IL-17F-induced IL-11 release in bronchial epithelial cells via MSK1-CREB pathway

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Asthma is a clinical diagnosis based on episodic symptoms and variable airway obstruction. It is also characterized by variable degrees of chronic inflammation and structural alterations in the airways (5, 39). These structural alterations, collectively called airway remodeling, encompass various changes in composition, content, and organization of many cellular and molecular constituents of the airway wall (31). The most important abnormalities are epithelial detachment, goblet cell hyperplasia, subepithelial thickening, hyperplasia and hypertrophy of airway smooth muscle, bronchial gland enlarge-
mitogen- and stress-activated protein kinase (MSK) 1. To gain further understanding of the function of IL-17F and its signal-
waying pathway, expression of IL-11 by IL-17F was investigated. Moreover, the effect of Th2 cytokines on IL-17F-induced IL-11 expression was investigated since Th2 cytokines, IL-4 and IL-13, have been associated with airway inflammation in asthma (24). Herein, we demonstrated for the first time that bronchial epithelial cells express IL-11 in response to IL-17F via the activation of MSK1-CREB pathway.

MATERIALS AND METHODS

Cell culture. Two different bronchial epithelial cells were used in this study. A bronchial epithelial cell line, BEAS-2B, was cultured in Hanks’ F-12/DMEM (Biofluids, Rockville, MD) with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 ng/ml streptomycin (Life Technologies-BRL, Gaithersburg, MD). Normal human bronchial epithelial cells (NHBEs) were purchased from Lonza (Walkersville, MD) and cultured in bronchial epithelial basal medium according to the manufacturer’s instructions. The cells were cultured for no more than three passages before the analysis.

Analysis of IL-11 gene and protein expression. Total RNA was extracted using RNaseasy (Qiagen, Chatsworth, CA) from 1 × 10⁶ cells at 2 h after stimulation with 10 and 100 ng/ml of IL-17F, and cDNAs were synthesized from 500 ng of total RNA and subjected for PCR. The sequences of PCR primers for IL-11: forward, 5’-AGCTCTCAGCTCCAGGGT-3’, reverse, 5’-TCACACCGCCGGAGTCT-TCAGC-3’; G3PDH: forward, 5’- ACCACGTCATGCGATCATC-3’, reverse, 5’-TCACCCACCTGTGCTGA-3’. The amplification reaction was performed for 30 cycles with denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The expected size for IL-11 was 343 bp, and for G3PDH it was 450 bp. PCR products were detected by ethidium bromide staining and quantitated by video densitometry using Image 1.61 software (NIH Public Software; National Institutes of Health, Bethesda, MD). The level of IL-11 gene expression was quantitated by calculating the ratio of densitometric readings of the band intensity for IL-11 and G3PDH from the same cDNA sample. The values are expressed as means ± SD (n = 3 experiments). IL-11 protein levels in the supernatants and cell lysates of IL-17F-stimulated cells were determined with a commercially available ELISA kit (Biosource, Camarillo, CA) according to the manufacturer’s instructions and expressed as the amount recovered per 10⁶ cells. The cell lysates were grown as described previously (20). The values are expressed as means ± SD (n = 6 experiments).

Effect of Th2 cytokines on IL-17F-induced IL-11 expression. BEAS-2B cells were treated with 100 ng/ml of each of the cytokines: IL-17F generated as described previously (21), IL-4, or IL-13 (all purchased from R&D Systems, Minneapolis, MN) or a combination of IL-17F (100 ng/ml) with either IL-4 (100 ng/ml) or IL-13 (100 ng/ml) for 24 h. IL-11 protein levels in the supernatants were determined as described above. The values are expressed as means ± SD (n = 6 experiments).

