Interleukin-17A modulates human airway epithelial responses to human rhinovirus infection

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Wiehler S, Proud D. Interleukin-17A modulates human airway epithelial responses to human rhinovirus infection. Am J Physiol Lung Cell Mol Physiol 293:L505–L515, 2007. First published June 1, 2007; doi:10.1152/ajplung.00066.2007.—Human rhinovirus (HRV) infections are associated with exacerbations of asthma and chronic obstructive pulmonary disease that are characterized by a selective neutrophil infiltration. IL-17A, a cytokine derived primarily from activated T cells, has been linked to neutrophilic inflammation of the airways. We hypothesized that IL-17A alters the response of HRV-infected epithelial cells to modulate airway inflammatory cell populations. IL-17A synergistically enhanced HRV-16-induced epithelial production of the neutrophil chemottractant IL-8, as well as human β-defensin-2 (HBD-2), a chemottractant for immature dendritic cells and memory T cells, but suppressed viral production of the eosinophil chemottractant, RANTES. These effects were not due to alterations of viral uptake or replication by IL-17A. The synergy between HRV-16 and IL-17A for IL-8 protein production was both dose- and time-dependent. IL-8 induction by IL-17A or HRV-16, alone and in combination, was reduced by inhibitors of the p38 and p44/42 MAPK pathways. By contrast, induction of HBD-2 depended on the activation of the p38 and JNK pathways. The ability of IL-17A to synergistically enhance HRV-induced IL-8 is mediated posttranscriptionally, since IL-8 promoter activation by the combination of the two stimuli was merely additive, whereas the combination of IL-17A and HRV-16 led to stabilization of IL-8 mRNA. Similarly, stimulation of HBD-2 promoter constructs by the combination of IL-17A and HRV-16 was no more than the sum of the individual responses. Further studies are needed to examine HBD-2 mRNA stability. Taken together, these data represent the first demonstration that IL-17A can modify epithelial responses to HRV in a manner that would be expected to favor the recruitment of neutrophils, immature dendritic cells, and memory T cells to the airways.

HUMAN RHINOVIRUS (HRV) infections are the primary cause of the common cold and are a major risk factor associated with exacerbations of asthma and chronic obstructive pulmonary disease (COPD) (18, 31, 44). The mechanisms by which HRV infection induces exacerbations of asthma and COPD are poorly understood, but it is known that airway inflammation is increased.

Although HRV can interact with several cell types in in vitro systems, the airway epithelial cell is the major site of HRV infection and, indeed, is the only cell type proven thus far to be infected in vivo. Moreover, it has been shown that virus also spreads to the lower airway epithelium (3, 35). In contrast to some other viruses, HRV does not induce overt epithelial cytotoxicity. Thus it is reasonable to hypothesize that increased airway inflammation in exacerbations of asthma and COPD is triggered by viral-induced alterations in epithelial cell biology. In support of this, numerous studies have demonstrated that HRV infection of epithelial cells in vitro leads to the generation of a wide variety of proinflammatory chemokines and cytokines, including IL-8, ENA-78 (CXCL5), IP-10 (CXCL10), RANTES (CCL5), IL-1, and IL-6 (8, 40, 43, 46–48), as well as host defense molecules, including human β-defensin-2 (HBD-2) and nitric oxide (38, 41). Several of these products also have been detected in nasal lavages during HRV infections of normal subjects in vivo, but, to date, there have been few studies examining the presence of these molecules in lower airway secretions during viral exacerbations of asthma or COPD.

Given the wide range of chemokines that can be generated from HRV-infected epithelial cells in vitro, it may be expected that a diverse array of inflammatory cell types may be recruited to the airways during viral exacerbations of asthma and COPD. Instead, HRV infections in asthmatics are associated with a selective recruitment of neutrophils and, to a lesser extent, a selective recruitment of lymphocytes to the airways (17, 36). Importantly, the degree of neutrophil degranulation correlates with symptom severity in virus-induced asthma exacerbations (50). Many acute exacerbations of asthma and COPD seen in the clinical setting are also characterized by increased airway neutrophilia consistent with a viral etiology (9, 33, 39, 51).

Although several chemoattractants could conceivably play a role in neutrophil recruitment to the airways, some, such as leukotriene B4, have not been detected during HRV infections. By contrast, substantial data suggest that IL-8 plays a major role in neutrophil chemotaxis during HRV infections. Not only has IL-8 been detected in airway lavage and sputum during HRV infections (12, 49), but also IL-8 levels correlate with sputum neutrophil counts in subjects experiencing exacerbations either of asthma or COPD (4, 9, 33).

