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Original Article

Soluble ST2 suppresses IL-5 production by human basophilic KU812 cells, induced by epithelial cell-derived IL-33

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hBE cell human bronchial epithelial cell

hBE33 cell

human bronchial epithelial cell containing

the IL-33 gene

sST2 soluble ST2

ABSTRACT

Background: Epithelial cell-derived IL-33 has an important role in the initiation and activation of innate allergic inflammation. IL-33 acts as a cytokine through the ST2 receptor (ST2L) and it stimulates the production of Th2 cytokines. Soluble ST2 (sST2) may regulate Th2 responses by neutralizing the activity of IL-33. Basophils express ST2L and produce IL-5 in response to IL-33. However, the role of the epithelial cell–basophil interaction and sST2 in IL-5 production remains unclear.

Methods: Cultured human bronchial epithelial (hBE33) cells, that contained the human IL-33 gene (i.e., hBE33 cells) and a human basophilic cell line, KU812 cells, were used to study the epithelial cell–basophil interaction in the production of IL-5 induced by HDM.

Results: At 15 min after incubation, HDM stimulated the rapid release of IL-33 from cultured hBE33 cells. IL-33 and the supernatant of HDM-treated hBE33 cells stimulated IL-5 production from KU812 cells. Anti-IL-33 antibody and anti-ST2 antibody treatment of KU812 cells suppressed IL-5 production, which had been induced by the supernatant of HDM-treated hBE33 cells. The hBE33 cells secreted sST2 in a time-dependent manner. The production of sST2 by KU812 cells co-cultured with hBE33 cells was significantly increased, compared with KU812 cells cultured with the supernatant of hBE33 cells. Soluble ST2 suppressed IL-5 production by KU812 cells, which was induced by the supernatant of HDM-treated hBE33 cells.

Conclusions: Epithelial cell-derived IL-33 promoted IL-5 production by KU812 cells. The subsequently produced sST2 has important roles in regulating Th2 responses.

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Introduction

IL-33, a ligand for ST2L (also called ST2, IL-1RL1, T1, Der4, or fit-1), is a member of IL-1 of family cytokines.¹ It is stored in the nucleus of various types of immune cells and nonimmune cells such as mast cells, macrophages, dendritic cells, endothelial cells, epithelial cells, smooth muscle cells and fibroblasts.^{2,3} Epithelial cells release IL-33 after exposure to allergens, microbial pathogens, and injury-induced stress.⁴ IL-33 has an important role in initiating and activating innate and adaptive Th2 immune responses, and is implicated in the pathogenesis of asthma,^{5,6,7} allergic rhinitis,⁸ allergic conjunctivitis,⁹ atopic dermatitis,^{10,11} and several autoimmune diseases.^{12–15} IL-33 stimulates the

production of Th2 cytokines such as IL-4, IL-5, and IL-13 through its receptor, ST2L,¹⁶ which is highly expressed on several immune cells such as basophils, type 2 innate lymphoid cells (ILC2s), mast cells, Th2 cells, and eosinophils.¹⁷

The ST2 gene produces a soluble secreted form of the ST2 protein (sST2) and a transmembrane form of the protein (ST2L). These proteins are transcribed from a dual promoter system to drive differential mRNA transcription.^{18,19} The sST2 molecule lacks the transmembrane and intercellular domains, and acts as a binding decoy receptor for IL-33. Thus, sST2 regulates IL-33 activity during inflammatory responses. In a mouse model of bronchial asthma, sST2 was produced predominantly in the lung, lymph nodes, thymus, spleen, and ovaries, and the concentration of serum sST2 is elevated after an allergen challenge.²⁰ The concentration of sST2 was also significantly elevated in the nasal secretion after an allergen challenge in patients with pollen allergy.²¹ In asthmatic mice, some research indicates that sST2 significantly inhibits the IL-33-induced production of IL-4, IL-5, and IL-13 by activated

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splenicocytes.²⁰ However, other cellular sources and the role of sST2 in the Th2 response remain unclear.

