Original Paper

β₂-Adrenoceptor Agonists Enhance Cytokine-Induced Release of Thymic Stromal Lymphopoietin by Lung Tissue Cells

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Abstract
Background: While β₂-adrenoceptor agonists (β₂-agonists) are widely used as bronchodilators in the treatment of asthma, there has been increasing concern that regular use of β₂-agonists may adversely affect the control of asthma. However, the molecular mechanisms of such undesirable effects of β₂-agonists are not fully understood. In this study, we examined the effects of β₂-agonists on cytokine-induced production of thymic stromal lymphopoietin (TSLP), an indispensable cytokine in the development of allergic diseases, by lung tissue cells. **Methods:** Normal human bronchial epithelial cells (NHBE), smooth muscle cells (BSMC) and fibroblasts (NHLF) were stimulated with the IL-4 and TNF-α cytokines, alone and in combination, and their production of TSLP was examined by ELISA. The effects of β₂-agonists (salmeterol, formoterol, salbutamol), intracellular cyclic adenosine monophosphate (cAMP)-elevating agents (8-bromo-cAMP, dibutyryl cAMP, forskolin) and a corticosteroid (fluticasone) on the cytokine-induced TSLP production were examined. **Results:** The following results were observed in all three types of lung tissue cells tested (that is, NHBE, BSMC and NHLF). Costimulation with IL-4 and TNF-α significantly induced TSLP production, and β₂-agonists further enhanced it via upregulation of intracellular cAMP. However, addition of a corticosteroid to the cytokines and β₂-agonist resulted in a marked decrease in TSLP production. **Conclusions:** β₂-Agonists significantly enhanced the cytokine-induced TSLP production by primary human lung tissue cells. This may be partly responsible for the undesirable clinical effects of continuous β₂-agonist monotherapy, and combination therapy with a corticosteroid might effectively inhibit TSLP-mediated allergic inflammation.

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Introduction

Asthma has been defined as a chronic inflammatory disease of the airways that is characterized by airway hyperresponsiveness, reversible airflow obstruction and

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airway remodeling. β_2-Adrenoceptor agonists (β_2-agonists) are widely used as bronchodilators in the treatment of asthma because of their potent bronchodilating effects on airway smooth muscle. In addition to being bronchodilators, they may also have anti-inflammatory properties, including inhibition of granulocyte functions [1]. However, there has been increasing concern that regular use of β_2-agonists may adversely affect the control of asthma [2, 3]. More specifically, continuous and repetitive β_2-agonist monotherapy has been considered to be associated with an increase in the degree of allergic inflammation [4], poor asthma outcomes [5] and an increase in the risk of asthma death [6, 7]. Although the precise molecular mechanisms underlying these undesirable effects of β_2-agonists are not fully understood, several studies have independently demonstrated that β_2-agonists have the potential to increase Th2 cytokine-mediated inflammation both in vivo and in vitro. For instance, Coqueret et al. [8] demonstrated that ovalbumin-sensitized mice treated with a daily injection of salbutamol showed increased anti-ovalbumin IgE levels in their serum, probably due to increased production of Th2 cytokines. Panina-Bordignon et al. [9] demonstrated that β_2-agonists prevented Th1 development by selectively inhibiting IL-12 production. More recently, Loza et al. [10] demonstrated that human Th2 cells express β_2-adrenergic receptor and that β_2-agonists augmented the accumulation of Th2 cells in human peripheral blood lymphocyte cultures subjected to bystander stimuli. These findings suggest a mechanism by which β_2-agonist monotherapy may favor Th2 immune responses, which are believed to be involved in the pathogenesis of asthma.

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine which was originally identified in the supernatant of a murine thymic stromal cell line [11]. Increasing evidence suggests that TSLP plays important roles in the pathogenesis of allergic diseases such as asthma and atopic dermatitis [12, 13]. The most clinically relevant role of TSLP is mediated by dendritic cells through induction of OX40 ligand expression on dendritic cells [14, 15]. Naïve T cells receiving antigen presentation from TSLP-primed dendritic cells develop into Th2 cells that produce IL-4, IL-5, IL-13 and TNF-α but not IL-10 [14–16]. These Th2 cells are now referred to as 'inflammatory Th2 cells' in consideration of their potential for releasing the pro-inflammatory cytokine, TNF-α, in addition to Th2 cytokines [17]. Furthermore, mice with transgenic overexpression of TSLP in the lung develop severe airway inflammation, including massive infiltration by inflammatory cells, goblet cell hyperplasia and airway hyperre-

sponsiveness [18]. Mice with transgenic overexpression of TSLP in skin keratinocytes develop severe dermatitis with itching, which is similar to the clinical features of atopic dermatitis in humans [13]. On the other hand, mice lacking the TSLP receptor exhibit strong Th1 responses and fail to develop an inflammatory lung response to antigens [12]. Thus, TSLP is an important cytokine that is necessary and sufficient for initiation of allergic inflammation. In addition, elevated expression of TSLP was also found in the airways of the patients with chronic obstructive pulmonary disease [19, 20].

