CREB regulates TNF-α-induced GM-CSF secretion via p38 MAPK in human lung fibroblasts

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ABSTRACT

Background: Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that mediates eosinophilic differentiation, migration and survival, causing respiratory tract inflammation. GM-CSF is also known to be secreted from respiratory tract structural cells. However, the mechanisms of GM-CSF secretion have not been well established.

Methods: Human fetal lung fibroblasts and human primary asthmatic lung fibroblasts were used for the study of tumor necrosis factor alpha (TNF-α)-induced GM-CSF secretion. GM-CSF secretion and mRNA expression were measured by enzyme-linked immunosorbent assay and quantitative real-time reverse transcription polymerase chain reaction, respectively. Knockdown of cAMP response element-binding protein (CREB) in fibroblasts was carried out by using specific small interfering RNAs of CREB.

Results: Among respiratory tract structural cells, pulmonary fibroblasts exhibited increased GM-CSF secretion and mRNA expression after stimulation with TNF-α in a concentration-dependent manner. Moreover, a p38 mitogen-activated protein kinase (MAPK) inhibitor controlled TNF-α-induced GM-CSF secretion, and rolumilast and rolipram, inhibitors of phosphodiesterase-4, suppressed TNF-α-induced GM-CSF secretion. Consistent with this, forskolin also completely blocked GM-CSF secretion, and similar results were observed in response to cAMP treatment, suggesting that cAMP signaling suppressed TNF-α-induced GM-CSF secretion in human lung fibroblasts. Furthermore, CREB was phosphorylated through p38 MAPK but not cAMP signaling after TNF-α stimulation, and GM-CSF secretion was inhibited by CREB knockdown. Finally, these effects were also demonstrated in human primary lung fibroblasts in a patient with asthma.

Conclusions: CREB signaled independent of cAMP signaling and was phosphorylated by p38 MAPK following TNF-α stimulation, playing a critical role in GM-CSF secretion in human lung fibroblasts.

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Introduction

Asthma is a type of allergic inflammation of the airways. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is thought to have a central role in the pathogenesis of asthma, which is characterized by eosinophilic inflammation of the bronchi. Although the pathogenesis of allergic inflammation is complicated, GM-CSF, which exhibits chemotactic activity in eosinophils, has been shown to play a pivotal role in allergic inflammation through the recruitment of eosinophils to the site of allergic inflammation. GM-CSF is essential for the maintenance of eosinophil activity, including eosinophil differentiation, migration, and survival, and is known to be secreted from bronchial epithelium cells (BECs), respiratory tract fibroblasts, and airway smooth muscle cells (ASMCs). The mechanisms of GM-CSF secretion have been investigated mainly using BECs, but not lung fibroblasts. In asthma, damage and detachment of the bronchial epithelium are observed. A similar state is worsened by the respiratory viral infection, and it is thought that lung fibroblasts and ASMCs under BECs in asthma produce various cytokines because it is exposed to various inflammatory mediators. Therefore, examining inflammatory cytokine secretion from lung fibroblasts was important for understanding asthmatic airway inflammation.

GM-CSF hypersecretion is associated with various pathologies of the respiratory tract, such as airway remodeling, chronic inflammation, and airway hypersensitivities, and p38 mitogen-activated protein kinase (MAPK) has been shown to be important for GM-CSF secretion as well as inflammation, cell cycle progression, differentiation, and apoptosis. p38 MAPK activity is induced by a variety of extracellular stimuli, such as pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α). However, the underlying mechanism through which p38 MAPK regulates GM-CSF secretion is unknown.