Effect of inhibitors on the expression of IL-11. For analysis of involvement of the Raf1-MEK-ERK1/2-MSKI pathway, the cells were treated in the presence or absence of the following kinase inhibitors at varying doses: MEFK1/2 inhibitors PD-98059 and U0126, MSKI inhibitors H89 and Ro-31-8220, and a vehicle control, DMSO (Me2SO), for 1 h before treatment with 100 ng/ml IL-17F (all purchased from Calbiochem, La Jolla, CA) (4, 6, 7, 36). The final concentration of Me2SO did not exceed 0.1% (vol/vol). On the other hand, MSKI is activated by other MAP kinase family members such as p38 and JNK (23, 41). Therefore, p38 inhibitor SB-202190 and JNK inhibitor SP-600125 were used (all purchased from Calbiochem) (1, 4). The cell supernatants were harvested at 24 h after stimulation for analyses by ELISA. IL-11 protein levels in the supernatants were determined as described above. The values are expressed as means ± SD (n = 4 experiments). The total number of cells and cell viability at the end of the culture period for each experiment were similar among all culture conditions, as determined by trypan blue exclusion assay, suggesting that the inhibition of IL-17F-induced IL-11 expression did not result from cytotoxicity of those inhibitors.

Detection of MSKI. For analysis of activation of MSKI, the cells were treated with IL-17F (100 ng/ml) in some cases with or without treatment with the MEK inhibitor PD-98059 or a vehicle control (Me2SO) for 1 h. Following treatment, the total cellular extracts (1 × 10⁶ cell equivalents/lane) were subjected to 4–20% Tris-glycine gel electrophoresis (NOVEX, San Diego, CA), followed by transfer onto polyvinylidene difluoride membranes (Bio-Rad, Tokyo, Japan) as previously described (20). The antibodies (Ab) used were rabbit anti-MSKI Ab and anti-phospho-MSKI Ab (Cell Signaling Technology, Beverly, MA).

Overexpression of Raf1 dominant negative vector. The plasmid encoding pCMV-RafS621A vector (dominant negative mutant of Raf1) was cloned into pCMV-Neo and a control vector were purchased from Clontech (San Diego, CA). The plasmids were prepared by using Qiagen plasmid DNA preparation kit. BEAS-2B cells were cultured on 100-mm plates and were transfected by an Effectene Reagent (Qiagen) according to the manufacturer’s instructions. The cells were selected with 500 ng/ml Geneticin (G418, Gibco/BRL). After selection, the cells were seeded into six-well culture plates. The cells were near confluent, and the cell supernatants were then harvested at 24 h after stimulation with 100 ng/ml IL-17F for analyses by ELISA. IL-11 protein levels in the supernatants were determined as described above. The values are expressed as means ± SD (n = 3 experiments).

Knockdown of MSKI and CREB with siRNA. Predesigned siRNAs for MSKI and CREB and control siRNAs were purchased from Ambion and BioLabs, respectively (Tokyo, Japan). siRNA transfection into the cells was performed according to the manufacturer’s instructions. The cells and cell supernatants were then harvested at 20 min and 24 h after stimulation with 100 ng/ml IL-17F and subjected to Western blotting and ELISA analyses, respectively. For analysis of the interrelationship between MSKI and CREB, Western blotting was performed as described above. The antibodies used were anti-CREB antibody and anti-phospho-CREB antibody (Cell Signaling Technology). IL-11 protein levels in the supernatants were determined as described above. The values are expressed as means ± SD (each n = 4 experiments).

Data analysis. The statistical significance of differences was determined by ANOVA. The values are expressed as means ± SD from independent experiments. Any difference with P values less than 0.05 was considered significant. When ANOVA indicated a significant difference, the Scheffé F-test was used to determine the difference between groups.