Various pro-inflammatory mediators, including cytokines and pathogen-associated microbial patterns, are known to induce TSLP production by bronchial epithelial cells [21], smooth muscle cells [19] and skin keratinocytes [22].

In this context, we examined the effects of β_2-agonists on cytokine-mediated TSLP production by human lung tissue cells such as epithelial cells, smooth muscle cells and fibroblasts and found that they significantly enhanced TSLP production by those cells. Our findings suggest that β_2-agonist-enhanced production of TSLP by lung tissue cells may be partly responsible for the undesirable clinical effects of continuous β_2-agonist monotherapy.

Materials and Methods

Reagents
Recombinant human IL-4 and TNF-α were purchased from PeproTech (Rocky Hill, N.J., USA). The following corticosteroid, β_2-agonists and cAMP-elevating agents were all purchased from Sigma-Aldrich Japan (Tokyo, Japan): fluticasone propionate (fluticasone), salmeterol xinafoate (salmeterol), formoterol fumarate (formoterol), salbutamol sulfate (salbutamol), N6,2′-O-dibutyryl adenosine 3′,5′-cyclic monophosphate sodium salt (db cAMP), 8-bromoadenosine 3′,5′-cyclic monophosphate sodium salt (8-Br cAMP) and forskolin.

Cell Culture and Stimulation
Primary normal human bronchial epithelial cells (NHBE), normal human bronchial smooth muscle cells (BSMC) and normal human lung fibroblasts (NHLF) were purchased from Lonza (Walkersville, Md., USA) and maintained exactly as recommended by the manufacturer. NHBE were cultured in flasks or plates coated with type I collagen (Iwaki, Tokyo, Japan). At least 24 h before stimulation, NHBE were cultured in SAGM BulletKit (Lonza) without hydrocortisone, epinephrine or retinoic acid. BSMC and NHLF were cultured in noncoated culture flasks or plates (Iwaki) in their respective optimized growth media, that is, SmGM-2 BulletKit and FGM-2 BulletKit (Lonza). All experiments described in this study were performed using second- or third-passage cells in 70–80% confluent monolayers. Further-

more, in order to eliminate effects of the different genetic and environmental backgrounds of the primary cell donors, we performed the same experiments with each type of cells originating from at least two different donors and obtained reproducible results.

The concentration of both cytokines, IL-4 and TNF-α, used in this study was 10 ng/ml, and other reagents were used at the indicated concentrations. After stimulation, cells or cell-free culture supernatants were harvested at 5 min, 6 h and 48 h for cAMP assay, quantitative real-time PCR and ELISA, respectively. These optimal concentrations and time points were determined in our preliminary experiments and by referring to previous reports [21].

The β_2-agonists, cAMP-elevating agents and corticosteroid were each added to the wells simultaneously with the cytokines in order to investigate whether they affected the production of TSLP. Salmeterol and salbutamol were dissolved in ethanol and distilled water, respectively, and other agents, including formoterol, cAMP-elevating agents and fluticasone, were dissolved in dimethyl sulfoxide as stock solutions. Therefore, cells were treated with the respective solvents at the highest concentration used in each experiment as vehicle controls.

Quantitative Real-Time PCR
Total RNA samples were extracted using RNeasy (Qiagen, Valencia, Calif., USA) and digested with RNase-free DNase I (Qiagen) in accordance with the manufacturer's instructions. First-strand cDNA was synthesized using an Oligo(dT) (12–18mers) primer (Invitrogen, Carlsbad, Calif., USA) and SuperScript II (Invitrogen) as the reverse transcriptase. Quantitative real-time PCR was performed as previously described [23]. Primer sets for two genes were synthesized at Fasmac (Kanagawa, Japan): TSLP (sense, 5′-GCCCAGGCTATTCGGAAACT-3′; antisense, 5′-CGACGCCACAATCCTTGTAATT-3′); β-actin (sense, 5′-CCCAGCCATGTACGTTGCTAT-3′; antisense, 5′-TCACCGGAGTCCATCACGAT-3′). To determine the exact copy numbers of the TSLP gene, quantified concentrations of the purified PCR product of TSLP were serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 5 ng of the total RNA samples were used for each real-time PCR. The mRNA expression levels were normalized to the β-actin level in each sample.