Basophils have crucial roles in IgE-dependent and IgE-independent allergic inflammation through their migration to the site of inflammation and the secretion of various cytokines, chemokines, and proteases. Human basophils produce large amounts of IL-4 in response to various stimuli, and IL-4 activates macrophages, innate lymphoid cells, fibroblasts and endothelial cells.²² In a murine study,²³ basophils were activated and recruited to the draining lymph nodes in response to the Th2-inducing allergen challenge, and a protease allergen (i.e., papain) stimulated basophils to produce Th2 cytokines such as IL-4 and IL-13, and thymic stromal lymphopoietin (TSLP). Basophils are also activated by IL-3.²⁴ Human basophils express high levels of ST2L, and respond to IL-33 by producing proinflammatory cytokines such as IL-4, IL-5, IL-13, and granulocyte macrophage-colony-stimulating factor (GM-CSF).^{6,25}

In the present study, to elucidate the roles of epithelial cell-derived IL-33 and sST2 on basophil-dependent IL-5 production, we examined the epithelial cell–basophil interaction induced by house dust mite (HDM) normal human bronchial epithelial cells that contained the human IL-33 gene (hBE33), and KU812 cells, which are a human basophilic cell line.

Methods

Reagents

Crude allergens of HDM were purchased from Greer Laboratories (Lenoir, NC, USA). Recombinant human IL-33, recombinant human ST2/IL-1 R4 Fc chimera (i.e., sST2), normal goat IgG, goat anti-human IL-33 antibody (IgG), and goat anti-human ST2/IL-1 R4 antibody (IgG) (i.e., ST2 antibody) were obtained from R&D Systems (Minneapolis, MN, USA). Recombinant human IL-18 was obtained from Medical and Biological Laboratories (Nagoya, Japan). Human FC fusion protein was obtained from Qoolabs (San Diego, CA, USA).

Cell culture and treatment

Human airway epithelial cells were kindly provided by Professor H. Kita (Mayo Clinic, Rochester, MN, USA). The full-length human IL-33 and a transposase gene were introduced into immortalized human bronchial epithelial (hBE) cells to achieve the stable expression of full-length IL-33 cells (i.e., hBE33 cells).²⁶ The hBE33 cells were maintained in supplemented basal epithelial growth medium (BEGM) (Lonza, Basel, Switzerland) and cultured in a 24-well tissue culture plate (Falcon; Corning, Inc., Corning, NY, USA). After incubating confluent cells, the hBE33 cells were incubated for up to 16 h with HDM (100 µg/mL) in serum-free Roswell Park Memorial Institute (RPMI) 1640 medium (Wako Pure Chemical Industries, Osaka, Japan).

A human chronic myelogenous leukemia cell line, KU812 cells, were obtained from American Type Culture Collection (Manassas, VA, USA). KU812 cells are an immature prebasophilic cell line²⁷ and possesses many characteristics of peripheral blood basophils.²⁸ KU812 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Biosera, Nuaille, France) at 37 °C in 5% carbon dioxide.

The KU812 cells (3×10^6 cells/well) were incubated with HDM (100 µg/mL), IL-18 (100 ng/mL), IL-33 (100 ng/mL), or with supernatants from HDM-stimulated hBE33 cells with or without sST2 (4.8 ng/mL or 4800 ng/mL) in 96-well round bottom plates (Falcon; Corning, Inc.) for 48 h. Human FC fusion protein (4800 ng/mL) was used as the negative control for sST2. In some

experiments, the supernatants from HDM-stimulated hBE33 cells were pretreated with IL-33 antibody (1.2 µg/mL) for 1 h, and/or KU812 cells were pretreated with ST2/IL-1 R4 antibody (2.4 µg/mL) for 1 h, before stimulating KU812 cells (3×10^6 cells/well) with the supernatants. KU812 cells were also stimulated by the supernatants from HDM-stimulated hBE33 cells with normal goat IgG (2.4 µg/mL) as the negative control. In some experiments, hBE33 cells and KU812 cells were co-cultured in RPMI medium with HDM (100 µg/mL) in a 24-well tissue culture plate (Falcon) for 48 h. In some experiments, hBE33 cells were plated onto collagen IV-coated 6.5 mm diameter pore Transwell-clear culture inserts (0.4 µm pore Size; Corning, Inc.) in BEGM. After hBE33 cells formed a confluent monolayer (approximately 1 week), KU812 cells used in the submersion culture were seeded and hBE33 cells on the apical surface were stimulated with HDM (100 µg/mL) in RPMI medium for 48 h.

In other experiments, recombinant human IL-33 (10 ng/mL) were pretreated with 0.1–100 ng/mL of recombinant sST2 for 2 h in RPMI medium at 37 °C in 5% carbon dioxide.