IL-17A is a proinflammatory cytokine that is a member of a larger gene family with six members (IL-17A–F). In humans, IL-17A is produced mainly by activated memory T cells of the CD45RO⁺CD4+ and CD45RO⁺CD8⁺ phenotypes (45). It exerts its actions via binding to the IL-17 receptor (IL-17R), a type 1 membrane receptor that is present on a wide range of cell types, including epithelial cells (54). Numbers of IL-17A-expressing T cells are increased in the airways of patients with asthma (30), and levels of IL-17A detected in sputum of patients with asthma and chronic bronchitis correlate with...
airways hyperresponsiveness (2). A growing body of evidence, particularly from murine models, indicates a role for IL-17A in host defense and neutrophil recruitment within the lungs. Intratracheal instillation of IL-17A induces significant neutrophil recruitment that is associated with increased expression of IL-8 (or rodent homologs) within the airways (26). Consistent with this, mice with homozygous deletion of IL-17R show markedly diminished recruitment of neutrophils to the lung after gram-negative pathogen exposure (57). Similarly, neutralization of IL-17A reduced airway neutrophilia in allergen-sensitized and -challenged mice, whereas lung eosinophilia was greater in allergen-challenged mice treated with anti-IL-17A (13). It is known that IL-17A exerts effects on human epithelial cells, including stimulating production of IL-8, which may contribute to the neutrophil recruitment induced by IL-17A (19, 23, 27, 37). Interestingly, IL-17A was the most powerful stimulus (among a wide panel of cytokines tested) for inducing production of HBD-2 from human epithelial cells (21). It has been postulated that HBD-2 plays an important role as a link between innate and specific immune responses to HRV infection by recruiting immature dendritic cells, which could capture and present viral antigens, and memory T cells (38).

The current studies were undertaken to test the hypothesis that IL-17A may modulate epithelial responses to HRV-16 in a manner that would selectively alter airway inflammatory responses to favor neutrophil, dendritic cell, and memory T cell recruitment. We showed that IL-17A enhanced HRV-induced expression of the neutrophil chemoattractant IL-8 and of HBD-2, a chemoattractant for immature dendritic cells and memory T cells, but suppressed viral induction of the eosinophil chemoattractant, RANTES.

MATERIALS AND METHODS

Materials. The following reagents were purchased from the indicated suppliers: Ham’s F-12 medium, Eagle’s minimal essential medium, HBSS, penicillin-streptomycin-ampicillin B, and l-glutamine from MediCorp (Montreal, Quebec, Canada); TRIZol reagent, sodium pyruvate, nonessential amino acids, gentamicin, FBS, dNTPs, oligo(dT), and Superscript II from Invitrogen Life Technologies (Burlington, Ontario, Canada); bronchial epithelial cell growth medium (BEGM) from BioWhittaker (Walkersville, MD); SB-203580, PD-98059, and SP-600125 from Calbiochem-Novabiochem (San Diego, CA); TaqMan master mix, 20× GAPDH, RANTES gene expression kit, RNase inhibitor, and reverse transcriptase from Applied Biosystems (Foster City, CA); Pfu polymerase from Stratagene (Foster City, CA); antibody pairs for RANTES, rhRANTES, and rhIL-17A from R&D Systems (Minneapolis, MN); the firefly luciferase reporter plasmid pGL3-basic, the renilla luciferase plasmid pRL-null, genomic DNA, and the dual-luciferase reporter assay system from Promega (Madison, WI); FuGENE 6, leupeptin, aprotinin, and pepstatin from Roche Diagnostics (Laval, Quebec, Canada); and antibodies for phospho-p44/42, phospho-p38, phospho-JNK, total p44/42, total p38, and total JNK from Cell Signaling Technology (Danvers, MA). All other chemical were purchased from Sigma-Aldrich (St. Louis, MO).

Virus and cell lines. The BEAS-2B cell line was a gift from Curtis Harris (National Cancer Institute, Bethesda, MD), whereas the human bronchial epithelial cell-1 (HBE-1) cell line was generously provided by Reen Wu (University of California, Davis, CA). HRV type 16 (HRV-16) and WI-38 cells were purchased from the American Type Culture Collection (Rockville, MD). Viral stock solutions of HRV-16 were generated by passage in WI-38 cells and were purified by centrifugation through sucrose to remove ribosomes and soluble factors as previously described (10). Viral titers were determined using WI-38 cells grown in 96-well plates as previously described (40).

Epithelial cell cultures. Primary HBE cells were obtained by protease digestion of nontransplanted normal human lung as previously described (6). Primary cells, BEAS-2B, and HBE-1 cells were grown on six-well culture plates in serum-free epithelial growth medium (BEGM). Twenty-four hours before stimulation with virus, cells were cultured in BEGM, from which hydrocortisone had been withdrawn, and this hydrocortisone-free medium was used for all experimental exposures.