ELISA
The TSLP protein concentrations in the cell-free supernatants were measured with a specific ELISA kit (R&D Systems, Minneapolis, Minn., USA) in accordance with the manufacturer's instructions.

cAMP Assay
The intracellular cyclic AMP (cAMP) levels in NHBE were measured by Parameter™ cAMP assay (R&D Systems) according to the manufacturer's instructions and a previous report [24]. Briefly, cells were stimulated with a combination of IL-4 and TNF-α in the presence and absence of 10^{-10} M salmeterol, 10^{-8} M salbutamol and/or 10^{-8} M fluticasone at 37°C for 5 min. The cells were then suspended in cell lysis buffer (R&D Systems), freeze-thawed several times with mixing, and centrifuged. The supernatants were used for determination of the cAMP concentrations. The optical density of each sample was measured using a microplate reader (Benchmark; Bio-Rad, Hercules, Calif., USA) set at 450 nm.

Statistical Analysis
All data were presented as the mean $\pm$ SD. Differences between groups were analyzed using analysis of variance with Bonferroni's post hoc test and were considered to be significant when p < 0.05.

Results

Cytokine-Induced Production of TSLP by Lung Tissue Cells
First, we found that IL-4, a Th2 cytokine, induced production of TSLP by NHBE (fig. 1a). Although TSLP was not detected in the culture supernatants of NHBE stimulated only with TNF-α, a pro-inflammatory cytokine, TNF-α synergistically enhanced the IL-4-induced production of TSLP, as previously reported by Kato et al. [21]. In contrast, TNF-α, but not IL-4, significantly and robustly induced production of TSLP by lung mesenchymal cells such as BSMC and NHLF (fig. 1b, c). IL-4 showed synergistic effects on the TNF-α-induced TSLP production by those cells.

Effects of β_2-Agonists on Cytokine-Induced TSLP Production
We next examined the effects of β_2-agonists on the cytokine-induced TSLP production by the cultured cells. When NHBE were stimulated with a combination of IL-4 and TNF-α, simultaneous addition of various concentrations of two long-acting β_2-agonists, that is, salmeterol and formoterol, and a short-acting β_2-agonist, salbutamol, showed significant enhancement of the cytokine-induced TSLP production (fig. 2a). Optimal concentrations of these β_2-agonists were employed, and then the mRNA expression of TSLP in NHBE was measured by quantitative real-time PCR. TSLP mRNA expression was significantly enhanced by 10^{-10} M salmeterol, 10^{-10} M formoterol and 10^{-8} M salbutamol (fig. 2b), suggesting that the enhancing effects of β_2-agonists on TSLP production were transcriptionally regulated. These enhancing effects of β_2-agonists on TSLP production were also observed in BSMC and NHLF, resulting in the release of appreciably greater amounts of TSLP (fig. 2c, d).

Effects of cAMP-Elevating Agents on Cytokine-Induced TSLP Production
To determine the role of intracellular cAMP in the enhancement of TSLP production by β_2-agonists, the cells were stimulated with three cAMP-elevating agents, that is, 8-Br cAMP, db cAMP and forskolin (an adenylate cy-
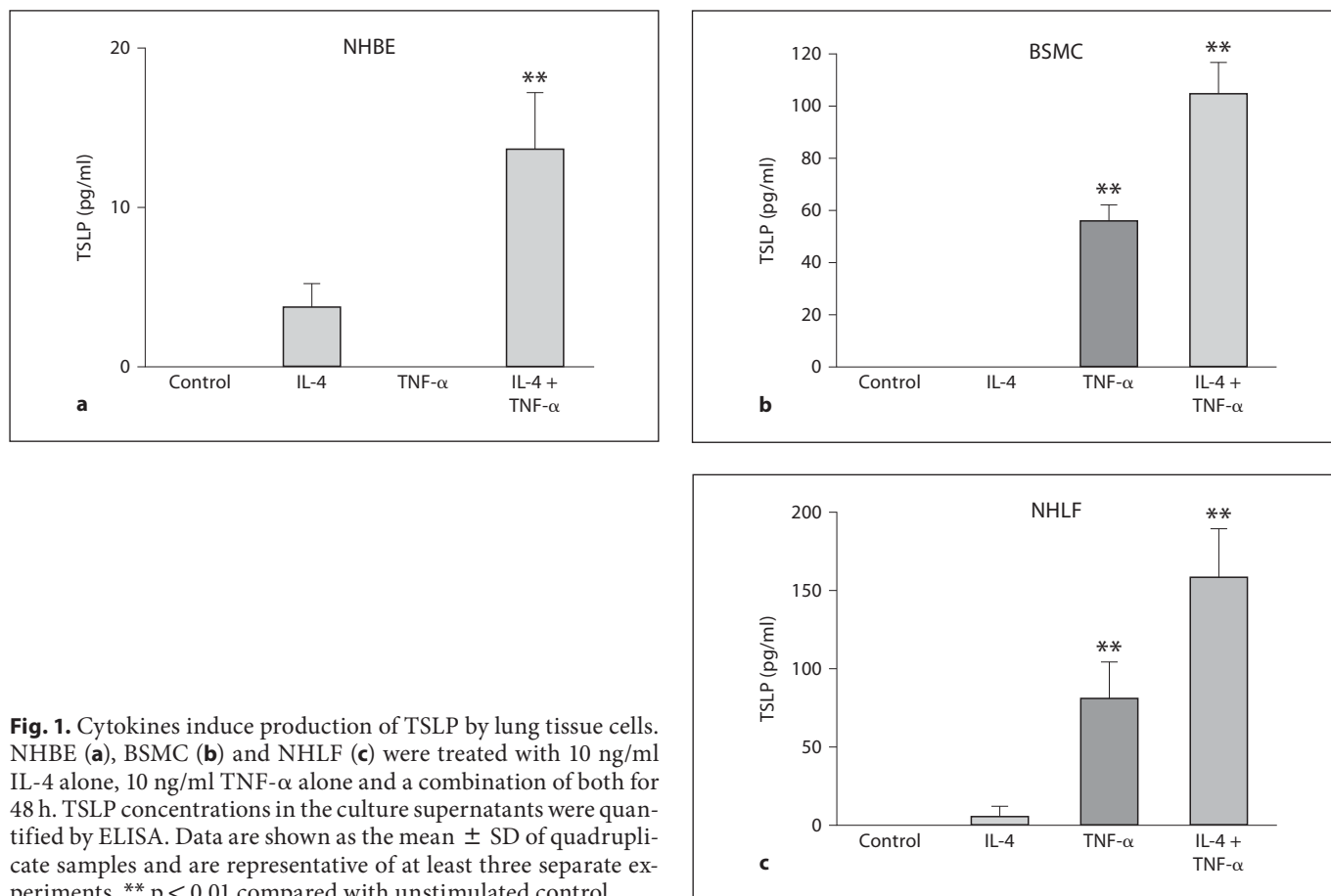
Fig. 1. Cytokines induce production of TSLP by lung tissue cells. NHBE (**a**), BSMC (**b**) and NHLF (**c**) were treated with 10 ng/ml IL-4 alone, 10 ng/ml TNF-α alone and a combination of both for 48 h. TSLP concentrations in the culture supernatants were quantified by ELISA. Data are shown as the mean ± SD of quadruplicate samples and are representative of at least three separate experiments. ** $p < 0.01$ compared with unstimulated control.