Many transcriptional effects of cAMP are mediated by the cAMP response element-binding (CREB) protein. CREB regulates several cellular functions, such as inflammation, cell proliferation, differentiation, adaptation, and survival. Increased CREB activity has been shown to be associated with the pathogenesis of asthma, alterations in cognitive memory, and the CREB activity has been shown to be associated with the pathogenesis of asthma, alterations in cognitive memory, and the CREB activity has been shown to be associated with the pathogenesis of asthma, alterations in cognitive memory, and the CREB activity has been shown to be associated with the pathogenesis of asthma, alterations in cognitive memory, and the CREB activity has been shown to be associated with the pathogenesis of asthma, alterations in cognitive memory, and the CREB activity has been shown to be associated with the pathogenesis of asthma, alterations in cognitive memory, and the CREB activity has been shown to be associated with the pathogenesis of asthma, alterations in cognitive memory, and the CREB activity has been shown to be associated with the pathogenesis of asthma, alterations in cognitive memory, and the CREB activity has been shown to be associated with the pathogenesis of asthma, alterations in cognitive memory, and the CREB activity has been shown to be associated with the pathogenesis of asthma, alterations in cognitive memory, and the CREB activity has been shown to be associated with the pathogenesis of asthma, alterations in cognitive memory, and the CREB activity has been shown to be associated with the pathogenesis of asthma, alterations in cognitive memory, and the CREB activity has been shown to be associated with the pathogenesis of asthma, alterations in cognitive memory, and the CREB activity has been shown to be associate..

Methods

Reagents

SB239063, SP600125, and Roflumilast were purchased from Sigma--Aldrich (St. Louis, MO, USA). Forskolin, LY294002, Rolipram, and PD98059 were purchased from WAKO Biochemical (Osaka, Japan). TNF-α was purchased from PeproTech (Rocky Hill, NJ, USA). Cell-permeable 8-bromo-cAMP was purchased from Cayman Chemical (Ann Arbor, MI, USA). SB239063, SP600125, Roflumilast, forskolin, LY294002, Rolipram and PD98059 were dissolved in dimethyl sulfoxide (DMSO). Unless otherwise stated, the cells were incubated with these agents or their vehicle under starvation conditions at 37 °C in an atmosphere containing 5% CO2 and 95% air.

Cell culture

Human fetal lung fibroblasts (HFL-1 cells; lung, diploid, human) and human bronchial epithelial cells (Beas-2B) were obtained from the American Type Culture Collection (Rockville, MD, USA). Human ASMCs and human primary asthmatic lung fibroblasts (AHLFs) were purchased from Lonza (Walkersville, MD, USA). In all experiments, cells were used between passages 2 and 8 and cultured in a humidified atmosphere of 95% air plus 5% CO2. HFL-1 cells were cultured in F-12 medium (Invitrogen, Grand Island, NY, USA) containing 10% FBS (Biological Industries, Israel). Beas-2B cells were cultured in bronchial epithelial cell basal medium (Lonza, Walkersville, MD, USA) supplemented with bovine pituitary extracts, hydrocortisone, epidermal growth factor, epinephrine, transferrin, insulin, retinoic acid and triiodothyronine. Before each experiment, Beas-2B cells were starved with DMEM (Sigma--Aldrich) supplemented with 0.1% fatty acid-free BSA. Human ASMCs were grown in Lonza custom smooth muscle growth media supplemented with human epidermal growth factor, insulin, human fibroblast growth factor β, and FBS. AHLFs were grown in Lonza custom fibroblast basal media-2 medium with additional Lonza SingleQuots supplements containing insulin, recombinant fibroblast growth factor β, and FBS.

Antibodies

Primary antibodies selectively recognizing phosphorylated forms of c-Jun N-terminal kinase (JNK; anti-phospho-JNK, Thr183/Tyr185), p38 MAPK (anti phospho-p38 MAPK, Thr180/Tyr182), ERK (anti-phospho-p44/42 MAPK, Thr202/Tyr204), AKT (anti-phospho-AKT, Ser473, Thr308), CREB (anti-phospho-CREB, Ser133) were purchased from Cell Signaling Technology (Beverly, MA, USA). Phosphorylation-state independent anti-JNK, anti-p38 MAPK, anti-ERK, anti-β-actin, and anti-CREB antibodies were also purchased from Cell Signaling Technology.