Viral infection and stimulation of epithelial cells. Subconfluent cell monolayers were washed with HBSS and exposed to doses of 10^3.5 to 10^5.5 50% tissue culture-infective dose (TCID50) U/ml HRV-16, 0.3–100 ng/ml IL-17A, or a combination of the two. Cells were incubated at 34°C for the times indicated, at which point supernatants and total cellular RNA were harvested for subsequent analysis. To determine whether MAPK inhibitors have any effect on protein expression, primary epithelial cells were preincubated with SB-203580, PD-98059, or SP-600125 (10 μM in each case) for 1 h. Cells were then exposed to HRV-16, 10 ng/ml IL-17A, or HRV-16 + IL-17A for 24 or 48 h in the presence of inhibitors. Supernatants were collected, and RNA was extracted with TRIZol. Assay of lactate dehydrogenase (Promega) established that the drugs were not cytotoxic under these conditions. At the concentrations selected, each of the MAPK inhibitors has been shown to be selective (7). Moreover, preliminary dose-response curves in both BEAS-2B and primary epithelial cells indicated that effects of SB-203580 and PD-98059 were optimal at 10 μM (data not shown). Experiments were also performed to determine whether IL-17A altered viral replication. In these experiments, primary epithelial cells were exposed for 1 h with HRV-16 alone or with HRV-16 and 10 ng/ml IL-17A, and cells were washed to remove free virus. Fresh media or 10 ng/ml IL-17A were added, supernatants were collected after 24 h, and viral titer was assayed using WI-38 cells.

Real-time RT-PCR. Gene expression for IL-8, HBD-2, and RANTES was measured using the Applied Biosystems model 7900 Sequence Detector. To ensure that there was no contribution of genomic DNA to amplification, samples were treated with DNase I (Ambion, Austin, TX) before use. For IL-8 and HBD-2, input RNA (400 ng) was reverse transcribed to cDNA, followed by PCR amplification in the presence of specific forward and reverse primers and fluorescently labeled probes specific for each gene of interest. Primers and probe for HBD-2 were as previously described by us (38). Primers and probe for IL-8 were forward primer 5’-CCTGGCCGTTGCTCTCTTG-3’, reverse primer 5’-TTAGCACTCTTTGCGAAAACCTG-3’, probe 5’-FAM-CCTTCTGTATTCTGCAGCTCTGTTGAA-TAMRA-3’. Expression of RANTES was assessed using a primer and probe gene expression kit available from Applied Biosystems. For RANTES, 1 μg of RNA was reverse transcribed using oligo(dT) and Superscript II, and 1 μl of cDNA was used as the template in the real-time PCR reaction. Analysis of the housekeeping gene GAPDH was performed on each sample using a primer and probe kit obtained from Applied Biosystems. For each gene of interest, we confirmed that the efficiency of amplification was comparable to that of GAPDH (data not shown), permitting us to assess fold induction of genes as previously described (41).

For studies of IL-8 mRNA stability, BEAS-2B cells were exposed to virus, IL-17A, or the combination of the two for 2 h, and then actinomycin D was added at a final concentration of 10 μg/ml. Cells were harvested for RNA isolation at 0, 30, 60, 120, and 180 min post-actinomycin D exposure. IL-8 mRNA levels were determined by real-time RT-PCR. Levels of GAPDH were also monitored in these experiments and showed no changes over the time periods assessed for any of the treatment groups.

ELISAs. Measurements of IL-8 and HBD-2 were performed by previously described ELISAs sensitive to 30 pg/ml and 20 pg/ml.
respectively (38, 47). Levels of RANTES protein were measured as per manufacturer’s protocol (R&D Systems). Sensitivity of the RANTES assay was 30 pg/ml.

Western blotting for MAPK pathways. To reduce background MAPK activation, subconfluent monolayers of BEAS-2B cells were preincubated in BEBM for 1 h and then stimulated with HRV-16, IL-17A, or a combination of the two in BEGM-4 (BEGM without hydrocortisone, epidermal growth factor, bovine pituitary extract, and epinephrine). After 30 min, 1 h, and 3 h of culture, supernatants were removed, and cells were washed with HBSS and lysed in ice-cold lysis buffer (1% Triton X-100, 5 mM EDTA, pH 7.4, 50 millimolars/ml aprotonin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 mM PMSF, 2 mM sodium orthovanadate, 20 mM sodium pyrophosphate, and 50 mM sodium fluoride) for 30 min on ice with gentle agitation. Cells were scraped, sonicated, and centrifuged. Protein concentrations for the Triton X-100-soluble lysates were determined by using a DC Protein Assay (Bio-Rad, Montreäl, Québec, Canada). Equivalent amounts of protein (30 µg per lane) were separated by SDS-PAGE, and proteins were then transferred to a polyvinyl difluoride membrane. Membranes were blocked with 5% skim milk for 1 h and probed with a 1:750 dilution of phospho-specific anti-p44/42, anti-p38, or anti-JNK antibody overnight at 4°C. Membranes were washed and then incubated for 1 h with a 1:750 dilution of horseradish peroxidase-conjugated anti-rabbit Ig antibody (General Electric Healthcare BioSciences, Piscataway, NJ). Proteins were visualized with ECL substrate reagent (General Electric Healthcare BioSciences). Equal loading was further determined by stripping each membrane and reprobing with an antibody against total p44/42, p38, or JNK protein.