clase activator). All three agents caused significant enhancement of cytokine-induced TSLP production by the lung tissue cells (fig. 3).

Effects of Corticosteroid on TSLP Production

We next examined the effects of a corticosteroid on the TSLP production. Simultaneous addition of various concentrations of fluticasone caused dose-dependent, significant inhibition of both cytokine-induced (closed squares) and salmeterol-enhanced (closed triangles) TSLP production by NHBE (fig. 4a, upper graph). Similar results were obtained in experiments using NHLF (fig. 4a, lower graph) and BSMC (data not shown).

Furthermore, in order to clarify how corticosteroids might inhibit TSLP production, we examined the effects of fluticasone and β2-agonists on the intracellular cAMP level in NHBE. Addition of salmeterol or salbutamol significantly increased the cAMP level after 5 min of incubation (fig. 4b). Addition of 10^{-8} M fluticasone showed no effect on the intracellular cAMP level whether in the

presence or absence of a β2-agonist (fig. 4b), suggesting that corticosteroid inhibition of TSLP production is not due to direct inhibition of cAMP signaling.

Discussion

To date, a number of clinical studies have found that regular use of a β2-agonist had an adverse effect on the control of asthma [2–7], and some basic studies showed that β2-agonists have the potential to increase Th2 cytokine-mediated inflammation [8–10], which may partly explain the undesirable clinical effects of continuous β2-agonist monotherapy. In the present study, to further elucidate the possible mechanistic role of β2-agonist-mediated allergic inflammation, we focused on the effects of β2-agonists on synthesis of TSLP, a cytokine that is necessary and sufficient for initiation of allergic inflammation.

