IL-17 enhances IL-1β-mediated CXCL-8 release from human airway smooth muscle cells

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Dragon S, Rahman MS, Yang J, Unruh H, Halayko AJ, Gounni AS. IL-17 enhances IL-1β-mediated CXCL-8 release from human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 292: L1023–L1029, 2007. First published December 22, 2006; doi:10.1152/ajplung.00306.2006.—Recent studies into the pathogenesis of airway disorders such as asthma have revealed a dynamic role for airway smooth muscle cells in the perpetuation of airway inflammation via secretion of cytokines and chemokines. In this study, we evaluated whether IL-17 could enhance IL-1β-mediated CXCL-8 release from human airway smooth muscle cells (HASMC) and investigated the upstream and downstream signaling events regulating the induction of CXCL-8. CXCL-8 mRNA and protein induction were assessed by real-time RT-PCR and ELISA from primary HASMC cultures. HASMC transfected with site-mutated activator protein (AP)-1/NF-κB CXCL-8 promoter constructs were treated with selective p38, MEK1/2, and phosphatidylinositol 3-kinase (PI3K) inhibitors to determine the importance of MAPK and PI3K signaling pathways as well as AP-1 and NF-κB promoter binding sites. We demonstrate IL-17 induced and synergized with IL-1β to upregulate CXCL-8 mRNA and protein levels. Erk1/2 and p38 modulated IL-17 and IL-1β CXCL-8 promoter activity; however, IL-1β also activated the PI3K pathway. The synergistic response mediating CXCL-8 promoter activity was dependent on both MAPK and PI3K signal transduction pathways and required the cooperation of AP-1 and NF-κB cis-acting elements upstream of the CXCL-8 gene. Collectively, our observations indicate MAPK and PI3K pathways regulate the synergy of IL-17 and IL-1β to enhance CXCL-8 promoter activity, mRNA induction, and protein synthesis in HASMC via the cooperative activation of AP-1 and NF-κB trans-acting elements.

HUMAN AIRWAY SMOOTH MUSCLE CELLS (HASMC) are key determinants of asthma owing to their ability to contract in response to inflammatory cell products (1). Because of their intrinsic phenotype plasticity, airway myocytes also exhibit a capacity for multifunctional behavior and are actively involved in local inflammation and fibrosis (15). Emerging evidence suggests that HASMC can also directly contribute to the pathogenesis of asthma by altering the interstitial extracellular matrix, expressing cell adhesion and costimulatory molecules as well as secreting multiple proinflammatory cytokines and chemokines (19, 26). These responses can perpetuate airway inflammation and act on the hyperplastic and hypertrophic growth of HASMC contributing to airway hyperresponsiveness (AHR) and development of airway remodeling (39).

IL-1β is a proinflammatory mediator released by activated airway macrophages and epithelium and is found in elevated levels in the bronchoalveolar lavage (BAL) fluid of asthmatic, chronic obstructive pulmonary disease, and acute respiratory distress syndrome patients (4, 7, 8). In vivo and in vitro assays have demonstrated that IL-1β can alter airway function by inducing cellular infiltrate, mucus hyperplasia, airway wall thickening, fibrosis, and enlargement of distal air spaces (21, 25). Effector mechanisms include secretion of IL-8 (CXCL-8), which has been demonstrated to modulate HASMC contraction and migration mediating airway responsiveness and remodeling in asthmatic patients (9, 14, 47). Most importantly, CXCL-8 is recognized to induce the mobilization of neutrophils into the airways, negatively affecting lung and bronchial epithelial function (32).

Neutrophil chemotactic factors such as CXCL-8, granulocyte/macrophage colony-stimulating factor (GM-CSF), leukotriene B4, and complement activation product are increased in BAL fluid from asthmatic individuals (5, 10); however, upstream factors mediating neutrophil recruitment are still elusive. IL-17, a pleiotropic T lymphocyte cytokine released from a distinctive T helper type 1 (Th1)/Th2 lineage subset (31), is hypothesized to orchestrate the granulocyte influx into the airways via the induction of CXCL-8 (23, 27). In humans, elevated IL-17 levels can be detected in sera, sputum, and BAL fluid from asthmatic patients and have been shown to correlate with BAL neutrophilia, myeloperoxidase levels, and AHR (3, 11, 18, 28, 33). Recently, the effect of IL-17 on TNF-α-stimulated lung structural cells has gained attention for its synergistic induction of neutrophilic mobilizing chemokines (22, 24, 41). Increases in CXCL-8 and IL-6 levels in vitro have equally been reported for these cytokines in primary HASMC (16, 17). However, the modulating effect of IL-17 on IL-1β-mediated CXCL-8 induction in HASMC has not been investigated.