IL-8 and HBD-2 plasmid construction. A 174-bp IL-8 promoter construct, corresponding to the sequence from -166 to +8 (relative to the transcriptional start site) of the 5′-flanking region of the human IL-8 gene, was generated from human genomic DNA using forward 5′-CGGGTACCCATCTGGATAGTTAAGGAAC-3′ and reverse 5′-CCCAAGCTTCCATGAGTCCGTTGG-3′ primers. The 174-bp promoter was cloned upstream of the inducible firefly luciferase gene in pGL3basic via KpnI and HindIII restriction sites (underlined). Mutations to the NF-κB, C/EBPα and activator protein-1 (AP-1) sites were generated separately in the 174-bp construct. Site-directed mutagenesis was performed using the following primers: NF-κB mutant, forward primer 5′-GCCAATCGTGGAGTTAAAATCGCATCAATGAAAGATGAAGGAGG-3′, reverse primer 5′-CTCATCTTCTATTGTCAGTAAATTCCACAGGTGTC-3′; CE/BP/ NF-κBIL-6, forward primer 5′-GAGGGGATGGGCTATGGAATCCATTG-3′, reverse primer 5′-GCAAATCGTGGAGTTAAAATCGCATCAATGAAAGATGAAGGAGG-3′, reverse primer 5′-CCCAAGCTTCCATGAGTCCGTTGG-3′, reverse primer 5′-CCCAAGCTTCCATGAGTCCGTTGG-3′, reverse primer 5′-CCCAAGCTTCCATGAGTCCGTTGG-3′. Boldface, lowercase characters denote mutation sites. All mutant constructs were cloned into pGL3basic. Successful generation of all constructs were confirmed by sequencing (University of Calgary DNA sequencing facility).

Experiments were also performed to determine whether inhibitors of the MAPK pathways could affect promoter activity. In these experiments, after overnight recovery from transfection, cells were pretreated with either 10 µM SB-203580, 10 µM PD-98059, 10 µM SP-600125, or vehicle control for 1 h and then stimulated as usual for 5 h in the presence of inhibitors.

Statistical analysis. For normally distributed data, appropriate one-way or repeated-measures ANOVA was used to assess significant differences with post hoc analysis using Fisher’s protected least significant difference tests. Alternatively, paired t-tests were used. For data that were not normally distributed, analysis was performed using Kruskal-Wallis or Friedman ANOVA followed by post hoc analysis using Wilcoxon matched pairs signed rank test. To determine whether there was synergy between HRV-16 and IL-17A, the sum of virus and IL-17A alone was compared with HRV-16 + IL-17A. Wilcoxon signed rank test was used to determine differences in real-time data, and a paired t-test was used for protein data. For all statistical tests, a P value ≤ 0.05 was assumed to be significant.

RESULTS

IL-17A modulates virally induced message and protein production from primary bronchial epithelial cells. IL-17A alone induced modest induction of IL-8 mRNA and protein from primary HBE cells and was a less effective stimulus in this regard than purified HRV-16 (Fig. 1, A and B). A striking and significant (P < 0.05) synergistic production of IL-8 was seen, however, when cells were exposed to the combination of HRV-16 and IL-17A for 24 h. Consistent with a previous report (55), IL-17A was an effective stimulus for production of HBD-2 mRNA and protein (Fig. 1, C and D). Although IL-17A was more effective at stimulating HBD-2 expression from primary bronchial epithelial cells than HRV-16 alone, the combination of the two stimuli cause a marked synergistic induction of HBD-2 mRNA and protein at 48 h post-stimulation. The synergistic response seen for IL-8 and HBD-2, however, is selective. IL-17A alone is an ineffective stimulus for inducing RANTES expression. Interestingly, however, it significantly inhibited HRV-16-induced RANTES gene and protein production at 48 h after exposure (Fig. 1, E and F).

We also examined the interaction of UV-inactivated HRV-16, which is incapable of replication, and IL-17A (data not shown). UV-treated HRV-16 still induced IL-8 protein production, although less effectively than intact virus in matched experiments (6,351 ± 1,960 pg/ml IL-8 with UV-HRV-16 and 13,159 ± 3,178 pg/ml IL-8 with HRV-16). Co-stimulation with UV-HRV-16 and IL-17A still induced a significant increase in IL-8 generation although, again, to a lesser extent than with fully functional HRV-16 (8,087 ± 2,022 pg/ml IL-8 with UV-HRV-16 + IL-17A and 24,145 ± 2,961 pg/ml with HRV-16 + IL-17A), indicating that synergistic induction of IL-8 is not dependent on replicating virus. We (38) have previously shown that HRV-16-induced production of HBD-2 is absolutely dependent on replicating virus. Consistent with this, stimulation of epithelial cells with a combination of UV-HRV-16 and IL-17A induced no more HBD-2 protein than IL-17A alone. RANTES expression is also replication dependent (43), and costimulation with UV-HRV-16 and IL-17A did not produce RANTES (data not shown).