RESULTS

To examine whether IL-17F is able to induce IL-11 expres-
sion, bronchial epithelial cells were stimulated with varying doses of IL-17F at five different time points, and the levels of IL-11 gene and protein expression were analyzed. While IL-11 gene was not expressed in control culture, IL-17F induced, in a dose-dependent manner, the gene expression of IL-11 at the 2-h time point in BEAS-2B cells (Fig. 1A). In the time course experiments, IL-11 gene expression peaked at the 2-h time point in IL-17F (100 ng/ml)-treated BEAS-2B cells (Fig. 1B) and returned to baseline 24 h after stimulation. IL-11 proteins were weakly detected in untreated BEAS-2B cells, but in the presence of IL-17F, the levels of IL-11 in cell lysates were significantly increased and peaked at the 12-h time point (Fig. 1C), whereas the levels of IL-11 in the supernatants peaked at 24 h and decreased at 48 h after stimulation (Fig. 1D). Simi-
larly, NHBEs also induced IL-11 expression in response to IL-17F and showed the same kinetics as BEAS-2B cells (Fig. 1E).

Next we investigated whether IL-17F modulates the expression of IL-11 in cells stimulated with Th2 cytokines (IL-4 and IL-13). As shown in Fig. 2, the Th2 cytokines (IL-4 and IL-13) did not induce IL-11 expression. However, both IL-4 and IL-13 showed an augmenting effect in combination with IL-17F compared with that seen in cells stimulated with IL-17F alone.

To investigate IL-17F-mediated signaling events leading to the induction of IL-11, the activation of the Raf1-MEK1/2-ERK1/2 pathway was investigated, since this pathway is a central upstream pathway of IL-17F (15, 16, 18, 19, 20). The results showed that pretreatment of the cells with each of the selective MEK inhibitors, PD-98059 (10 μM) and U0126 (5 μM), and Raf1 kinase inhibitor I (1 nM) significantly decreased the levels of IL-17F-induced IL-11 expression, whereas a 1-h pretreatment of the cells with vehicle alone (DMSO) did not affect IL-11 expression. In addition, the protein levels of IL-11 were unchanged in IL-17F-treated cells in the presence of varying doses of a p38 kinase inhibitor, SB-202190, and a JNK inhibitor, SP-600125 (Fig. 3). While induction of IL-11 is only partially inhibited by PD-98059, U0126, or Raf1 kinase inhibitor I, even at relatively high doses (50 μM, 10 μM, and 10 nM, respectively), the combination of 10 μM PD-98059 and 1 nM Raf1 kinase inhibitor I inhibited, to a significantly greater degree, the production of IL-11 (Fig. 3).

Next, the involvement of Raf1 kinase in IL-17F-induced IL-11 expression was further confirmed by the use of a Raf1 dominant negative mutant with serine to alanine substitution at position 621. The results showed that overexpression of Raf1 dominant negative mutants in BEAS-2B cells significantly inhibited IL-17F-induced IL-11 expression (Fig. 4), whereas the cells transfected with a control vector showed no significant change in the level of IL-11 expression. Therefore, these results suggest that IL-17F expresses IL-11 through Raf1-mediated pathway.

To date, the downstream signaling pathway of IL-17F has not been fully understood. In this study, a transient phosphor-
ylation of MSK1 was observed upon stimulation of the cells with IL-17F, reaching the maximum at 20 min after stimulation (Fig. 5A). To establish the interrelationship between ERK1/2 and MSK1, the cells were treated with a MEK inhibitor, PD-98059, before the stimulation with IL-17F, since the current study and a previous report (20) have demonstrated that IL-17F activates only ERK1/2 in bronchial epithelial cells. As shown in Fig. 5B, pretreatment of the cells with PD-98059 markedly inhibited the phosphorylation of MSK1, indicating that ERK1/2 is a critical upstream kinase responsible for activation of MSK1.

