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IL-13 induces translocation of NF-κB in cultured human bronchial smooth muscle cells

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ABSTRACT

Background and purpose: Interleukin-13 (IL-13), a major Th2 cytokine, plays an important role in bronchial asthma, including mucus production, inflammation and airway hyperresponsiveness. Although IL-13 through its binding to IL-4 receptor α (IL-4Rα/IL-13Rα1 uses the canonical signal transducer and activator of transcription 6 (STAT6)-signaling pathway to mediate these tissue responses, recent studies have demonstrated that other signaling pathways may also be involved in. In the present study, whether IL-13 induces an activation of nuclear factor (NF)-κB, inflammatory transcription factor, was investigated in human bronchial smooth muscle cells (hBSMCs). *Methods:* Nuclear proteins were extracted from cultured hBSMCs treated with tumor necrosis factor (TNF)-α (10 ng/mL) or IL-13 (100 ng/mL), and assayed for activated NF-κB and STAT6 by Western blotting. *Result:* Treatments with TNF-α and IL-13 induced a translocation of NF-κB to nuclei in hBSMCs. In addition, coincubation with BMS-345541 (0.3 μM), an inhibitor of NF-κB (IκB) kinase (IKK) inhibitor, markedly inhibited the translocation of NF-κB. *Conclusion:* Our results suggest for the first time that IL-13 activates NF-κB in hBSMCs.

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1. Introduction

T helper 2 (Th2) cytokines such as interleukin-4 (IL-4), IL-5 and IL-13 are important mediators of bronchial asthma, a complex chronic disease of the airways, which is characterized by airway inflammation, airway remodeling and airway hyperresponsiveness (AHR). In murine models of the asthmatic response, IL-13 has been shown to be both necessary and sufficient for the generation of asthma-like deleterious tissue alterations [1–4]. The mechanisms of these responses have not been fully defined. However, because IL-13 induces a greater number of genes in airway smooth muscle as compared with other lung cell types, including smooth muscle myosin light chain (MLC), phospholipase A_2 and IL-13 receptor α1 (IL-13Rα1), airway smooth muscle cells have the capacity to respond to direct stimulation by IL-13 [5].

IL-13 binds to a polymeric receptor that contains IL-4Rα and IL-13Rα1 subunits. The majority of the studies of this receptor have focused on its ability to induce signal transducer and activator of transcription 6 (STAT6) tyrosine phosphorylation and subsequent signaling. In murine models, IL-13 has been shown to mediate many of its asthma-relevant responses through the activation of the STAT6 pathway in bronchial epithelial cells [6]. However, other signal transduction pathways also contribute to the pathogenesis of asthma-like Th2 responses including airway hyperresponsiveness and inflammation [7,8]. Extracellular signal-regulated kinase (ERK) 1/2 mitogen activated protein kinase (MAPK) activation is required for optimal IL-13 stimulation of specific chemokines, matrix metalloproteinases (MMPs), and protease inhibitors in the murine lung [9].

The nuclear transcription factor NF-κB, typical of p50 and p65 heterodimer, regulates over 150 genes involved in immune and inflammatory responses. Current evidence suggests an essential role in NF-κB overactivation for increased expression of many inflammatory genes and for airway inflammation in asthma [9,10]. One well established mechanism of NF-κB suppression is the export of nuclear NF-κB by inhibitor of NF-κB (IκB) a, thereby rapidly repressing NF-κB activation [11]. In general, NF-κB has been known to be activated through phosphorylation and degradation of IκBα by tumor necrosis factor (TNF)-α. However, it is reported that NF-κB is activated by not only TNF-α but also IL-1β, UV-light and stress and has an important role in the immune response including inflammation [12–14].

We hypothesized that NF-κB plays a critical role in the pathogenesis of IL-13-induced signal transduction. To test this hypothesis, we investigated whether IL-13 induces an activation of NF-κB in human bronchial smooth muscle cells (hBSMCs). As the results, both TNF-α and IL-13 induced activations of NF-κB and STAT6, respectively. Interestingly, IL-13 also induced an activation of NF-κB, which was demonstrated by the NF-κB translocation to nuclei. These phenomena were significantly inhibited by combination with BMS-345541, IκB kinase (IKK) inhibitor. Therefore, our results

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suggest that both the IL-13 signaling and TNF-α signaling regulate the activation of NF-κB in hBSMCs.