We previously established that IL-17 induces CXCL-8 expression in primary HASMC (35). In this study, we demonstrate a role for IL-17 in synergizing IL-1β-induced CXCL-8 release from primary HASMC. The synergistic effect of combining IL-17 with IL-1β is regulated at the transcriptional level and is dependent on the cooperative function of activator protein (AP)-1 and NF-κB cis-acting elements. Erk1/2, p38 MAPKs, and the phosphatidylinositol 3-kinase (PI3K) pathway mediate the upstream signal transduction of CXCL-8 promoter activity. Collectively, our observations indicate IL-17...
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Reagents and Antibodies. Recombinant human IL-17 and IL-1β were purchased from R&D (Minneapolis, MN). DMEM, Ham’s F-12 media, antibiotics (penicillin, streptomycin), dNTP, SuperScript reverse transcriptase, and Taq polymerase were purchased from (GIBCO-BRL, Grand Island, NY). The p38 MAPK inhibitor SB-203580 (4-[4-fluorophenyl]-2-[4-pyridy]-Imidazole) MEK1/2 extracellular signal-regulated kinase (Erk-1/2) inhibitor U0126, and the PI3K inhibitor Wortmannin were purchased from Calbiochem (Mississauga, Ontario, Canada). FBS was purchased from Hyclone Laboratories (Logan, UT), and unless stated otherwise, all other reagents were obtained from Sigma Chemical (Oakville, Ontario, Canada).

Preparation of Bronchial HASMC. Bronchial HASMC were obtained from macroscopically healthy segments of the main bronchi after lung resection from surgical patients in accordance with procedures approved by the Human Research Ethics Board of the University of Manitoba. Primary HASMC cultures were established and performed as previously described (35). HASMC were grown at 37°C in 5% CO₂ in DMEM with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). In all experiments, cells were used at passages 2–5.

Cell stimulation and ELISA assay. Confluent HASMC were grown arrested by FBS deprivation for 48 h in Ham’s F-12 media containing 5 µg/ml human recombinant insulin, 5 µg/ml human transferrin, 5 ng/ml selenium, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were then stimulated in fresh FBS-free media with human recombinant IL-17 (0.1, 1, 10, and 100 ng/ml) or IL-1β (0.1, 1, 10, and 100 ng/ml) in combination or vehicle (PBS). Supernatants were collected at different time points (24 and 48 h), centrifuged for 7 min at 4°C to remove cellular debris, and stored at −80°C before ELISA. To test the effect of mRNA synthesis inhibitor actinomycin D (Act D) on CXCL-8 mRNA or protein release, 5 µg/ml was added to serum-deprived HASMC 30 min before stimulation with IL-17 (1 ng/ml), IL-1β (1 ng/ml), or both (40). Supernatants were recovered at 24 and 48 h and processed as described above. Immunoreactive CXCL-8 was quantified by ELISA using matched antibodies from Pierce Endogen (Biosource International, Mississauga, Ontario) according to basic laboratory protocols. The sensitivity limit of CXCL-8 was 10 pg/ml.

Real-time RT-PCR analysis. Confluent HASMC were grown arrested by FBS deprivation and were stimulated in fresh FBS-free media containing IL-17 (1 ng/ml), IL-1β (1 ng/ml), both, or vehicle (PBS) for 6 h. Cells were then harvested, and RNA was purified using the guanidinium isothiocyanate method (6). Relative levels of CXCL-8 mRNA were analyzed by quantitative real-time RT-PCR analysis using the Light-Cycler (Roche) as previously described (35). Briefly, DNA standards were prepared from PCR using cDNA of cells stimulated with IL-1β (1 ng/ml). The amount of extracted DNA was quantified by spectrophotometry and expressed as copy numbers, and serial dilutions were used to generate the standard curve. Product quantification by spectrophotometry and expressed as copy numbers, and calculation of the percentage of each cDNA species was performed as previously described (13). The amplification of target genes in stimulated cells was calculated by first normalizing to the amplification of GAPDH and then expression of the normalized values as fold increase over the value obtained with unstimulated control cells.

