INTRODUCTION

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine that was originally identified as a factor derived from a thymic stromal cell line that could support the growth of a mouse B cell line\(^1\). However, many studies indicate that TSLP derived from epithelial cells is a critical factor for the development of Th2-type allergic inflammation. For example, transgenic mice expressing TSLP in keratinocytes or in lung epithelial cells have been shown to develop atopic dermatitis- or asthma-like inflammation in the skin or lungs.
respectively. Moreover, TSLP receptor-deficient mice also fail to develop an inflammatory lung response to inhaled antigen.

Epithelial cells have evolved receptors like the protease-activated receptors (PARs), and Toll-like receptors (TLRs) to sense invasion and penetration. The activation of these receptors will induce NF-κB activation and the release of innate pro-Th2 cell cytokines like interleukin (IL)-25, IL-33 and TSLP. These signals program dendritic cells (DCs) to mount Th2-cell-mediated immunity, and license the innate type2 cell response by activation of type2 innate lymphoid cells (ILC2), basophils, eosinophils, and mast cells. A current evidence exists for TSLP, in that a human anti-TSLP monoclonal immunoglobulin, which binds human TSLP and prevents receptor interaction, reduces allergen-induced bronchoconstriction and indexes of airway inflammation [exhaled nitric oxide, peripheral blood and sputum eosinophilia] before and after allergen challenge.

TSLP is a master regulator of allergic inflammation in humans and mice. Despite its well-known importance in allergic responses, the roles of TSLP in cancer have only recently been identified. Cancer creates an immunosuppressive microenvironment to escape immune system. It has been reported that CD4+CD25+ regulatory T cells (Tregs), which could suppress the activity of immune cells and help the tumor cells to escape the host immune system. One study indicated that the expression of TSLP protein was significantly increased in tumor tissue compared with that in benign lesion and non-cancer lung tissue. Furthermore, the number of Tregs in the tumor microenvironment correlated with the expression of TSLP in tumor tissue.

A previous study demonstrated high levels of TSLP mRNA to be present, not only in epithelial cells, but also in stromal cells, such as lung fibroblasts.

However, the regulation of TSLP expression in stromal cells has been largely unexplored in contrast to the extensive number of studies on the regulation of epithelial TSLP expression. In particular, it is still unclear whether human lung fibroblasts express TSLP at the protein level and, if so, precisely how such human lung fibroblast-derived TSLP expression is regulated also remains to be elucidated.

The purpose of this study was to investigate the regulation of TSLP expression in human lung fibroblasts. The results indicate that TSLP expression in human lung fibroblasts is induced by TNF-α/NF-κB or ATP and also is negatively regulated by TGF-β or FGF-2. Furthermore, we provide evidence suggesting that there is a functional interaction between lung fibroblasts and bronchial epithelial cells in the regulation of TSLP expression via these mediators.

**Materials and Methods**

**Reagents**

Recombinant human TNF-α, IL-4, IL-13, TGF-β1, activin A, BMP-2, FGF-2, and human and mouse TNF-α type I receptor-Fc chimeras were purchased from R&D Inc. (Minneapolis, MN). Lipopolysaccharide (LPS), the synthetic ligand polyinosinic: polycytidylic acid (polyI:C), BAY 11-7082, suramin, and the non-hydrolyzable ATP analog ATPγS were purchased from Sigma Aldrich, Inc. (St. Louis, MO). Cigarette smoke extract (CSE) was prepared from a major cigarette brand in Japan (tar content: 10 mg) as previously described and then it was stored at –20°C until use.