It is widely accepted that TSLP is expressed predominantly in epithelial cells of the lung, intestine and skin
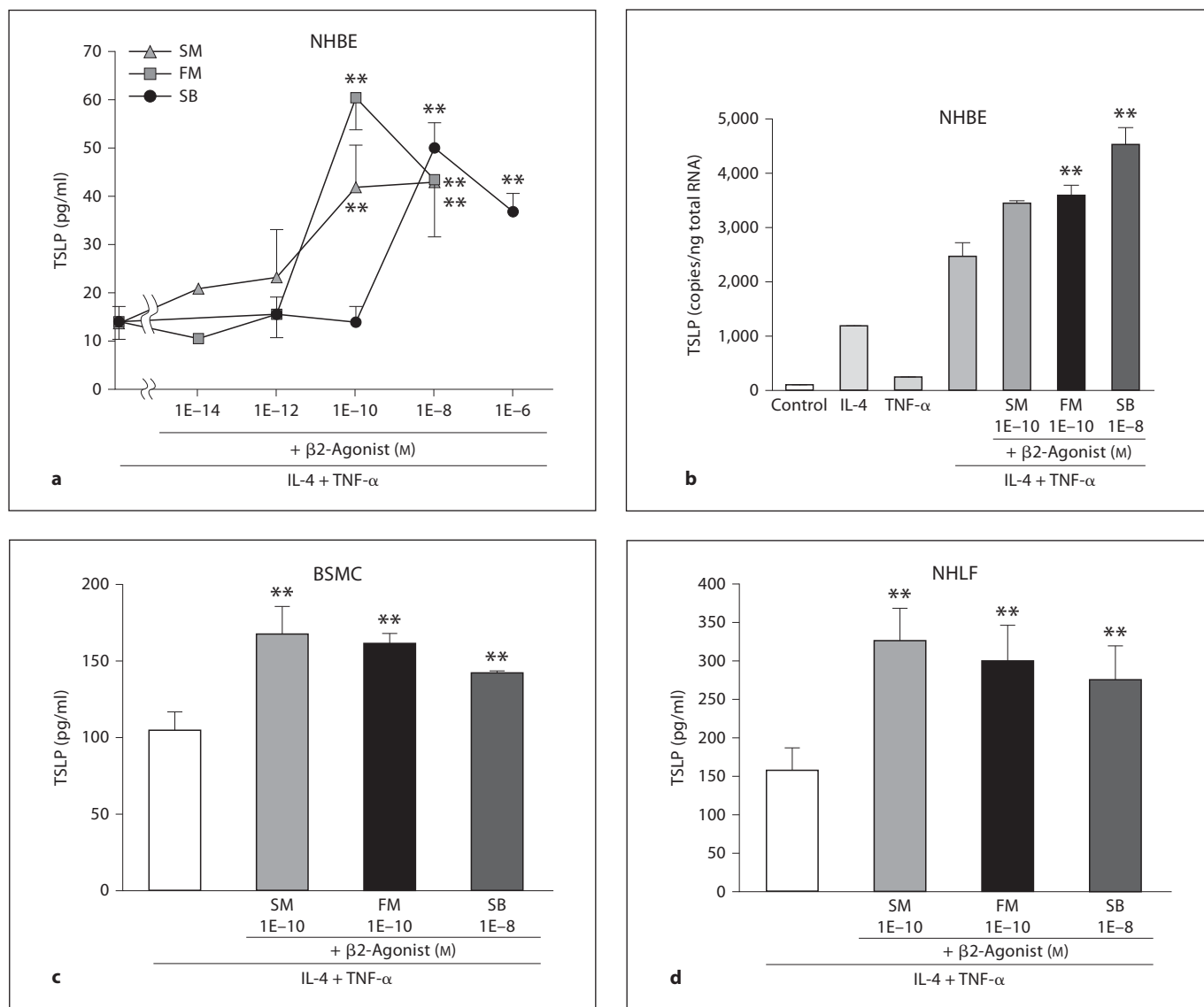
Fig. 2. β_2-Agonists enhance cytokine-induced TSLP production by lung tissue cells. **a** NHBE were treated with different concentrations of two long-acting β_2-agonists, salmeterol (SM) and formoterol (FM), or a short-acting β_2-agonist, salbutamol (SB), in the presence of 10 ng/ml IL-4 and 10 ng/ml TNF-α for 48 h. TSLP concentrations in the culture supernatants were quantified by ELISA. **b** NHBE were treated with cytokines at 10 ng/ml in the presence and absence of the indicated concentrations of each β_2- agonist (SM, FM, SB) for 6 h. The copy numbers of TSLP mRNA are shown. BSMC (**c**) and NHLF (**d**) were treated with the indicated concentrations of each β_2-agonist (SM, FM, SB) in the presence of 10 ng/ml IL-4 and 10 ng/ml TNF-α for 48 h. TSLP concentrations in the culture supernatants were quantified by ELISA. All data are shown as the mean $\pm$ SD of quadruplicate samples and are representative of at least three separate experiments. ** $p < 0.01$ compared with IL-4 plus TNF-α.

keratinocytes [16, 25]. We confirmed an earlier report [21] that a combination of IL-4 and TNF-α synergistically induced TSLP production by NHBE (fig. 1a). Unlike NHBE, lung mesenchymal cells such as smooth muscle cells (BSMC) and fibroblasts (NHLF) produced TSLP in response to TNF-α, but not IL-4 alone. However, like