IL-17A does not modify viral replication in primary bronchial epithelial cells. To determine whether IL-17A exerts its effects by altering viral uptake or replication, viral titer was determined by stripping each membrane and reprobing with an antibody against total p44/42, p38, or JNK protein.

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with HRV-16 or with HRV-16 and IL-17A. Cells were washed, and fresh medium, or medium containing IL-17A, was placed on the cells for an additional 24 h. Viral titers were not altered by IL-17A regardless of whether it was present only during viral exposure, only during subsequent incubation, or during both phases (Fig. 2).

The effects of IL-17A on viral generation of IL-8 are both dose- and time-dependent in BEAS-2B cells. The BEAS-2B epithelial cell line has been used extensively as a model system for studies of the production of epithelial chemokines, including IL-8, in response to a range of stimuli, including HRV (11, 34, 47). This cell line also has been effectively used for transfection studies using IL-8 promoter constructs (5, 24). In the current studies, we chose to utilize this cell line for experiments requiring large numbers of cells and for IL-8 promoter construct studies, as we were unable to consistently transfect these constructs into primary cells. To determine whether BEAS-2B cells were appropriate for use in IL-8 promoter experiments, we confirmed that synergistic induction of IL-8 by HRV-16 and IL-17A also was observed in this cell line, and also performed comprehensive dose-response and kinetics studies. We also established that IL-17A did not alter viral uptake or replication in BEAS-2B cells (data not shown).

BEAS-2B cells stimulated with increasing doses of IL-17A induced a modest increase in IL-8 protein production ($110 \pm 23$ pg/ml with 0.3 ng/ml IL-17A to $317 \pm 36$ pg/ml with 10 ng/ml) at 24 h (Fig. 3A). HRV-16 infection alone induced $5,546 \pm 778$ pg/ml IL-8 protein. The effects of IL-17A on viral generation of IL-8 were dose dependent ($P < 0.001$ by ANOVA). Post hoc analysis showed that responses were statistically greater than the sum of the two stimuli alone for concentrations of IL-17 of 0.3 ng/ml or greater. Generation of
IL-8 by each stimulus was time dependent ($P < 0.001$ by ANOVA) with significant synergy between IL-17A and HRV-16 seen as early as 3 h postexposure (Fig. 3B). Unstimulated cells produced $<105$ pg/ml IL-8 protein at all time points examined.

IL-17A does not modify virally induced activation of the MAPK pathways as assessed by Western blotting in BEAS-2B cells. A relatively low dose of HRV-16 ($3 \times 10^3$ TCID$_{50}$ units) led to the activation of all three MAPK pathways (Fig. 4). Phosphorylation of p38, p44/42, and JNK were induced within 30 min after exposure to HRV and maintained until 3 h postinfection. By contrast, IL-17A alone did not increase p44/42 or JNK phosphorylation over unstimulated conditions nor did it modify p44/42 or JNK activation in virally infected epithelium. At 30 min, there was slight activation of the p38 pathway with IL-17A, but phosphorylation of p38 was not observed at 1 and 3 h post-stimulation. Again, there was no appreciable enhancement in the p38 phosphorylation profile when cells were stimulated with HRV-16 alone or HRV-16 + IL-17A.

Effects of MAPK inhibitors on IL-8 protein production from primary epithelial cells stimulated with HRV-16 and IL-17A alone and in combination. Although no effects of IL-17A on p44/42 or JNK activation were observed by Western blotting, it is possible that low level activation may not be detectable by this method. To further define the role of MAPK pathways in the synergistic induction of IL-8 by HRV-16 and IL-17A, therefore, we used selective inhibitors of these pathways. The inhibitors were first confirmed to have no effect on cell viability or rhinovirus replication (data not shown). In primary epithelial cells, inhibitors of either the p38 (SB-203580) or p44/42 (PD-98059) pathways significantly inhibited IL-8 protein production induced by either HRV-16, IL-17A, or a combination of the two stimuli (Fig. 5). An inhibitor of the JNK pathway (SP-600125) had no effect on induction of IL-8 by any of the stimuli used. An identical pattern of response to each MAPK inhibitor was seen when levels of IL-8 mRNA were examined. Moreover, both SB-203850 and PD-98059 showed identical inhibitory effects using each stimuli alone and in combination in BEAS-2B cells (data not shown).

Effects of HRV-16 and IL-17A on IL-8 promoter activation in BEAS-2B cells are additive, not synergistic. To determine whether synergy between IL-17A and HRV-16 was seen at the level of transcription, we performed studies using several IL-8 promoter-luciferase constructs transfected into the BEAS-2B cell line. We first examined varying lengths of the IL-8 promoter upstream of the transcriptional start site. Three promoter constructs were generated ranging from 720 to 174 bp in

![Graph showing IL-8 protein production](http://ajplung.physiology.org/)

**A**. BEAS-2B cells were infected with HRV-16 in the presence or absence of IL-17A. Cells were exposed for an initial 1-h period to the stimuli shown. They were then washed, and the stimuli shown were added for a 2nd 24-h period. Values are means ± SE ($n = 5$). TCID$_{50}$, 50% tissue culture-infective dose.