**Cell Culture**

Primary normal human adult lung fibroblasts
and normal human adult bronchial epithelial cells were purchased from TaKaRa Bio. (Shiga, Japan). Human lung fibroblasts were cultured in a serum-free fibroblast growth medium, FBM (Lonza, Walkersville, MD), containing FGF-2, insulin, GA-1000, and fetal bovine serum, at 37°C in a humidified atmosphere in the presence of 5% CO₂. Human bronchial epithelial cells were cultured in a serum-free bronchial epithelial cell growth medium, BEBM (Lonza), containing epidermal growth factor, insulin, hydrocortisone, epinephrine, transferin, retinoic acid, triiodothyronine, BSA-FAF, gentamycin, amphotericin B, and bovine brain pituitary extract at 37°C in a humidified atmosphere in the presence of 5% CO₂. Hydrocortisone was not added to the culture medium in some experiments. The experiments were performed using the cells at the third or fourth passage. Cells from the fetal lung fibroblast cell line MRC-5 were cultured in Minimum Essential Medium-α (MEMα) (Gibco/Invitrogen, Carsbad, CA) containing 10% FCS and antibiotics. MRC-5 is used in order to conduct a preliminary experiment.

**ELISA**

The amounts of TSLP in the culture supernatants were measured by ELISA using the human TSLP sandwich ELISA development kit (R&D, Minneapolis, MN) according to the manufacturer’s instructions.

**Quantitative Real-Time PCR**

Quantitative real-time RT-PCR with specific primers and probes for human TSLP and GAPDH (Applied Biosystems, Foster City, CA) was performed using the AB7300 real-time PCR system (Applied Biosystems) as previously described.²⁵

**Cell Viability Assay**

The cells (1 x 10 cells/well) were cultured with or without the indicated doses of the cytokines for 24 hours in a flat-bottom 96-well microtiter plate. Cell viability was then determined by a WST assay using the Tetra Color ONE kit (Seikagaku Corporation, Tokyo, Japan) according to the manufacturer’s instructions.

**ATP Measurements**

Primary human bronchial epithelial cells were cultured in a serum-free bronchial epithelial cell growth medium without hydrocortisone for 24 hours and the culture supernatants were collected and stored at −80°C until they were assayed. The ATP content was measured using the ATP Bioluminescence Assay Kit CLS II (Roche, Basal, Switzerland) according the manufacturer’s instructions.

**Data Analysis**

Values represent the mean ± SD. The statistical analysis was performed using Student’s t-test. A value of P < 0.05 was considered to be significant.

**RESULTS**

**TNF-α Induces TSLP Production in Human Lung Fibroblasts**

To investigate the regulation of TSLP expression in lung fibroblasts, the effects of various cytokines and environmental factors on TSLP expression were examined by ELISA in primary human lung fibroblasts.

Among the evaluated variables (TNF-α, IL-13, TGF-β, LPS, and polyI:C), TNF-α induced TSLP production and IL-13 enhanced TNF-α-induced TSLP production in human lung fibroblasts (Fig. 1A), although IL-13 alone did not induce TSLP production. Furthermore, TNF-α
Fig. 1. TNF-α induces TSLP production in human lung fibroblasts.
A. Human lung fibroblasts (2.5 x 10^5 cells/well) were cultured in the presence or absence of TNF-α (10 ng/ml), TGF-β (10 ng/ml), IL-13 (10 ng/ml), polyI:C (1 μg/ml), LPS (1 μg/ml), or the indicated ligand combinations for 24 hours. The culture supernatants were collected and the TSLP concentrations were measured by ELISA.
B. MRC-5 cells (2.5 x 10^5 cells/well) were cultured in the presence or absence of TNF-α (10 ng/ml), TGF-β (10 ng/ml), IL-13 (10 ng/ml), polyI:C (1 μg/ml), LPS (1 μg/ml), or the indicated ligand combinations for 24 hours. The culture supernatants were then collected and the TSLP concentrations were measured by ELISA.
C. MRC-5 cells (5 x 10^5 cells/well) were cultured in the presence or absence of TNF-α plus IL-4 (10 ng/ml for each cytokine) for 24 hours. RNA was then extracted from the cells and real-time RT-PCR was performed for TSLP and GAPDH.
D. Human lung fibroblasts (2.5 x 10^5 cells/well) were cultured in the presence or absence of TNF-α (10 ng/ml) with or without 1 or 10 μM BAY 11-7082 for 24 hours. The culture supernatants were collected and the TSLP concentrations were measured by ELISA.
E. Human lung fibroblasts were cultured with 10 ng/ml TNF-α with or without 1 or 10 μM BAY 11-7082 for 24 hours and cell viability was subsequently measured by a WST assay.
Values represent the mean ± SD (n = 3). *p < 0.05 in comparison to the corresponding control. Similar results were obtained at least in three independent studies. ND: not detected.
induced TSLP production in synergy with IL-4 or IL-13 in the MRC-5 human fetal-derived lung fibroblast cell line (Fig. 1B). These findings were also confirmed at the mRNA level in MRC-5 cells (Fig. 1C and data not shown). In addition, 10 μM of BAY 11-7082, an inhibitor of IκB phosphorylation, inhibited TNF-α-induced TSLP production in human lung fibroblasts without affecting the cell viability (Fig. 1D and E). Collectively, these results indicated that TNF-α induced TSLP production in primary human lung fibroblasts in a NF-κB dependent manner and IL-4 or IL-13 enhanced TNF-α-induced TSLP production. The latter phenotype was also observed in MRC-5 cells.