NHBE, those cells produced greater amounts of TSLP as a result of synergistic effects between IL-4 and TNF-α (fig. 1b, c). Of note, these mesenchymal cells produce appreciable amounts of TSLP compared to NHBE, although the levels are not completely comparable. Nevertheless, these results suggest the possibility that lung mesenchy-
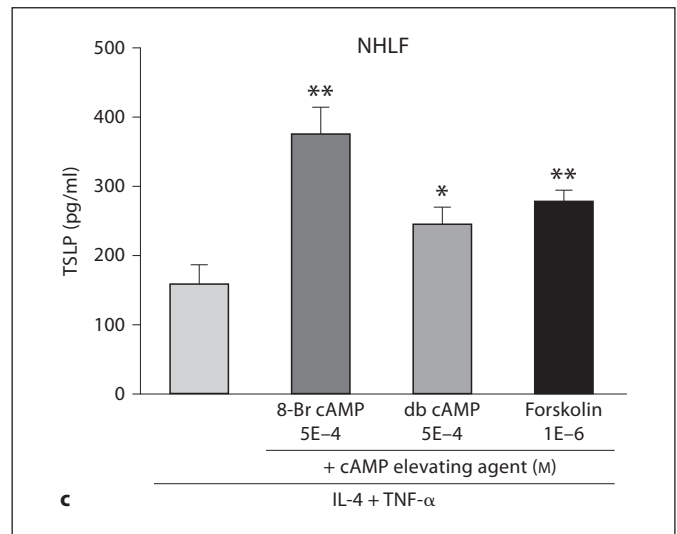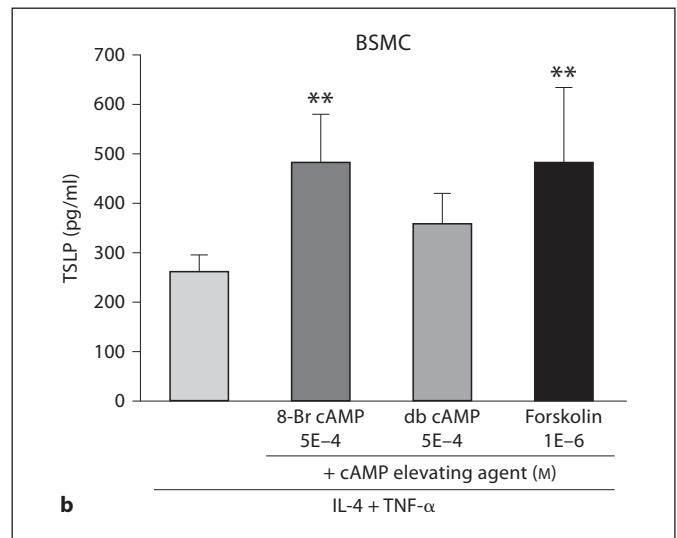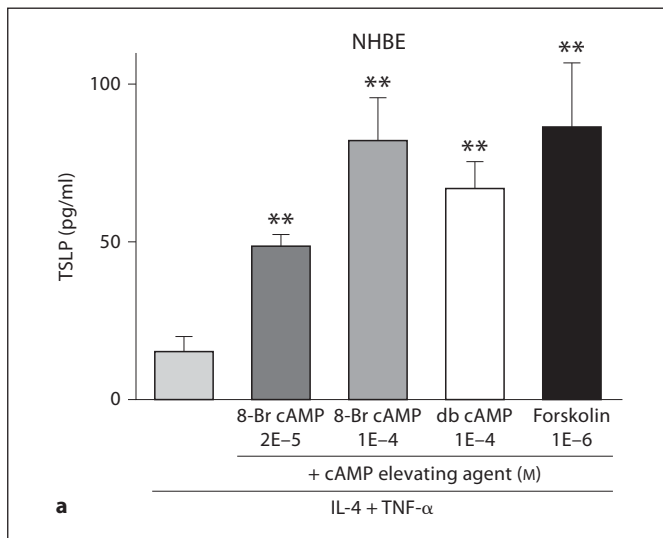
Fig. 3. Intracellular cAMP-elevating agents enhance cytokine-induced TSLP production by lung tissue cells. NHBE (**a**), BSMC (**b**) and NHLF (**c**) were treated with 10 ng/ml IL-4 and 10 ng/ml TNF-α in the presence and absence of the indicated concentrations of each cAMP-elevating agent (8-Br cAMP, db cAMP, forskolin) for 48 h. TSLP concentrations in the culture supernatants were quantified by ELISA. Data are shown as the mean ± SD of quadruplicate samples and are representative of at least three separate experiments. * $p < 0.05$ and ** $p < 0.01$ compared with IL-4 plus TNF-α.

mal cells are, like epithelial cells, important cellular sources of TSLP.