Enzyme-linked immunosorbent assay (ELISA)

TNF-α-induced secretion of GM-CSF and interleukin (IL)-8 in the supernatant was quantified by ELISA using a DuoSet ELISA Development System (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Cells were seeded in 12-well plates and cultured in complete medium for 36–48 h and then starved for 24 h. The cells were stimulated with 10 ng/ml TNF-α in the presence or absence of various inhibitors. Lower detection limit of ELISA for GM-CSF and IL-8 were approximately 10 pg/ml and 20 pg/ml, respectively.
RNA isolation and reverse transcription polymerase chain reaction

Cells were stimulated with TNF-α for 12 h, and mRNA was extracted and purified using an RNeasy Mini kit (Qiagen, Hilden, Germany). The mRNAs were treated with DNase (Qiagen) and converted to cDNA using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA).

Quantitative RT-PCR (RT-qPCR)

TaqMan probes for RT-qPCR were purchased from Applied Biosystems. The mRNA expression levels of human GM-CSF and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured by RT-qPCR using TaqMan gene expression assays on a sequence detection system model 7700 (Applied Biosystems). All data were standardized to GAPDH as previously described.33

Transfection with small interfering RNA (siRNA)

Nonsilencing control siRNA (AATTCTCCGAACGTGTCACGT) and siRNAs targeting CREB, i.e., CREB#4 (CACCCACAGATTCCACATTAG), CREB#5 (AACCAAGTTGTGTTCAAGCT), and CREB#6 (AACCTGGATTCCCAAAAGCGAAG) were purchased from QiaGen. siRNA transfections were carried out by reverse transfection according to the manufacturer's protocol (Invitrogen).32 Briefly, siRNA (2.0 nM for HFL-1 and 0.5 nM for AHFL) was incubated with RNAiMAX reagent (Invitrogen) in Opti-MEM (GIBCO) for 15−30 min, mixed with the cells suspended in F-12 medium supplemented with 10% FBS, and then cultured in 6- or 12-well plates. After incubation for 36−48 h, the cells were starved with culture medium supplemented with fatty acid-free BSA without FBS and incubated for the indicated times.

Western blotting

Cells were incubated with 10% trichloroacetic acid for 10 min, and the physiological reactions in the cells were then terminated. The cells were washed with 1× phosphate-buffered saline once and with water once to remove trichloroacetic acid. The cells were then lysed using 2× SDS sample buffer (40 mM Tris-HCl, pH 6.8, 1.6% SDS, 0.016% bromophenol blue, 24% glycerol). Cell lysates were separated by SDS-polyacrylamide gel electrophoresis using 5−20% gradient gels (Wako Chemicals, Tokyo, Japan) at 100 V. Proteins in the gels were transferred to nitrocellulose membranes, which were subsequently blocked with blocking buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, and 5% dried milk powder) for 60 min. Membranes were incubated with primary antibodies overnight at 4 °C. After five washes for 5 min each, membranes were incubated with horseradish peroxidase-labeled secondary antibodies (1:3000 in blocking buffer) for 60 min at room temperature, followed by another nine washes for 5 min each. Signal detection was carried out using Supersignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, MA, USA).

Cell proliferation assay

The effects of CREB siRNA on cell proliferation were evaluated using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. The cells treated with siRNA were incubated at a density of 2×10^4 cells per well in 96-well plates. Next, cells were incubated for 72 h and the Cell Counting Kit-8 reagents (10 μL) were added to the medium. The cells were further incubated for 2 h, and the absorbance at 450 nm, which reflected the amount of formazan dye generated by active

![Fig. 1.](image)