Next, to determine whether MSK1 affects IL-17F-induced IL-11 expression, effects of MSK1 inhibitors were investigated. Pretreatments with MSK1 inhibitors Ro-31-8220 and H89 significantly blocked IL-17F-induced IL-11 expression (Fig. 6), suggesting that MSK1 regulates this expression. Finally, to further confirm whether MSK1 plays a role in IL-17F-induced IL-11 expression, total MSK1 expression was first diminished in the cells by transfecting with siRNA targeting MSK1 (Fig. 7A). Next, the interrelationship between MSK1 and CREB was investigated by using siRNAs targeting MSK1, since CREB is located downstream of MSK1 (40). Knockdown of MSK1 in cells abrogated the IL-17F-induced activation of CREB (Fig. 7B). This suggests that CREB is located downstream of MSK1. As shown in Fig. 7C, the markedly inhibited the phosphorylation of MSK1, indicating that ERK1/2 is a critical upstream kinase responsible for activation of MSK1.
involvement of MSK1 and CREB for induction of IL-11 expression by IL-17F was then analyzed. Induction of IL-11 expression by IL-17F was significantly inhibited in cells transfected with siRNA targeting MSK1, whereas no significant difference was seen in wild-type cells and cells transfected with a control siRNA.

DISCUSSION

In this report, we demonstrated that IL-17F induces expression of IL-11 in bronchial epithelial cells through the activation of the Raf1-MEK-ERK1/2-MSK1-CREB signaling pathway, providing a novel functional linkage between two cytokines that are involved in airway inflammation and remodeling. Bronchial epithelial cells have been shown to be a major cell source of IL-11 (10). Bronchial epithelial cells did not induce IL-11 in response to IL-4 and IL-13. However, IL-4 and IL-13 augmented IL-17F-induced IL-11 expression. These findings suggest that IL-17F is a novel inducer of IL-11 and induces airway remodeling and inflammation either alone or in combination with Th2 cytokines.

IL-11 plays a key role in airway remodeling and inflammation in asthma. A significantly increased expression of IL-11 mRNA and protein within the airways of subjects with severe asthma is seen when compared with those with mild asthma (28). The number of cells expressing IL-11 mRNA was inversely correlated to the FEV1 (28). IL-11 induces subepithelial fibrosis, accumulation of fibroblasts, myofibroblasts and myocytes, and deposition of type I and III collagen (35). Furthermore, a more recent report demonstrated that IL-17F is a novel inducer of IL-11 and is involved in pathogenesis of asthma via the induction of IL-11. Uncovering interactions between IL-17F and IL-11 may be valuable in understanding and treating asthma.

IL-17F is derived from activated CD4+ T cells, basophils, and mast cells, which are important regulatory cell types for allergic airway inflammation (21). Upregulated expression of IL-17F is seen in the bronchoalveolar lavage cells from asthmatics following segmental allergen challenge (21). Similarly, IL-17F is also clearly upregulated in the mouse model of asthma (34). Moreover, IL-17F has been shown to be able to induce pulmonary neutrophilia and produces an additive effect on antigen-induced allergic inflammatory responses (29). These findings suggest a role of IL-17F in the enhancement of allergic airway inflammation. Recently, we have demonstrated that IL-17F is a candidate gene for asthma susceptibility (12, 22). Moreover, IL-17F is able to induce several cytokines and chemokines in bronchial epithelial cells, vein endothelial cells, and fibroblasts (13, 15, 16, 18, 19, 20, 32). A recent report demonstrated that IL-17F also acts on eosinophils, one of the most important inflammatory cells in allergic airway inflammation and remodeling, to induce several cytokines and chemokines such as IL-1β, IL-6, IL-8, GROα, and MIP-1β (3). These findings suggest that these cell types play crucial roles in asthma in response to IL-17F. The potential involvement of IL-17F in allergic inflammation is, therefore, likely mediated, in part, through the induction of IL-11. The release of IL-11 from

Fig. 7. Regulation of IL-17F-induced IL-11 by MSK1-CREB in BEAS-2B cells. The validation of its blocking by siRNA for MSK1 was performed by Western blotting. The results shown are representative of 3 separate experiments. A: effect of siRNA for MSK1 on IL-17F-induced phosphorylation of CREB. Western blotting analysis was performed with antibodies against t-CREB and p-CREB. The results shown are representative of 4 separate experiments. B: values are expressed as means ± SD (n = 4). *P < 0.05 vs. non-transfected cells.