2. Materials and methods

2.1. Chemicals

All biochemicals were of analytical grade and were purchased from commercial suppliers: rhTNF-α (Peprotech, Paris, France), rhIL-13 (Peprotech, Paris, France) and BMS-345541 (Sigma, MO, USA).

2.2. Cell culture

Human bronchial smooth muscle cells (hBSMCs, Cambrex, MD, USA) were maintained in SmBM (Cambrex) medium supplemented with 5% fetal bovine serum (FBS), 1 mg/mL hFGF-B, 0.5 mg/mL hEGF, 5 mg/mL insulin and gentamycin/amphotericin B. The cells plated at a density of 2.7×10^4 per one well of a 6-well dish in FBS. Cells were serum deprived for 24 h using serum-free medium, which consists of SmBM supplemented with gentamycin/amphotericin B. The culture technique was used in experiments involving stimulation with 10 ng/mL TNF-α, 100 ng/mL IL-13 or sterile phosphate buffered saline (PBS) as vehicle control for 30 min or 1 h. BMS-345541, IKK inhibitor, was coincubated with TNF-α or IL-13.

2.3. Nuclear protein extraction

Cells were washed in PBS and lysed in 15 mM KCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.6), 2 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.1% (v/v) Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2.5 μg/ml leupeptin, and 5 μg/ml aprotinin for 10 min on ice. Nuclei were pelleted by centrifugation at 14,000*g* for 20 s at 4 °C. Proteins were extracted from nuclei by incubation at 4 °C with vigorous vortexing in buffer (420 mM NaCl, 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 25% (v/v) glycerol, 1 mM DTT, 0.5 mM PMSF, 2.5 μg/ml leupeptin, and 5 μg/ml aprotinin). Nuclear debris was pelleted by centrifugation at 13,000*g* for 30 min at 4 °C, and the supernatant extract was collected and stored at −85 °C until use.

2.4. Western blot analyses

To determine the levels of p65 in nucleus of hBSMCs and the phosphorylated levels of STAT6 in hBSMCs, the samples (10 mg of total protein per lane) were subjected to 10 or 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), and the proteins were then electrophoretically transferred to a poly vinylidene fluoride (PVDF) membrane. After blocking with 3% skim milk or 3% gelatin, the PVDF membrane was incubated with polyclonal rabbit anti-NF-κB p65 (1:1000 dilution; Biolegend, San Diego, CA, USA), polyclonal rabbit anti-STAT6 (1:1000 dilution; Santa Cruz Biotechnology, Inc., CA, USA), or polyclonal rabbit anti-phosphorylated STAT6 antibody (1:1000 dilution; Santa Cruz). Then the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:2500 dilution; Amersham Biosciences, Co., NJ, USA), detected by an enhanced chemiluminescent system (Amersham Biosciences) and analyzed by a densitometry system. Detection of house-keeping gene was also performed on the same membrane by using polyclonal rabbit anti-histone H1 (1:1000 dilution; Santa Cruz), or monoclonal mouse anti-β-actin (1:5000 dilution; Santa Cruz) to confirm the same amount of proteins loaded.