**Fig. 1.** TNF-α-induced GM-CSF secretion from respiratory tract structural cells. **(A)** TNF-α-induced GM-CSF secretion from the respiratory tract structural cells. The cells were starved for 24 h prior to TNF-α stimulation. The cells were incubated with 10 ng/mL TNF-α for 24 h. GM-CSF levels in the supernatants were analyzed using ELISAs. **(B)** GM-CSF mRNA expression induced by TNF-α in respiratory tract structural cells. The cells were treated as in (A) and then harvested for mRNA purification. The y-axis shows the relative ratio of GM-CSF mRNA expression after TNF-α stimulation divided by the GAPDH mRNA expression. **(C)** Time-dependence of TNF-α-induced GM-CSF secretion from HFL-1 cells. **(D)** Concentration-dependent effects of TNF-α-induced GM-CSF secretion from HFL-1 cells. Cells were starved for 24 h and stimulated with TNF-α at the indicated times and concentrations. The supernatants were centrifuged and subjected to GM-CSF ELISA. * P < 0.05 as compared with HFL-1 cells; n = 3 independent experiments.
dehydrogenases in living cells, was measured using a microplate reader.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism software, version 6 (GraphPad Software, San Diego, CA, USA). All values were expressed as the means ± standard errors of the means (SEMs) from the indicated number of experiments. The data were compared using the Student’s t-tests for multiple comparisons. Differences with P values of 0.05, after adjustment for the number of comparisons, were considered statistically significant.

**Results**

**TNF-α-induced GM-CSF secretion from human lung fibroblasts**

First, we examined TNF-α-induced GM-CSF secretion using airway structural cells. As shown in Figure 1A, HFL-1 cells exhibited increased secretion of GM-CSF after TNF-α stimulation. Airway structural cells did not show significant GM-CSF secretion without TNF-α stimulation. Moreover, GM-CSF mRNA expression was higher in HFL-1 cells than in BEAS-2B human bronchial epithelial cells (Fig. 1B). Next, we examined the mechanisms of GM-CSF secretion using HFL-1 cells. TNF-α-induced GM-CSF secretion from HFL-1 cells occurred in a concentration- and time-dependent manner (Fig. 1C, D). Accordingly, we used 10 ng/mL TNF-α for 24 h, as previously reported in human pulmonary epithelial cells.10

**TNF-α-induced GM-CSF secretion and CREB phosphorylation by p38 MAPK in HFL-1 cells**

To elucidate the mechanism of TNF-α-induced GM-CSF secretion, we examined the effects of various MAPK inhibitors and PI3-K inhibitors on TNF-α-induced GM-CSF secretion. TNF-α-induced GM-CSF secretion was partially inhibited by PD98059 (an ERK inhibitor), SB239063 (a p38 MAPK inhibitor) and LY294002 (a PI-3 kinase inhibitor), as shown in Figure 2A. To determine the time course of this phosphorylation event after TNF-α stimulation, we performed western blot analysis using phosphorylation-specific antibodies. TNF-α stimulated the phosphorylation of p38 MAPK (Fig. 2B). p38 MAPK has also been shown to phosphorylate CREB at Ser133 in human vascular smooth muscle cells.23 Consistent with this, TNF-α stimulation also induced the phosphorylation of CREB in a pattern similar to those of p38 MAPK phosphorylation (Fig. 2B). To further investigate whether CREB phosphorylation was mediated by p38 MAPK in HFL-1 cells, we examined CREB phosphorylation after TNF-α stimulation in the presence of the p38 MAPK inhibitor, SB239063. As shown in Figure 2C, TNF-α-induced CREB phosphorylation was completely inhibited by treatment with SB239063. Thus, we next focused on the role of CREB in TNF-α-induced GM-CSF secretion.