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epithelial cells by IL-17F may contribute both to the development of airway remodeling and to inflammation in asthma. The IL-17F-IL-11 axis provides new insight into the molecular mechanisms involved in airway remodeling and inflammation. So far, asthma has been believed to be an example of Th2-mediated airway inflammation. Recent reports demonstrated that a new CD4+ T cell subset, Th17 cells, are involved in the pathogenesis of asthma (38). Current and previous data show that Th2 cytokines are able to augment the function of IL-17F (16). These findings suggest that both Th2 and Th17 cytokines orchestrate many features of asthma. The combination of Th2 cytokines and IL-17F may thus perpetuate allergic inflammation and cause airway remodeling via enhancement of IL-11 expression.

Unlike IL-17A, little information has been available regarding the signaling pathway of IL-17F. Similar to IL17A, IL-17F utilizes a heterodimer of IL17RA and IL17RC as its receptor (37). In the upstream signaling pathway, we have previously demonstrated that IL-17F activates the Raf1-MEK1/2-ERK1/2 pathway (15, 16, 18, 19, 20). Similarly, IL-17F induced IL-11 expression through this pathway. This pathway has been shown to be a central one for IL-17F-induced cytokine and chemokine expression in bronchial epithelial cells. Therefore, ERK1/2 may serve as a crucial signaling molecule for IL-17 family members, since IL-17A also activates ERK1/2 in bronchial epithelial cells (17). These findings suggest that regulation of the Raf1-MEK1/2-ERK1/2 pathway may constitute a useful therapeutic target for IL-17 family cytokine-associated diseases. Moreover, we have demonstrated that p90RSK-CREB pathway is an important downstream signaling pathway for IL-17F (16). However, inhibition of p90RSK did not completely abrogate biological activity of IL-17F, suggesting an additional signaling pathway to CREB (23). Here, we identified that MSK1-CREB is a novel signaling pathway of IL-17F. MSK1 is located downstream of the Raf1-MEK1/2-ERK1/2 cascade, since a MEK inhibitor, PD-98059, blocked the phosphorylation of MSK. Similarly, CREB is the downstream signaling molecule of MSK1, since the siRNA targeting MSK1 inhibited the activation of CREB. Moreover the activation of MSK1 is essential for IL-11 expression by IL-17F, since MSK1 inhibitors Ro-31-8220 and H89 and siRNA targeting MSK1 blocked its expression. These data suggest the candidacy of the MSK1-CREB pathway as a potential pharmacological target in IL-17F-induced airway inflammation. On the other hand, it is reported that ERK1/2 and p38MAPK are able to activate MSK1 in skeletal myoblasts in response to growth factors and cellular stress (23). However, IL-17F did not phosphorylate p38MAPK (20), and a p38MAPK inhibitor, SB-202190, did not elicit a significant response in the present study. These data suggest that MSK1 is regulated by ERK1/2 in the case of IL-17F in bronchial epithelial cells.

In conclusion, this study has revealed that IL-17F induces an airway remodeling-related cytokine, IL-11, in bronchial epithelial cells, and induces IL-11 via the activation of the Raf1-MEK1/2-ERK1/2-MSK1-CREB pathway. The respective role of IL-17F and IL-11 in allergic airway diseases has been suggested, and our study provides evidence for a functional linkage between these two cytokines, further strengthening their role in the regulation of airway inflammation. IL-17F may contribute to amplification and persistence of allergic airway inflammatory processes. Activation of the IL-17F-IL-11 axis in asthma may have important therapeutic implications and contribute to the development of airway remodeling and inflammation.

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