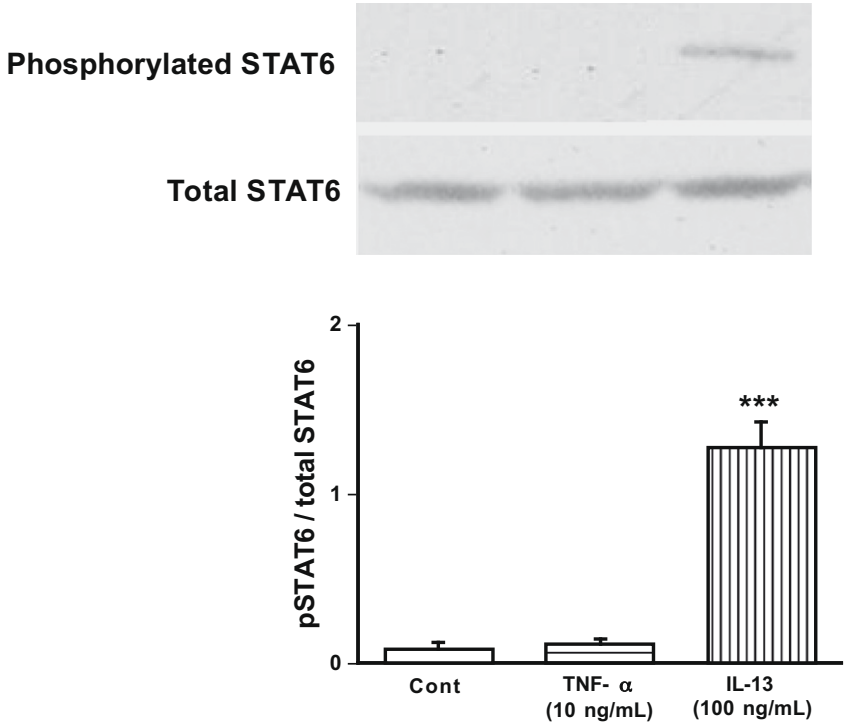

Fig. 1. Phosphorylation of signal transducer and activator of transcription 6 (STAT6) induced by TNF-α (10 ng/mL, 1 h) or IL-13 (100 ng/mL, 1 h) in cultured human bronchial smooth muscle cells (hBSMCs). Total proteins of hBSMCs were assayed for phosphorylated STAT6 by immunoblottings. *(Upper photo)* Typical blots for phosphorylated and total STAT6. The phosphorylation levels of STAT6 are summarized in the *lower panel*. IL-13 induced phosphorylation of STAT6, but TNF-α did not. ($n = 4$, $^{***}p < 0.001$ vs. control by one-way ANOVA with *post-hoc* Bonferroni/Dunn).

2.5. Statistical analyses

All the data were expressed as the mean with S.E.M. Statistical significance of difference was determined by unpaired Student's *t*-test or two-way analysis of variance (ANOVA) with *post-hoc* Bonferroni/Dunn (StatView for Macintosh ver. 5.0, SAS Institute, Inc., NC). A value of $p < 0.05$ was considered as significant.

3. Result

To determine whether TNF-α and IL-13 are capable of activating STAT6 and NF-κB in hBSMCs, the levels of STAT6 phosphorylation and nuclear translocation of p65 were assessed by immunoblottings.

As shown in Fig. 1, IL-13 caused distinct phosphorylation of STAT6 in the hBSMCs: the level of phosphorylated STAT6 in the IL-13 (100 ng/mL)-treated cells was significantly increased as compared with that in the vehicle-treated control cells ($p < 0.001$). On the other hand, TNF-α (10 ng/mL) had no effect on the level of STAT6 phosphorylation (Fig. 1).

Fig. 2 shows the effects of TNF-α and IL-13 on the level of nuclear p65 in hBSMCs. As widely reported in various types of cells, TNF-α (10 ng/mL) caused a significant increase in nuclear p65 level (Fig. 2; $p < 0.01$), i.e., translocation of p65 to nuclei, in hBSMCs. Interestingly, a significant increase in nuclear p65 was also observed in the cells treated with 100 ng/mL of IL-13 (Fig. 2; $p < 0.05$), suggesting that IL-13 is capable of activating not only STAT6 but also NF-κB in hBSMCs.

Next, the effect of an IκB kinase (IKK) inhibitor, BMS-345541, on the IL-13-induced translocation of p65 was investigated to determine the role of IKK in the IL-13-induced activation of NF-κB. As shown in Fig. 3, the TNF-α-induced translocation of p65 was completely blocked by BMS-345541 (0.3 μM; $p < 0.01$). This concentration of BMS-345541 also inhibited the IL-13-induced translocation of p65 completely, indicating that the p65 translocation induced by IL-13 is mediated by an activation of IKK in hBSMCs.

4. Discussion

IL-13 is a Th2 cytokine that has emerged as a critical regulator of inflammatory immune responses, with key roles in asthma and parasite immunity [15,16]. IL-13 can be detected in the bronchial tissue [17], nasal lavage fluid [18], and induced sputum [17] of asthmatics. Following segmental allergen challenge, bronchoalveolar lavage (BAL) fluid contains IL-13 mRNA [19] and IL-13 protein [20], indicating that the cytokine is generated in the lung in response to respiratory provocation. Transgene pulmonary overexpression of IL-13 in mice is associated with several key pathological features of airways inflammation and remodeling, also observed in patients with chronic severe asthma, including lymphocyte and eosinophil accumulation, mucus cell metaplasia, subepithelial fibrosis and AHR [1]. Several studies indicate that IL-13 may cause AHR independently of inflammation and it is plausible that the effect may result from a direct effect on airway smooth muscle (ASM). ASM cells express functional IL-13 receptors and IL-13 is also linked to an augmented ASM contractility in rabbit tracheal strips [21,22].