**B**. ELISA analysis of IL-8 protein release at 3, 6, 12, and 24 h from HRV-16-, IL-17A-, and HRV-16 + IL-17A-stimulated BEAS-2B cells ($n = 4$). Control levels were below 105 pg/ml IL-8 protein at all time points. Asterisks indicate a significant ($P < 0.05$) difference between HRV-16 + IL-17A and the sum of HRV-16 and IL-17A alone. Values are means ± SE.
length. We found no difference in fold activation of IL-8 promoter activity by IL-17A, HRV-16, or the combination of the stimuli between any of the truncated constructs indicating that the 174-bp construct contains all of the transcription factor recognition sequences needed for full promoter activation (data not shown). Previous studies have shown that the AP-1, NF-kB, and NF-IL6 recognition sequences in the promoter have been associated with IL-8 activation in several cell types (25, 28, 56). To determine whether HRV-16 and IL-17A may activate the promoter using different transcriptional activation pathways, we generated constructs in which the NF-kB, NF-IL6, and AP-1 sites were individually mutated within the 174-bp construct. Consistent with data on induction of endogenous mRNA and protein, IL-17A was a weak activator of the wild-type 174-bp IL-8 promoter (Fig. 6A). HRV-16 was a more robust stimulus, inducing ~14-fold activation. Importantly, the combination of IL-17A and HRV-16 induced no more than an additive response. Mutation to the NF-kB site significantly inhibited promoter activation by HRV-16, IL-17A, and the combination of the two stimuli. A modest but significant inhibition was observed for HRV-16 alone and in combination with IL-17A when the NF-IL6 site was altered. No effect was seen with the mutated AP-1 construct.

Given that MAPK inhibitors modulated induction of IL-8 mRNA and protein in response to HRV-16 and IL-17A alone and in combination, we assessed whether these inhibitors had any effect on activation of the wild-type 174-bp IL-8 promoter construct. There was no effect of any of the inhibitors on promoter activation in response to HRV-16, IL-17A, or the combination of the two stimuli (Fig. 6B). Taken together, these data indicate that synergistic induction of IL-8 in response to HRV-16 and IL-17A, and inhibitory effects of p38- and p44/42 MAPK-dependent pathways on induction by these stimuli alone and in combination, are not mediated at the level of transcription.

The combination of HRV-16 and IL-17A results in increased stability of IL-8 mRNA compared with either stimulus alone in BEAS-2B cells. Given that the synergistic production of IL-8 by HRV-16 and IL-17A did not occur at the level of transcription, we assessed the effects of the two stimuli alone and in combination on mRNA stability using actinomycin D chase experiments. Preliminary data showed that synergistic induction of steady state mRNA levels was first observed at 2 h after viral exposure (data not shown), consistent with the initial synergy of protein at 3 h (Fig. 3). Thus we used a 2-h
stimulation before addition of actinomycin. In cells stimulated with IL-17A alone, mRNA levels decreased by 50% by 50 min after addition of actinomycin D. When cells were exposed to HRV-16 alone, 50% decay occurred by 70 min after addition of actinomycin D. When the two stimuli were used in combination, however, mRNA was significantly more stable with 50% decay being observed only after 165 min (Fig. 7).

**Effects of MAPK inhibitors on HBD-2 production from primary epithelial cells stimulated with HRV-16 and IL-17A alone and in combination.** The role of MAPK pathways in the regulation of epithelial production of HBD-2 has not been previously examined. To determine whether the signaling pathways involved in synergistic induction of HBD-2 from primary epithelial cells stimulated with HRV-16 and/or IL-17A were the same as those involved in IL-8 generation, inhibitors of the MAPK pathways were used. Inhibition of the p38 pathway with SB-203580 led to a significant decrease in HBD-2 protein in response to IL-17A and HRV-16 and IL-17A in combination (Fig. 8). In contrast to data observed for IL-8 generation, however, SP-600125, the JNK pathway inhibitor, significantly reduced HRV-16-, IL-17A-, and HRV-16 + IL-17A-stimulated HBD-2 expression, whereas blockade of the p44/42 pathway had no effect on HBD-2 protein generation. An identical pattern of response to each MAPK inhibitor was seen when levels of HBD-2 mRNA were examined (data not shown). Thus generation of IL-8 and HBD-2 depend on differential utilization of MAPK pathways.