The effects of cAMP and cAMP-elevating agents on TNF-α-induced GM-CSF secretion in HFL-1 cells

CREB has been shown to function downstream of protein kinase A (PKA) signaling.15 Therefore, we next examined the effects of cell-permeable 8-bromo-cAMP on TNF-α-induced GM-CSF secretion. As shown in Figure 3A, TNF-α-induced GM-CSF secretion was inhibited by cAMP in a concentration-dependent manner. The PKA inhibitor H89 did not block TNF-α-induced GM-CSF secretion, indicating that TNF-α stimulation did not activate PKA signaling. Recently, the cAMP-elevating agent Rolflumilast, a phosphodiesterase (PDE)-4 inhibitor, has been developed for use in the treatment of severe COPD.36,37 Therefore, we next tested the effects of cAMP-elevating agents, including Rolflumilast and Rolipram, on TNF-α-induced GM-CSF secretion. Interestingly, both Rolflumilast and Rolipram partly inhibited TNF-α-induced GM-CSF secretion, and forskolin, a potent cAMP-elevating agent, inhibited TNF-α-mediated GM-CSF secretion in a concentration-dependent manner (Fig. 3B). Since cAMP/PKA signaling is thought to phosphorylate CREB at Ser133, we examined the effect of cell-permeable 8-bromo-cAMP on CREB phosphorylation in HFL-1 cells. As shown in Figure 3C, stimulation of cAMP did not significantly increase CREB phosphorylation in the absence of TNF-α. In the presence of TNF-α, cAMP did not significantly increase CREB phosphorylation, while cAMP inhibited TNF-α-induced GM-CSF secretion. The results indicate that cAMP inhibits TNF-α-induced GM-CSF secretion without changing CREB phosphorylation in HFL-1 cells.

Knockdown of CREB inhibited TNF-α-induced GM-CSF secretion from HFL-1 cells and AHHFs

To explore the role of CREB downstream of p38 MAPK signaling after TNF-α stimulation, we examined the effects of CREB knockdown on TNF-α-induced GM-CSF secretion using CREB-specific siRNAs. All CREB siRNAs specifically knocked down CREB protein expression (Fig. 4A). Moreover, knockdown of CREB expression inhibited TNF-α-induced GM-CSF secretion (Fig. 4B). Importantly,
cell proliferation was not significantly influenced by CREB siRNA transfection (Fig. 4C) indicating that the transfection procedures were not cytotoxic. We further examined the effects of CREB knockdown on IL-8 secretion in HFL-1 cells. TNF-α-induced IL-8 secretion was not influenced by transfection with CREB-specific siRNA (Fig. 4D). Next, we examined TNF-α-induced GM-CSF mRNA expression in HFL-1 cells after treatment with CREB siRNAs. CREB knockdown inhibited TNF-α-induced GM-CSF mRNA expression as well as GM-CSF secretion, while CREB knockdown did not influence TNF-α-induced IL-8 mRNA expression (Fig. 5A–C). These results suggested that CREB regulated TNF-α-induced GM-CSF secretion via p38 MAPK, and that cAMP signaling inhibited TNF-α-induced GM-CSF secretion in human lung fibroblasts.

Finally, we examined the effects of CREB siRNA on TNF-α-induced GM-CSF secretion in AHLFs. AHLFs as well as HFL-1 exhibited increased GM-CSF and IL-8 secretion after TNF-α stimulation. As shown in Figure 6A, CREB phosphorylation was induced by TNF-α stimulation and inhibited by the p38 MAPK inhibitor, SB239063. Knockdown of CREB was achieved by transfection with CREB siRNA in AHLFs (Fig. 6B). Importantly, knockdown of CREB inhibited TNF-α-induced GM-CSF secretion (Fig. 6C) and slightly inhibited cell proliferation (Fig. 6D). However, CREB knockdown did not affect IL-8 secretion (Fig. 6E).

Discussion

To date, the signaling molecules involved in mediating GM-CSF secretion have not been identified well. In this study, we aimed to elucidate the mechanisms regulating GM-CSF secretion. Our results showed that CREB played a critical role in p38 MAPK-dependent, TNF-α-induced GM-CSF secretion, providing important insights into these processes.