Pretreatment of Human Lung Fibroblasts with TGF-β, but not Activin and BMP, Inhibits TNF-α-induced TSLP Production

TGF-β is an important negative regulator of asthmatic airway inflammation. Therefore, the effects of TGF-β on both TNF-α and TNF-α plus IL-13-induced TSLP production were examined in human lung fibroblasts. The simultaneous treatment of human lung fibroblast cells with TGF-β and either TNF-α or TNF-α plus IL-13 did not affect TNF-α- or TNF-α plus IL-13-induced TSLP production (Fig. 1A and data not shown). However, the pretreatment of human lung fibroblast cells with TGF-β 24 hours before stimulation with TNF-α- or TNF-α plus IL-13 almost completely abrogated TSLP production induced by these cytokines (Fig. 2A). The pretreatment of human lung fibroblasts with TGF-β did not affect the cell viability based on a WST assay (Fig. 2B), thus suggesting that the inhibition was not due to a reduced cell viability.

In contrast, either simultaneous or prior treatment with TGF-β-related family proteins, activin A and BMP-2, did not affect the TNF-α-
induced TSLP production in human lung fibroblasts (Fig. 2C and data not shown). The dose of either activin A or BMP-2 did not affect the cellular viability (Fig. 2D). These results suggest that the pretreatment of human lung fibroblasts with TGF-β, but not activin A and BMP-2, inhibited TNF-α-induced TSLP production.

**Pretreatment of Human Lung Fibroblasts with FGF-2 Inhibits TNF-α-induced TSLP Production**

The replacement of primary lung fibroblast growth medium with MEMα enhanced TNF-α plus IL-13-induced TSLP production in human lung fibroblasts (Fig. 3A). This suggested that some factors in the primary lung fibroblast growth medium could suppress TSLP production. As expected, FGF-2, which was in the fibroblast growth medium, inhibited TNF-α or TNF-α plus IL-13-induced TSLP production when added to MEMα medium 24 hours before the stimulation (Fig. 3B). These inhibitory effects of FGF-2 were not observed when FGF-2 was simultaneously added to MEMα medium with either TNF-α or TNF-α plus IL-13 (data not shown). The pretreatment of human lung fibroblasts with FGF-2 did not affect the cell viability based on a WST assay (Fig. 3C). These results suggested that the pretreatment of human lung fibroblasts with FGF-2 inhibited TNF-α-induced TSLP production.

**Conditioned Medium Obtained from PolyI:C- or CSE-stimulated Bronchial Epithelial Cell Cultures Induced TSLP Production in Human Lung Fibroblasts in a TNF-α-dependent Manner**

TNF-α is produced by bronchial epithelial cells upon stimulation with either polyI:C or CSE17, 18. Human bronchial epithelial cells did not produce TSLP upon stimulation with 1 μg/ml polyI:C or 5% CSE; however, the cells did produce TSLP upon stimulation with 25 μg/ml...
polyI:C without hydrocortisone in the medium, which was consistent with the results from a previous study\textsuperscript{19} (Fig. 4A).

