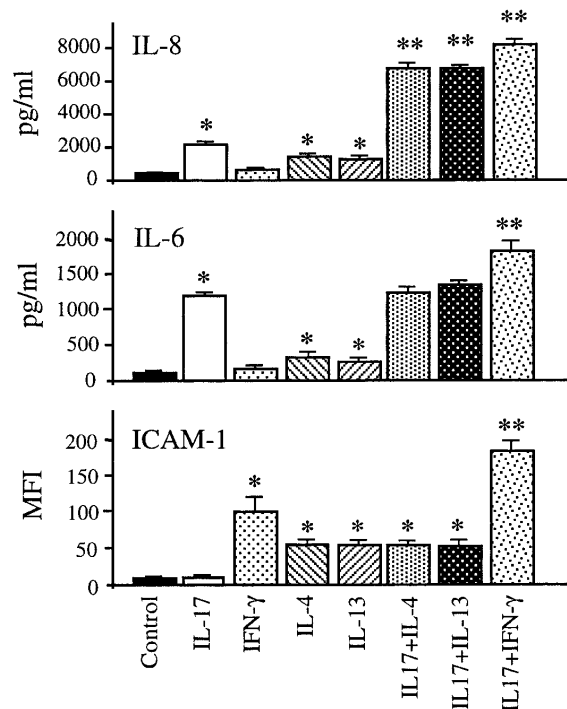


FIG 4. Effect of individual or combined cytokine stimulation. Experimental protocols and culture conditions are described in the Materials and Methods section. Significant differences between 2 experimental cultures are indicated. * $P < .05$ vs medium control. ** $P < .05$ vs cultures stimulated with individual cytokine; $n = 3$.

ment in the airways is mediated by the release of IL-8 and is inhibited by neutralizing anti-IL-8 Ab.^{13,14} Through use of transformed bronchial epithelial cell line, 16HBE14o-, a recent study showed that the IL-17-induced release of IL-8 was inhibited by both MAP p38 inhibitor SB202190 and MEK 1/2 inhibitor PD98059.¹⁶

In our study, the involvement of ERK kinase, but not of p38 or JNK, in IL-17-induced IL-6/IL-8 production was demonstrated through use of PBECs. This discrepancy might be the differential expression and activity of MAP kinases in transformed and primary epithelial cells. It is noted, however, that induction of IL-6/IL-8 is not completely inhibited by MEK 1/2 kinase inhibitor, PD98059, suggesting that 1 or more additional signaling pathways are also involved in IL-6/IL-8 production induced by IL-17 in primary epithelial cells. Work is ongoing to address this issue.

Of interest is the finding that IL-17 augments the expression of ICAM-1 on bronchial epithelial cells only when they are stimulated with IFN- γ and that the expression of IL-6 is enhanced when the cells are stimulated only with a combination of IL-17 and IFN- γ . These findings are consistent with those seen in IFN- γ -induced expression of ICAM-1 in keratinocytes in the presence of IL-17,^{13,14} but they are at variance with the finding that IFN- γ induces IL-6 production in keratinocytes.¹⁴ Previous studies have demonstrated that IL-17 alone did not affect ICAM-1 expression in keratinocytes but synergized with IFN- γ .^{13,14} IL-17 synergistically increased ICAM-1 surface expression and significantly induced soluble ICAM-1 production when IFN- γ was present.¹⁰ Similarly, IL-17 showed a synergistic effect with IFN- γ in IL-6, IL-8, GM-CSF, and growth-regulated oncogene α expression in keratinocytes.^{13,14} Additional evidence provided in our current study demonstrated an augmenting effect of IL-17 on IL-4- and IL-13-induced IL-8 production. When viewed together, these findings suggest a complex interaction between IL-17 and each of the T_H1 and T_H2 cytokines.

The mechanism by which IL-17 interacts with other cytokines in augmenting cytokine production and ICAM-1 expression is currently unclear. It is known that ICAM-1 is significantly expressed when signal transducers and activators of transcription 1 (STAT-1) and nuclear factor κ B are activated in fibroblasts.¹⁷ It is known that IFN- γ activates STAT-1 and that IL-17 activates STAT-1 and nuclear factor κ B in monocytes and macrophages, respectively.¹⁸⁻²⁰ The combination of these cytokines might stimulate the transcription of ICAM-1 expression synergistically; further work is needed to address these issues. IL-17R mRNA is detected in various cell types, including a bronchial epithelial cell line, A549.²¹ Treatment of the cells with IFN- γ did not affect expression of IL-17R in keratinocytes¹⁴ or in bronchial epithelial cells in this study, suggesting that the synergistic effect of IFN- γ and IL-17 is not exerted at the IL-17R level.

The expression of IL-17 has been found in various disease states. For example, increased numbers of IL-17 mRNA-expressing mononuclear cells have been found in cerebral spinal fluids from patients with multiple scler-

osis, and the highest number of IL-17⁺ cells was found in blood during clinical exacerbations.³ In addition, immunostainings of synovium in patients with rheumatoid arthritis showed that IL-17-producing cells were found in the T cell-rich area,⁵ and expression of IL-17 was detected in kidney biopsy specimens from patients experiencing graft rejection.⁹ Furthermore, increased numbers of IL-17-expressing cells were found in bronchial biopsy samples of patients with asthma.²² These findings, together with the fact that CD4⁺ T cells are the major cell source of IL-17 and the fact that subsets of T cells with T_H1 and T_H2 cytokines are involved in different inflammatory responses, suggest a role of IL-17 in leukocyte recruitment and the expression of inflammatory responses. It is tempting to speculate that IL-17 might play a general proinflammatory role through its specific activity and through a combined activity with other cytokines of both T_H1 and T_H2 subsets in the expression of inflammation.

In conclusion, this study indicates that IL-17 alone or in combination with either 1 T_H1 cytokine (IFN- γ) or 2 T_H2 cytokines (IL-4 and IL-13) modulates the function of bronchial epithelial cells via—at least in part—activation of ERK1/2 in PBECs, suggesting a potential role of IL-17 in the pathogenesis of airway inflammation.

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