IL-13 first binds to the IL-13Rα1 chain on the surface of cells. IL-4Rα is recruited upon IL-13 interaction with IL-13Rα1, to form the high affinity receptor complex [23]. Heterodimerization of cell surface IL-13Rα1 and IL-4Rα receptor chains initiates IL-13 signaling via recruitment of janus kinase (JAK) 1, JAK2 and tyrosine kinase (Tyk) 2, resulting in the phosphorylation of STAT6, a critical step in IL-13 and IL-4 dependent signaling [24].

The present study demonstrated for the first time that not only TNF-α (10 ng/mL) but also IL-13 (100 ng/mL) induces translocation

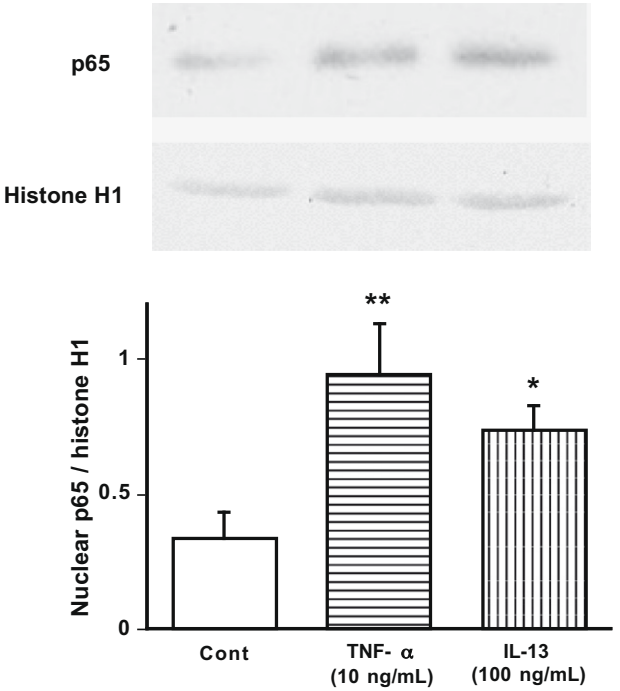

Fig. 2. Translocation of p65 to nuclei induced by TNF-α (10 ng/mL, 30 min) or IL-13 (100 ng/mL, 30 min) in cultured human bronchial smooth muscle cells (hBSMCs). Nuclear proteins of hBSMCs were assayed for p65 by immunoblottings. *(Upper photo)* Typical blots for p65 and histone H1. The expression levels of p65 are summarized in the *lower panel*. TNF-α or IL-13 induced translocation of NF-κB to nuclei. ($n = 4$, $^{*}p < 0.05$, $^{**}p < 0.01$ vs control by one-way ANOVA with *post-hoc* Bonferroni/Dunn).