Interestingly, the conditioned medium obtained from human bronchial epithelial cell cultures without any treatments induced TSLP production in human lung fibroblasts (Fig. 4B). The conditioned medium obtained from 1 \( \mu \)g/ml polyI:C- or 5% CSE-stimulated bronchial epithelial cell cultures enhanced the TSLP production in human lung fibroblasts (Fig. 4B). The neutralization of TNF-\( \alpha \) activity using a human, but not a mouse, TNF-\( \alpha \) type I receptor-Fc chimera inhibited the enhancement of TSLP production. These results suggested that the conditioned medium obtained from 1 \( \mu \)g/ml polyI:C- or 5% CSE-stimulated human bronchial epithelial cell cultures induced TSLP production in human lung fibroblasts in a TNF-\( \alpha \)-dependent manner.

**Human Lung Fibroblasts Incubated with the Conditioned Medium Obtained from Un-stimulated Bronchial Epithelial Cell Cultures Produced TSLP, which Depends on ATP Released from the Bronchial Epithelial Cells**

As described above, the conditioned medium obtained from human bronchial epithelial cell cultures without any treatment induced TSLP production in human lung fibroblasts in a TNF-\( \alpha \) independent manner (Fig. 4B). Various chemical inhibitors were added to the cultures to identify the factors responsible for the basal TSLP expression. Among them, suramin, a broad-range purinergic receptor antagonist, inhibited the TSLP production by human lung fibroblasts incubated with the culture medium derived from un-stimulated bronchial epithelial cells at 100 \( \mu \)M (Fig. 5A). We confirmed that 100 \( \mu \)M suramin did not affect the cell viability using a WST assay (data not shown). Consistently, the culture medium derived from un-stimu-

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**Fig. 4.** Conditioned medium obtained from polyI:C- or CSE-stimulated bronchial epithelial cell cultures induced TSLP production in human lung fibroblasts in a TNF-\( \alpha \)-dependent manner.

A. Human bronchial epithelial cells (2.5 \( \times \) 10\(^5\) cells/well) were cultured with 1 \( \mu \)g/ml or 25 \( \mu \)g/ml polyI:C or 5% CSE in the growth medium without hydrocortisone for 24 hours. The culture supernatants were collected and the TSLP concentrations were measured by ELISA.

B. Human lung fibroblasts (2.5 \( \times \) 10\(^5\) cells/well) were cultured in 300 \( \mu \)l of the conditioned medium obtained from human bronchial epithelial cells as described in A (1 \( \mu \)g/ml polyI:C or 5% CSE cultures) and 300 \( \mu \)l of MEM\( \alpha \) for 24 hours (total 600 \( \mu \)l volume) in the presence of 10 \( \mu \)g/ml human TNF type I receptor-Fc chimera or mouse TNF type I receptor-Fc chimera. The culture supernatants were subsequently collected and the TSLP concentrations were measured by ELISA. Values represent the mean \( \pm \) SD (n = 3). \(*p < 0.05\) in comparison to the corresponding control. Similar results were obtained at least in three independent studies. ND: not detected.
lated bronchial epithelial cells contained substantial amounts of ATP (Fig. 5B). Furthermore, the stimulation of human lung fibroblasts with the non-hydrolyzable ATP analog, ATPγS (a stable form of ATP), induced TSLP production in a dose-dependent manner (Fig. 5C). These results suggest that increased TSLP production in human lung fibroblasts which were incubated with a conditioned medium obtained from unstimulated bronchial epithelial cell cultures depends on ATP constitutively released from the bronchial epithelial cells.