CXCL-8 mRNA stability. Growth-arrested HASMC were stimulated with IL-1β (1 ng) or in combination with IL-17 (50 ng) for 10 h before the addition of 5 µg/ml Act D. Total cellular RNA was then extracted at the indicated time points post-Act D incubation, and CXCL-8 mRNA expression was quantified by semiquantitative real-time RT-PCR. CXCL-8 mRNA copy numbers were normalized to the respective GAPDH values. Results are presented as the % mRNA remaining compared with the initial 10-h culture time point. One-phase exponential decay constants (k) were calculated by nonlinear regression of the % mRNA remaining vs. time of Act D treatment using GraphPad Prism software (v.4.0) as previously described (43).
To verify whether the effect of IL-17 on IL-1β-mediated CXCL-8 protein expression depended on mRNA neosynthesis, confluent serum-deprived HASMC were pretreated with Act D and stimulated with IL-17 (1 ng/ml), IL-1β (1 ng/ml), or both. After 48 h, secreted protein levels from supernatants of IL-17 and IL-1β conditions were significant compared with vehicle (13.0 ± 0.3- and 1,382.0 ± 184.0-fold, respectively, Fig. 2B, *P < 0.001). Pretreatment with Act D before cytokine stimulation completely abrogated the induction of CXCL-8 (P < 0.001). In addition, CXCL-8 mRNA rate of decay was reduced by 2.1 ± 0.1-fold in the combined treated condition compared with IL-1β (Fig. 2C, 0.867 ± 0.127 vs. 0.4150 ± 0.207, P < 0.05). This effect resulted in a significant 2.0 ± 1.1-fold increase in CXCL-8 transcript levels at the 12-h time point over the IL-1β-treated condition (P < 0.05). Together, these results suggest that the synergy between IL-17 and IL-1β in HASMC can occur via both transcriptional and posttranscriptional mechanisms.

IL-17 enhances IL-1β-mediated CXCL-8 promoter activity. To investigate whether IL-17 modulates CXCL-8 promoter activity, HASMC were transiently transfected with the proximal CXCL-8 promoter fused to the luciferase reporter gene followed by stimulation with suboptimal concentrations (0.1 and 1 ng/ml) of IL-17, IL-1β, or in combination. IL-17 alone induced a 1.8- and 4.2-fold increase at 0.1 and 1 ng/ml, respectively, compared with vehicle (Fig. 3). Similarly, IL-1β at 0.1 and 1 ng/ml led to a 10.0- and 23.7-fold increase in reporter gene activity compared with vehicle. A significant synergistic effect on reporter gene activity was observed at 1 ng/ml of IL-17 in combination with IL-1β (1.6-fold increase compared with IL-1β alone, Fig. 3). The combination of both cytokines at different concentrations did not yield better synergistic effects than those shown at 1 ng/ml (data not shown).
SB-203580-treated IL-17 or IL-1β alone values. Interestingly, inhibition with U0126 decreased CXCL-8 promoter activity by 42.7 ± 2.8%, up 2.6 ± 0.4-fold compared with U0126-treated IL-17 or IL-1β alone (Fig. 5C). Wortmannin decreased CXCL-8 promoter activity by 24.2 ± 4.1% but remained 1.6 ± 0.4-fold over wortmannin-treated IL-1β alone. These data suggest that signaling via the MAPK and PI3K pathways is important for the synergy between IL-17 and IL-1β in the activation of the CXCL-8 promoter.

DISCUSSION

Increasing attention has been drawn to the synthetic properties of airway smooth muscle (ASM) for their contribution in the inflammatory component of airway disorders. Besides directing bronchomotor tone, ASM can release a multitude of cytokines that can contribute to the recruitment and activation of inflammatory cells in the airways (26). Of note, CXCL-8, a neutrophil chemokine, and IL-1α, a T lymphocyte-derived cytokine mediating neutrophilic inflammation, play important roles in the recruitment of neutrophils to the airways (27). Previously, we investigated the involvement of HASMC in regulating IL-17-mediated neutrophil recruitment by analyzing the basal expression levels of cell surface IL-17A receptor (IL-17RA) and mRNA in primary HASMC (35). We demonstrated a key role for AP-1 and NF-kB cis-acting elements for the induction of CXCL-8 by stimulation with IL-17.