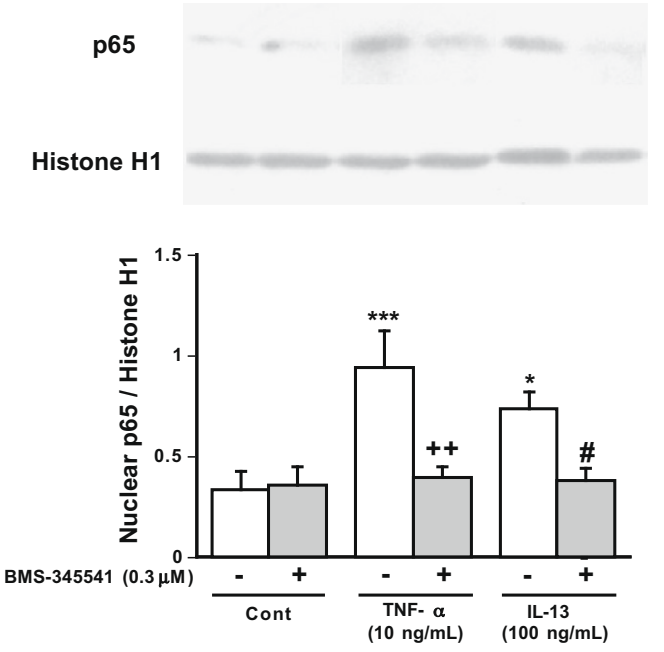

Fig. 3. Effects of BMS-345541, an IκB kinase (IKK inhibitor, on translocation of p65 to nuclei induced by TNF-α (10 ng/mL) or IL-13 (100 ng/mL) in cultured human bronchial smooth muscle cells (hBSMCs). Nuclear proteins of hBSMCs were assayed for p65 by immunoblottings. *(Upper photo)* Typical blots for p65 and histone H1. The expression levels of p65 are summarized in the *lower panel*. BMS-345541 inhibited TNF-α- or IL-13-induced translocation of p65 to nuclei. ($n = 4$, $^{*}p < 0.05$, $^{***}p < 0.001$ vs. control, $++p < 0.01$ vs. TNF-α alone and $\#p < 0.05$ vs. IL-13 alone by two-way ANOVA with *post-hoc* Bonferroni/Dunn).

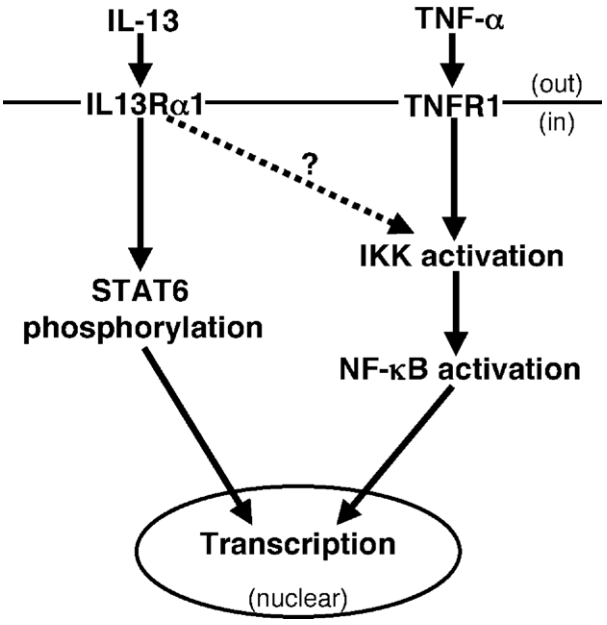

Fig. 4. Putative mechanisms of activation of signal transducer and activator of transcription 6 (STAT6) and NF-κB by IL-13 and TNF-α in hBSMCs. The current findings suggest that, in addition to the activation of STAT6 pathway, IL-13 also activate NF-κB via an activation of IκB kinase (IKK, although the signaling between IL-13 receptors and IKK is not yet clear now.

of NF-κB to nuclei in hBSMCs. A complex composed of IKKs (IKKα, IKKβ and IKKγ) is an essential component in NF-κB-signaling mechanisms participating in IκBα phosphorylation. Although IKKα and IKKβ are catalytic subunits, IKKγ is a regulatory subunit. IKKβ is essential for activation of the classical NF-κB pathway through phosphorylation of serine residues 32 and 36 of IκBα, resulting in the ubiquitination and subsequent degradation of IκBα by the 26S proteasome. The degradation of IκBα exposes a nuclear translocation sequence facilitating translocation of NF-κB to the nucleus. In spite of treatment with TNF-α or IL-13, the expression of total cellular p65 failed to show any change. Therefore, it is our judgment that upregulation of p65 in nuclei occurred by translocation of NF-κB. Svetlana et al. [25] had demonstrated an activation of NF-κB in mice when IL-13 was overexpressed in lung epithelia. In addition, Il-13-induced eosinophilia, inflammation, fibrosis, airway obstruction and alveolar remodeling were inhibited by interference with NF-κB activation [25]. In our works, IL-13 also activated NF-κB, and IL-13-induced translocation of NF-κB to nuclei was completely inhibited by BMS-345541 (0.3 μM), IKK inhibitor in hBSMCs. Because the TNF-α induced activation of NF-κB is dependent on IKKβ, we suggested that BMS-345541 (0.3 μM) was perfectly inhibited IKK activity. Therefore, our result indicated that IL-13 activates NF-κB via IKKβ.

In conclusion, we suggest for the first time that IL-13 activates NF-κB via an activation of IKK in hBSMCs (Fig. 4). Therefore, because the inhibition of NF-κB activity suppressed not only TNF-α induced signaling but also IL-13 induced signaling partly, NF-κB inhibitor can be a therapeutic target for bronchial asthma.

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