**Effects of HRV-16 and IL-17A on HBD-2 promoter activation in primary bronchial epithelial cells and in HBE-1 cells are additive, not synergistic.** BEAS-2B cells did not generate HBD-2 in response to any stimulus tested. In agreement with this lack of response, no activation of HBD-2 promoter constructs was observed with any stimulus when these constructs were transiently transfected into this cell line (data not shown). Given that the HBE-1 cell line has previously been used to study HBD-2 generation and promoter activation in response to IL-17A (21), we evaluated this cell line and found that HBD-2 was produced by both IL-17A and HRV-16 and that synergy was observed when the two stimuli were used in combination (data not shown). We, therefore examined HBD-2 promoter activation both in this cell line and in primary epithelial cells
In light of the lack of synergy at the promoter levels, we also sought to examine HBD-2 mRNA stability in HBE-1 cells. Unfortunately, the time course required for these experiments was substantially longer than for IL-8, and technical difficulties arose with actinomycin D cytotoxicity. Alternative approaches, therefore, will need to be developed to examine this issue.

**DISCUSSION**

IL-17A is present in increased amounts in several inflammatory airway diseases including asthma, COPD, bacterial pneumonia, and cystic fibrosis (2, 29, 30, 57), and strong evidence implicates IL-17A in regulating neutrophilic recruitment to the airways. Viral exacerbations of asthma and COPD also are characterized by a neutrophilic infiltration of the airways (32), although recruitment of memory T cells and dendritic cells also occur. We now provide the first evidence to support the concept that IL-17A in the airways of patients with...
asthma or COPD may help regulate inflammatory responses during viral exacerbations by modulating the response of human airway epithelial cells to infection with HRV.

Consistent with previous reports (19, 23, 27, 37), we found that IL-17A directly induced IL-8 from primary HBE cells and from BEAS-2B cells, although, under the conditions employed, it induced less IL-8 than HRV-16 alone. The combination of the two stimuli, however, caused striking synergistic induction of IL-8 mRNA and protein. The ability of IL-17A to increase HRV-induced production of IL-8 was dose- and time-dependent, with synergy first observed at 2–3 h. Although IL-17A has been reported to augment TNF-α- or IL-1-induced production of IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) from other cell types (14), our data represent the first demonstration of the ability of IL-17A to modulate epithelial cell responses to HRV infection.

The ability of IL-17A to enhance viral production of IL-8 is selective because, whereas IL-17A alone was unable to induce production of RANTES from primary epithelial cells, it significantly suppressed production of RANTES in response to HRV-16 infection. These data are consistent with reports that IL-17A can downregulate TNF-α-induced production of RANTES from fibroblasts (1, 42). Suppression of HRV-16-induced production of RANTES in the setting of enhanced production of IL-8 would strongly favor neutrophilic recruitment into the airways.

The ability of IL-17A to alter responses to HRV-16 is not due to modulation of the interaction of HRV-16 with epithelial cells. Viral titers were not altered when IL-17A was present only during a 1-h exposure to HRV-16 and not during the subsequent incubation, indicating that viral binding and uptake were not affected. Consistent with this, we did not find any effect of IL-17A on expression of the major rhinovirus receptor, ICAM-1, on epithelial cells (data not shown). IL-17A did not alter viral titer even when present during both viral exposure and subsequent incubation, indicating that it does not affect viral replication.

Because studies have suggested that both HRV infection and IL-17A induce production of chemokines, including IL-8, from human epithelial cells via activation of MAPK pathways (11, 22, 27), we examined whether the synergistic production of IL-8 by IL-17A and HRV-16 could be explained by synergistic activation of these pathways. Although HRV infection led to phosphorylation of p38, p44/42, and JNK, IL-17A was a relatively ineffective stimulus with only modest activation of p38 and no effects on p44/42 and JNK by Western blotting. Moreover, IL-17A did not enhance virally induced MAPK phosphorylation over the 3-h time period studied, even though synergistic production of IL-8 occurred within this time frame. None of the earlier studies implicating MAPK pathways in the effects of IL-17A in bronchial epithelial cells looked directly at MAPK phosphorylation but relied solely on the use of MAPK pathway inhibitors. Using such inhibitors, we showed that IL-8 production by HRV-16 alone, IL-17A alone, and the combination of the two were inhibited by blocking the p38 and p44/42 MAPK pathways. By contrast, inhibition of the JNK pathway had no effect on responses to any of the stimuli. Given that inhibition of IL-17A-induced IL-8 production by SB-203580 and PD-98059 agrees with an earlier report (27), it is surprising that p44/42 phosphorylation was not detectable in IL-17A-stimulated cells. Although PD-98059 is among the more selective kinase pathway inhibitors (7), it has been reported to also inhibit the activation of ERK5 and thus could be exerting its actions via this pathway (20). Alternatively, phosphorylation of p44/42 at a level not detectable by Western blotting may explain this effect. Regardless of this, no firm conclusion can be drawn regarding the role of specific MAPK pathways in the synergistic induction of IL-8 by the combination of HRV-16 and IL-17A.