In this study, we further investigated the upstream signaling mechanisms and the synergistic effects of the proinflammatory cytokine IL-1β, which mediates a critical role in promoting airway inflammation. We demonstrate that IL-17 enhances IL-1β-mediated CXCL-8 mRNA expression and protein levels from primary HASMC. This effect is regulated by p38, Erk1/2 MAPKs, and the PI3K pathways and is dependent on the cooperation of the AP-1 and NF-kB cis-acting elements upstream of the CXCL-8 gene.

AP-1 and NF-kB cis-acting elements have previously been identified as primary binding sites for the transcription and superinduction of CXCL-8 (29, 35, 37). The molecular mec-

Together, these results demonstrate the synergy observed between IL-17 and IL-1β is mediated at the CXCL-8 promoter level. IL-17 synergizes with IL-1β and requires intact AP-1 and NF-κB response elements. To ascertain which transcription factor binding sites were functionally important for the synergy of IL-17 with IL-1β-mediated CXCL-8 production, mutated AP-1, NF-κB binding sites, or double mutant AP-1/NF-κB CXCL-8 promoter luciferase constructs were transiently transfected into HASMC. Following stimulation with IL-17, IL-1β, or both (1 ng/ml), luciferase activity was significantly reduced in AP-1- and NF-κB-mutated constructs; however, it was completely abrogated in the double mutant compared with the wild-type construct (from 330.3 ± 41.5-fold increase over mock control to 33.1 ± 2.9 for the AP-1 mutant, 0.8 ± 0.1 for the NF-κB mutant, and 0.1 ± 0.1 for the double mutant, Fig. 4). These data suggest that AP-1 is important for CXCL-8 induction by IL-17; however, NF-κB is necessary for both cytokines. Deletion of both binding sites led to the abrogation of the CXCL-8 promoter activity indicating that the cooperation between AP-1 and NF-κB cis-acting elements are essential for the induction of the synergistic response.

IL-17 enhances IL-1β-mediated CXCL-8 promoter activity through p38, Erk1/2 MAPK, and the PI3K pathway. To further define the molecular mechanism by which IL-17 and IL-1β cooperate, we used selective pharmacological inhibitors of p38 (SB-203580), MEK1/2 (U0126), and PI3K (wortmannin). Treatment of HASMC with SB-203580 and U0126 before stimulation with IL-17 alone caused a significant inhibition of CXCL-8 promoter activity by 40.1 ± 20.7% and 78.6 ± 3.8% compared with control values in the absence of the respective inhibitor (P < 0.05, Fig. 5A). In contrast, inhibition of PI3K with wortmannin had no effect on IL-17-induced CXCL-8 promoter activity in HASMC (Fig. 5A). Pretreatment with SB-203580, U0126, or wortmannin significantly decreased IL-1β-induced CXCL-8 promoter activity by 28.8 ± 14.8%, 77.8 ± 2.9%, and 51.8 ± 11.4%, respectively (Fig. 5B).

The combination of both cytokines with SB-203580 decreased CXCL-8 promoter activity by 32.5 ± 5.1%, similar to
anisms regulating the function of IL-17 in enhancing IL-1β or TNF-α-induced CXCL-8 and IL-6 protein expression have been proposed to occur posttranscriptionally via mRNA stabilization (16, 41). CXCL-8 mRNA rate of decay is decreased 4.6-fold when cotreated with IL-17 and TNF-α simultaneously compared with TNF-α, resulting in higher yields of mRNA levels 12 h poststimulation (17). Similarly, when IL-17 was combined with IL-1β, we observed a 2.1-fold increase in the CXCL-8 mRNA half-life compared with that of the IL-1β-stimulated condition (Fig. 2C). However, identification of common transcriptional regulatory elements in IL-17 target genes such as 24p3/lipocalin 2 has also been demonstrated to be regulated at the transcriptional level (38). Results from our luciferase assay also suggest that IL-17 enhances AP-1 and NF-κB promoter binding sites in a cooperative fashion to synergize IL-1β-mediated induction of CXCL-8. Therefore, the synergistic effect upheld by IL-17 on IL-1β-mediated CXCL-8 production stems from transcriptional and posttranscriptional regulatory mechanisms.