**DISCUSSION**

Epithelial cell-derived TSLP is considered to be a master switch to develop allergic inflammation such as asthma\(^1\). Therefore, the regulation of TSLP expression in epithelial cells (eg. bronchial epithelial cells and keratinocytes) has been extensively studied\(^1, 13, 19–23\). In contrast, the regulation of TSLP expression in stromal cells, including lung fibroblasts, has been largely unexplored. This study showed that human adult lung fibroblasts produced TSLP in response to TNF-\(\alpha\) and this TSLP production was synergized with IL-4 or IL-13 and inhibited by TGF-\(\beta\), which was consistent with previous studies in human bronchial epithelial cells and keratinocytes\(^1, 19–23\). Therefore, epithelial cells and lung fibroblasts may possess, at least in part, common regulatory mechanisms for TSLP expression. In addition, FGF-2 or ATP inhibited or stimulated TSLP production, respectively, in human lung fibroblasts, which has not been addressed before. Lung fibroblasts might have a unique regulatory mechanism for TSLP production because FGF-2 pretreatment did not affect polyI:C-induced TSLP production in human bronchial epithelial cells (data not shown).

The finding that only pretreatment with TGF-
Regulation of lung fibroblast TSLP expression

β or FGF-2 inhibited TNF-α-induced TSLP production (Figs. 2 and 3) suggests that the TSLP inhibition was mediated by molecular events induced by TGF-β or FGF-2. This TSLP inhibition by TGF-β or FGF-2 did not appear to be associated with the differentiation of fibroblasts into myofibroblasts because we did not observe any mRNA expression of the myofibroblast differentiation marker α-smooth muscle actin in human lung fibroblasts at 24 hours following TGF-β or FGF-2 treatment (data not shown). However, precisely how TGF-β and FGF-2 inhibit the TNF-α-induced TSLP production in human lung fibroblasts thus remains to be investigated.

Conditioned medium obtained from bronchial epithelial cell cultures stimulated with asthma-associated environmental factors, such as polyI:C- (a mimic of double-stranded RNA virus) or CSE- (cigarette smoke), induced TSLP production in a TNF-α-dependent manner (Fig. 4). These findings suggest a possible functional interaction between human lung fibroblasts and epithelial cells in a microenvironment associated with asthma development.

Conditioned medium obtained from un-stimulated bronchial epithelial cell cultures induced TSLP production in human lung fibroblasts and this basal TSLP production depended on the ATP production released from human bronchial epithelial cells (Fig. 5). ATP acts as a danger signal and it is also suggested to play an important role in the development of asthma. Therefore, it is possible that epithelial- or immune cells-derived ATP may be involved in the development of asthma via TSLP production from fibroblasts. However, the precise cellular source and regulation of endogenous ATP in asthma remains a matter of speculation because Idzko et al. observed that the endogenous lung ATP levels only increased after an allergen challenge in mice or patients with asthma.

Endogenous ATP levels in the culture supernatants obtained from un-stimulated bronchial epithelial cell cultures were low (-nM) when compared with that (μ-mM) to induce TSLP in human lung fibroblasts (Fig. 5B and C). It is known that extracellular ATP is rapidly degraded by ubiquitous ecto-ATP/ADPases (CD39). In addition, it is also known that the intracellular concentration of ATP is in the range of 5–10 mM, and cell death or even ‘cellular stress’ will release large amounts of ATP into the pericellular space. Therefore, the low endogenous ATP levels in the culture supernatants obtained from un-stimulated bronchial epithelial cell cultures may be a result of rapid degradation of ATP at the time of the measurements and may not reflect ATP levels to stimulate TSLP production in lung fibroblasts.

In summary, human lung fibroblasts as well as human bronchial epithelial cells can produce substantial amounts of TSLP in response to TNF-α, which is negatively regulated by either TGF-β or FGF-2. In addition, ATP is a novel stimulator for TSLP production in human lung fibroblasts. Furthermore, the current results suggest that there may be a functional interaction between lung fibroblasts and bronchial epithelial cells in the regulation of TSLP expression via these mediators. These results suggest that not only epithelial- but also lung fibroblast-derived TSLP might be involved in the pathophysiology of asthma.

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CONFLICT OF INTERESTS

The authors declare no financial or commercial conflict of interest.

REFERENCES