Although β_2-agonists act mainly on airway smooth muscle as bronchodilators, they are also known to express anti-inflammatory effects on granulocytes [1], epithelial cells [26] and fibroblasts [27]. However, we unexpectedly found that simultaneous treatment with salmeterol, formoterol and salbutamol significantly enhanced cytokine-induced TSLP production by each of the tested lung tissue cells (that is, NHBE, BSMC and NHLF; fig. 2). It is well known that binding of β_2-agonists to β_2-adrenoceptors activates adenylate cyclase, resulting in generation of intracellular cAMP. We therefore examined the role of intracellular cAMP in the enhancement of

TSLP production and found that each of the tested cAMP-elevating agents (db cAMP, 8-Br cAMP and forskolin) significantly enhanced cytokine-induced TSLP production by the lung tissue cells (fig. 3). These results suggest that the enhancing effects of β_2-agonists on TSLP production were mediated via upregulation of intracellular cAMP in these cells.

Some other cAMP-elevating agents, such as cholera toxin and prostaglandin E_2, are widely recognized as Th2 adjuvants that can directly alter the characteristics of dendritic cells [28] and T cells [29]. Birch pollen-derived phytoprostanes, which are prostaglandin E_2-related molecules, are also known to favor Th2 responses by blocking IL-12 expression in activated dendritic cells [30]. Our
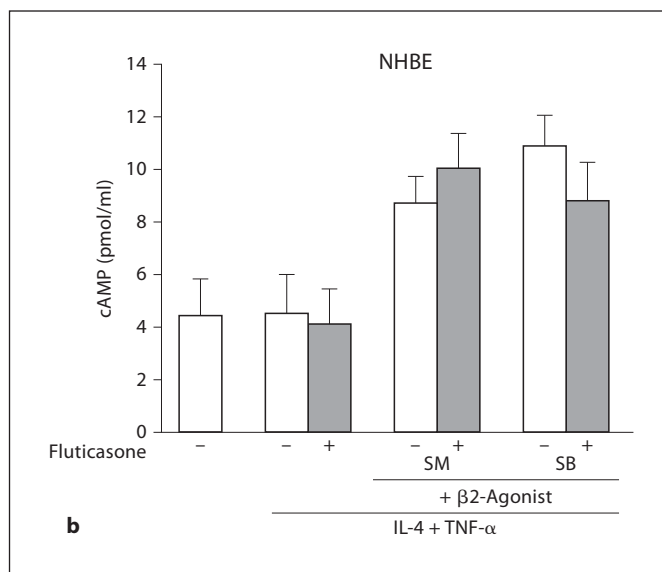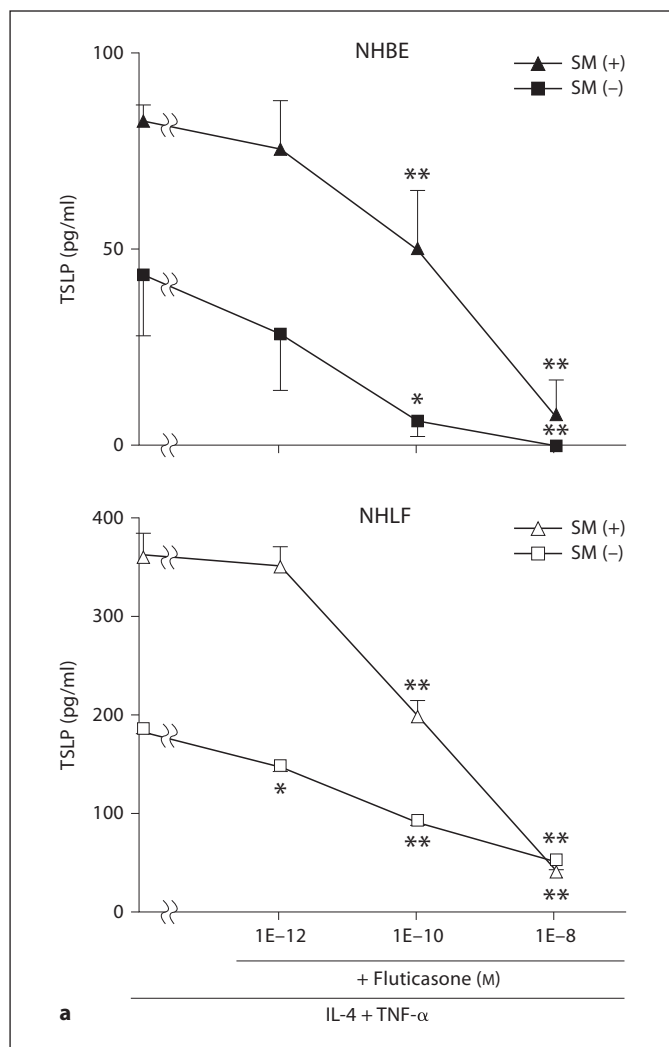
Fig. 4. Fluticasone inhibits TSLP production without affecting the intracellular cAMP level. **a** NHBE (upper graph) and NHLF (lower graph) were treated with 10 ng/ml IL-4 and 10 ng/ml TNF-α with and without 10^{-10} M salmeterol (SM) for 48 h. The effects of simultaneous addition of the indicated concentrations of fluticasone on the TSLP production are shown. Data are shown as the mean $\pm$ SD of quadruplicate samples and are representative of at least three separate experiments. * p < 0.05; ** p < 0.01 compared to without fluticasone. **b** NHBE were treated with 10 ng/ml IL-4 and 10 ng/ml TNF-α with and without a β_2-agonist (10^{-10} M SM, 10^{-8} M SB) for 5 min. The effects of simultaneous addition of 10^{-8} M fluticasone on the intracellular cAMP levels are shown. Data are shown as the mean $\pm$ SD of triplicate samples and are representative of three separate experiments.