Other groups have investigated the inflammatory effects of IL-17 on HASMC mediator release. To date, only CXCL-8, eotaxin/CCL-11, and 8-isoprostane, a biomarker of oxidative stress, have been identified as inducible products from IL-17 stimulation on HASMC in vitro (34, 35, 42, 46). Although IL-6 superinduction and protein synthesis have been observed in cotreated conditions of IL-17 and TNF-α, IL-17 alone or in combination with IL-1β fails to synergize IL-6 or GM-CSF production after 24 h (16). This immunoregulatory function of HASMC differs from other lung structural cells from which IL-17 has been demonstrated to induce the release of proinflammatory cytokines IL-6 and IL-11, α-chemokines CXCL-8, and growth related oncogene-α (Gro-α), growth factor G-CSF, and hyperresponsive responses of IL-6 and CXCL-8 to TNF-α (22, 28, 30, 41). These observations suggest IL-17 may selectively enhance synthetic functions of HASMC depending on the nature of the inflammatory stimulus.

MAPKs and NF-κB activation are downstream effector functions of both the IL-1 type I receptor and IL-17RA signaling pathways (2, 12). We sought to investigate the signal transduction pathways regulating AP-1- and NF-κB-induced CXCL-8 promoter activity. Similarly to Wuyts et al. (45), our results demonstrate a critical function for Erk1/2 since inhibition of upstream MEK1/2 kinase with U0126 inhibited 79 and 78% of CXCL-8 promoter activity by IL-17 or IL-1β compared with the respective controls. Interestingly, when both cytokines were used in combination, CXCL-8 promoter activity was increased 1.8-fold, suggesting these cytokines synergize in activating alternative pathways mediating CXCL-8 promoter activity. p38 MAPK pathway is a potential candidate responsible for mediating the synergistic effects of IL-17 and IL-1β since it has been demonstrated to regulate AP-1 transcriptional activity in human vascular smooth muscle cells and suppress mRNA destabilization by targeting AU-rich elements in epithelial cells (44). Our results demonstrate that inhibition of p38 with SB-203580 decreased IL-17- or IL-1β-mediated CXCL-8 promoter activity by 40 and 21%, respectively, but the combination of both cytokines did not combine to further decrease or increase CXCL-8 promoter activity. Together, these results suggest p38 may hold a regulatory function in enhancing CXCL-8 promoter activity.

Recently, IL-6 and CXCL-8 production from rheumatoid arthritis synovial fibroblasts in response to IL-17 has been shown to signal via a NF-κB, PI3K/Akt-dependent pathway (20). Since the PI3K pathway also activates AP-1 and NF-κB trans-acting elements in response to IL-1 (36), we sought to determine whether this pathway regulated IL-17 and IL-1β synergistic induction of CXCL-8. In contrast to IL-1β, inhibition with wortmannin did not affect IL-17-mediated CXCL-8 promoter activity, suggesting this pathway is not involved for the induction of CXCL-8 by IL-17 in HASMC. Interestingly, the combination of both cytokines increased CXCL-8 promoter activity, implicating that MAPKs, amid other signaling pathways, have a critical role in mediating the synergistic response. In conclusion, we demonstrate IL-17 and IL-1β can individually, or in concert, synergize to enhance HASMC CXCL-8 promoter activity, mRNA induction, and protein synthesis via the cooperative activation of AP-1 and NF-κB trans-acting elements. Inhibition of Erk1/2 in either IL-17 or IL-1β signal transduction pathways led to the greatest impact on CXCL-8 promoter activity. p38 appeared to mediate a significant function in modulating the synergistic effect at the transcriptional level, and the PI3K pathway induced by IL-1β was essential.
for the full induction of the synergistic response. Together, HASMC in the presence of IL-17 and IL-1β may actively participate in the recruitment of neutrophils into the airways and could therefore represent a key modulating cell type in the perpetuation of airway inflammation.

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