Although CREB has been shown to be activated by p38 MAPK in previous studies in vascular endothelial and smooth muscle cells, the physiological significance of CREB activation by p38 MAPK remains unclear. Hashimoto et al. reported that p38 MAPK regulates TNF-α-induced GM-CSF production in human bronchial epithelial cells. Consistent with these findings, our results using human lung fibroblasts also revealed that the activity of p38 MAPK was particularly involved in TNF-α-induced GM-CSF secretion. Although SB compounds, such as the inhibitor used in this study, compete for the ATP binding of p38 MAPK and inhibit signaling downstream of p38 MAPK, we observed partial inhibition of TNF-α-induced p38 MAPK phosphorylation in our study, consistent with the results of other studies. This effect may be explained by reduced autophosphorylation of p38 MAPK upon treatment with the SB compound. On the other hand, p44/42 MAPK and AKT inhibitors partially blocked TNF-α-induced GM-CSF secretion without affecting CREB phosphorylation. Previous studies reported that PD98059 inhibits MEK5/ERK5 and cyclooxygenase 2, while LY294002 inhibits PI-3 kinase. Since TNF-α did not stimulate both p44/42 MAPK and AKT phosphorylation in HFL-1 cells, other kinases affected by these kinase inhibitors may be involved in the TNF-α-induced GM-CSF secretion. However, further investigations are needed.

Inhibition of GM-CSF secretion in airway structural cells may be an important strategy for the treatment of asthma. In this study, we examined the inhibitory mechanism of TNF-α-induced GM-CSF secretion.
secretion. Although previous investigators have shown that cAMP-elevating agents, such as Rolipram, inhibit TNF-α-induced GM-CSF secretion in human bronchial epithelial cells,8 the effects of this inhibitor on human lung fibroblasts had not been clarified. Here, we found that HFL-1 cells exhibited greater increases in GM-CSF production than Beas-2B cells after TNF-α stimulation. The amount of GM-CSF secretion from Beas-2B cells in this study was similar to those in previous reports.8,10 In HFL-1 cells, cAMP-elevating agents, such as forskolin, Rolipram, and Roflumilast, inhibited TNF-α-induced GM-CSF secretion. The inhibitory effects of these cAMP-elevating agents on TNF-α-induced GM-CSF secretion were similar to those described in previous reports.8,43 These results indicated that cAMP signaling is an important inhibitory mechanism for GM-CSF secretion.

CREB is a target in the cAMP signaling pathway15 and can also be targeted by other kinases, including p38 MAPK.44 However,
because of the opposing effects of CAMP and p38 MAPK on TNF-α-induced GM-CSF secretion, the mechanisms mediating CREB signaling after TNF-α stimulation are unclear. Our results of the time-dependent effects of TNF-α stimulation on CREB and p38 MAPK phosphorylation supported that TNF-α-dependent CREB phosphorylation was induced through p38 MAPK signaling rather than CAMP signaling; thus, CREB upregulated TNF-α-induced GM-CSF secretion. This may be a possible mechanism explaining the increased CREB phosphorylation in the submucosa, where pulmonary fibroblasts are present, in patients with asthma,20 and inhibitory effect of cAMP/PKA pathway on TNF-α-induced GM-CSF secretion may be caused by CREB phosphorylation at another site or the blockage in the downstream of CREB phosphorylation in HFL-1 cells. Further investigations are required to support these hypotheses.

In summary, our results showed that p38 MAPK was essential for TNF-α-induced GM-CSF secretion. Furthermore, the activation of CREB was critical for intracellular regulation of GM-CSF secretion. This study also demonstrated that cAMP-elevating agents had inhibitory effects of TNF-α-induced GM-CSF secretion. Therefore, inhibition of the p38 MAPK and CREB pathways may attenuate eosinophilic inflammation and may have applications in the prevention or treatment of asthma. Further studies of the signal pathways activated by various cytokines may yield deeper insights into the signaling mechanisms mediating GM-CSF secretion.

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Conflict of interest

The authors have no conflict of interest to declare.

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