results suggest that β_2-agonists and other intracellular cAMP-elevating agents may favor Th2 responses by enhancing TSLP production, in addition to previously reported mechanisms (for example, direct inhibition of IL-12 production by dendritic cells).

Elevation of the intracellular cAMP level results in activation of cAMP-dependent kinase (protein kinase A, PKA). Although we investigated the effects of PKA inhibitors on β_2-agonist-enhanced TSLP production, neither H-89 (N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride) nor 5-24 showed any effect (data not shown), indicating that PKA is not involved in β_2-agonist-enhanced TSLP production. In fact, it was recently reported that various signaling pathways other than the classic cAMP-PKA pathway are involved in β_2-agonist-induced signaling [31]. In the future,

it will be necessary to clarify the molecular mechanisms by which β_2-agonists specifically enhance cytokine-induced TSLP production.

It should be noted that we have shown here for the first time that β_2-agonists enhanced TSLP production by airway smooth muscle cells and lung fibroblasts as well as bronchial epithelial cells (fig. 2, 3). We suppose that the production of TSLP by these lung tissue cells is particularly important because dendritic cells have to migrate through these airway interstitial cells to lymphopoietic tissues in order to present antigen information to naïve T cells. Therefore, enhanced TSLP production by lung tissue cells in response to β_2-agonists may lead to exacerbation of allergic airway inflammation, and this may partly explain the undesirable clinical effects of continuous β_2-agonist monotherapy.

According to the recently updated guidelines for asthma management [32], the preferred treatment regimen for patients with intermittent asthma is an inhaled short-acting β_2-agonist, and the next step regimen is additional treatment with an inhaled corticosteroid. Therefore, we examined the effects of a corticosteroid, fluticasone, on the β_2-agonist-induced increase in TSLP production and found that it significantly inhibited TSLP production by NHBE and NHLF (fig. 4a). Importantly, simultaneous treatment at the highest concentration of fluticasone (10^{-8} M), which can still be considered clinically feasible, almost completely abrogated not only the cytokine-induced TSLP production but also the enhancement by the β_2-agonists. The inhibitory effect of this corticosteroid on TSLP production did not appear to be due to direct inhibition of cAMP signaling (fig. 4b), suggesting that corticosteroids inhibit TSLP synthesis by acting on the downstream signaling pathway of cAMP.

To date, several mechanisms have been proposed to explain the synergistic action between corticosteroids and β_2-agonists: induction and protection of β_2-adrenoceptors by corticosteroids [33], enhancement of translocation of glucocorticoid receptors into the nucleus by β_2-agonists [34] and posttranscriptional regulation to suppress expression of inflammatory genes [35]. Our results may shed new light on the mechanisms by which combination therapy using an inhaled β_2-agonist and an inhaled corticosteroid shows synergistic clinical efficacy in patients with asthma.

It remains to be clarified whether the enhancing effect of β_2-agonists on cytokine production is specific to TSLP or not. Koyama et al. [26] demonstrated that TNF-α-induced production of granulocyte-macrophage colony-stimulating factor (GM-CSF), CCL5 and IL-8 by a human bronchial epithelial cell line, BEAS-2B, was significantly inhibited by procaterol, a β_2-agonist. We confirmed that the cytokine-induced production of GM-CSF and CCL5, but not IL-8, by NHBE was significantly suppressed by β_2-agonist treatment (data not shown). On the other hand, it was reported that rhinovirus-induced IL-6 production by NHBE was increased by salmeterol [36], and we also found that the cytokine-induced production of IL-6 as well as TSLP by NHBE was significantly enhanced by simultaneous treatment with β_2-agonists (data not shown). Thus, β_2-agonists are able to crucially modulate the production of various inflammatory mediators through mechanisms that need to be further elucidated.

In conclusion, we focused on the effects of β_2-agonists on the in vitro synthesis of TSLP, which is a key cytokine in the development of allergic diseases. We found that β_2-agonists significantly enhanced cytokine-induced TSLP production by cultured primary human lung tissue cells. This enhancement may be partly responsible for the undesirable clinical effects of continuous β_2-agonist monotherapy, and our other findings suggest that combination therapy with a corticosteroid might effectively inhibit TSLP-mediated allergic inflammation.

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