Activation of IL-8 promoter by the combination of IL-17A and HRV-16 was no more than additive compared with the responses to the two individual stimuli. Moreover, whereas inhibitors of the p38 and p44/42 MAPK pathways reduced induction of both mRNA and protein for IL-8, inhibitors of all three major MAPK pathways had no effect on promoter activation by HRV-16 or IL-17A alone or in combination. IL-8 promoter activation by IL-17A depended on activation of NF-κB but did not require either NF-IL6 or AP-1 recognition sites, consistent with reports that IL-17A-induced production of CCL20 from epithelial cells (22) and IL-8 from airway smooth muscle cells (52) also depended on NF-κB activation. As previously reported, promoter activation by HRV-16 was mediated via both NF-κB and NF-IL6 sites (58) but unaffected by mutation of the AP-1 site. Taken together, these data show that synergistic induction of IL-8 by HRV-16 and IL-17A is not regulated solely at the transcriptional level.

In contrast to the lack of synergy at the promoter, the stability of IL-8 mRNA was enhanced in the presence of the combination of HRV-16 and IL-17A compared with the presence of either stimulus alone. Consistent with this, IL-17A reportedly increases stability of TNF-α-induced IL-8 mRNA in human airway smooth muscle cells (15). As yet, however, no studies have identified the molecular basis for such an effect. Given that inhibitors of the p38 and p44/42 MAPK pathways modulated induction of IL-8 mRNA and protein without affecting transcriptional activation, it is of interest that the p38 MAPK pathway previously has been reported to stabilize IL-8 mRNA in another system (16). Additional studies using IL-8 3′-untranslated region (3′UTR) β-globin constructs will be necessary to clearly delineate the molecular mechanisms regulating IL-8 mRNA stability in response to HRV-16 and IL-17A.

HBD-2 is a potent chemoattractant for memory T cells and for immature dendritic cells bearing the CCR6 chemokine receptor. As such, it may play an important role in linking innate and adaptive immunity during viral infections (53). Because both HRV-16 and IL-17A have been shown to induce epithelial cell production of HBD-2 (21, 38), we also evaluated whether production was further modulated by the combination of the two stimuli. The combination of IL-17A and HRV-16 led to a striking synergistic response, producing levels of HBD-2 protein higher than we have ever recorded previously from epithelial cell cultures. The induction of HBD-2 is regulated differently than IL-8. Inhibition of the p38 MAPK pathway significantly inhibited induction of HBD-2 induced by IL-17A alone or in combination with HRV-16, whereas induction of HBD-2 by HRV-16 alone was not inhibited. In contrast to effects on IL-8, however, PD-98059 was without effect on induction of HBD-2 by IL-17A and HRV-16 either alone or in combination, but inhibition of the JNK pathway reduced HBD-2 production by all stimuli. Inhibition of HBD-2 production in response to IL-17A by SP-600125 suggests that activa-
tion of the JNK pathway occurs at a level below the sensitivity of Western blotting. HBD-2 induction by the combination of HRV-16 and IL-17A in the presence of either SB-203580 or SP-600125 was still more additive compared with levels induced by either stimulus alone in the presence of the same inhibitors, suggesting that although p38 and JNK pathways play a role in HBD-2 production, they do not underlie the synergistic response observed with HRV-16 + IL-17A.

HBD-2 promoter activation by the combination of IL-17A and HRV-16 was no more than additive compared with responses to the two stimuli individually in either the HBE-1 cell line or primary epithelial cells transiently transfected with the HBD-2 promoter. In contrast to IL-8, which has multiple adenosine/uridine-rich motifs associated with mRNA instability in the 3′UTR of its mRNA, analysis of the first 500 bp of 3′UTR of HBD-2 mRNA did not indicate the presence of such motifs. Consistent with this, HBD-2 mRNA is more stable than IL-8 mRNA, and we were not able to perform actinomycin D experiments over the time course required due to problems with actinomycin D cytotoxicity. Generation of β-globin constructs will be necessary, therefore, to determine whether effects on mRNA stability contribute to synergistic induction of HBD-2.

In conclusion, we provide the first demonstration that IL-17A can profoundly modulate responses of epithelial cells to infection with HRV-16. Synergistic induction of IL-8 and HBD-2, in the face of reduced RANTES production, would be expected to lead to marked recruitment of neutrophils, memory T cells, and dendritic cells in the airways and could potentially explain the selective inflammatory cell profile seen during viral exacerbations of airway disease. We also demonstrated differential involvement of specific MAPK pathways in the generation of IL-8 and HBD-2 by HRV-16 and IL-17A and showed that synergistic production of IL-8 is regulated at the level of mRNA stability, although as yet undefined posttranscriptional mechanisms also may play a role in synergistic induction of HBD-2. Further investigations are needed to define the molecular basis for these posttranscriptional effects.

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REFERENCES

MODULATION OF RHINOVIRUS RESPONSES BY IL-17A