Detection of IL-33, IL-5, and sST2

Cell-free supernatants were collected and stored at –30 °C until use. Enzyme-linked immunosorbent assay (ELISA) kits were used to determine the concentrations of IL-33, IL-5 (R&D systems), and sST2 (Elabscience Biotechnology, Wuhan, China). The sensitivity limits of IL-33, IL-5, and sST2 kits were 23.4 pg/mL, 3.9 pg/mL, and 31.25 pg/mL, respectively.

Statistical analysis

All data are expressed as the mean $\pm$ the standard error of the mean from the indicated number of replicates. Two-sided differences between two samples were analyzed with the Mann–Whitney *U* test. Data of three-group comparisons were evaluated by Kruskal–Wallis one-way analysis. Values of *p* < 0.05 were significant.

Results

Rapid release of IL-33 from epithelial cells in response to HDM

Figure 1 shows the time course release of IL-33 from hBE33 cells. HDM induced the immediate release of IL-33 at

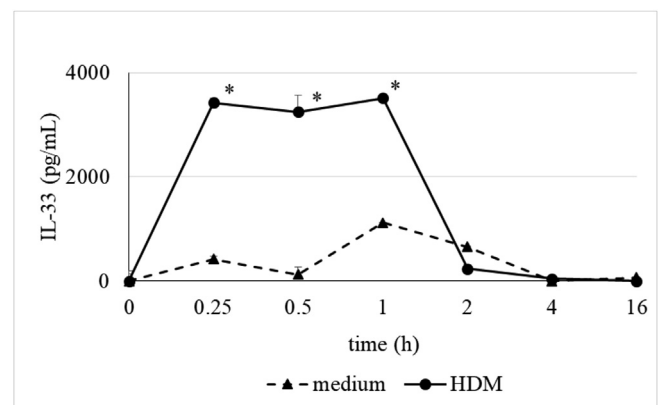


Fig. 1. IL-33 is rapidly released from epithelial cells in response to HDM. The hBE33 cells were incubated with medium or with HDM (100 µg/mL) for up to 16 h. The IL-33 concentration in the cell-free supernatants was measured using ELISA. **p* < 0.05, compared to cells with medium alone (*n* = 4).

15 min. The IL-33 level peaked at 1 h and then rapidly declined to the baseline level by 2 h after incubation. Commercial human primary airway epithelial cells derived from nasal scrapings or bronchial brushings contain small and varying amounts of IL-33 protein. Therefore, we used new human airway epithelial cells to evaluate the effects of various pharmacologic agents on IL-33 release.

KU812 cell, a human basophilic cell line, production of IL-5 in response to IL-33 exposure

Figure 2a shows the effects of HDM, IL-18, and IL-33 on IL-5 secretion by KU812 cells. Recombinant IL-33 significantly stimulated IL-5 production after incubation for 48 h. Recombinant IL-18 and HDM had no effect on IL-5 production. IL-5 production by KU812 cells, after stimulation with IL-33, increased in a time-dependent manner for up to 48 h (data not shown).

We next examined the effects of the supernatant of HDM-treated epithelial cells on IL-5 production from KU812 cells. The hBE33 cells were treated with HDM for 0 h, 1 h, 4 h, and 16 h. The supernatants were then collected. The supernatant of HDM-treated hBE33 cell collected at 1 h significantly stimulated the IL-5 production by KU812 cells. The IL-5 production from KU812 cells were not statistically difference between incubated in 96 well round bottom plates and 24 well flat bottom plates (data not shown). However, the supernatants collected at 4 h and 16 h, in which IL-33 concentration was decreased, showed no effect on IL-5 production (Fig. 1, 2b). Figure 2c shows the effects of IL-33 antibody and ST2 antibody on the HDM-treated supernatants-induced IL-5 production by KU812 cells. The HDM-treated supernatants of epithelial cells collected at 1 h were pretreated with IL-33 antibody for 1 h, and/or KU812 cells were pretreated with ST2 antibody for 1 h. Normal goat IgG was used as the negative control. The IL-33 antibody and ST2 antibody both significantly suppressed the supernatants-induced IL-5 production by KU812 cells (Fig. 2c). These results indicated that IL-33 released by HDM-treated hBE33 cells is involved in the production of IL-5 by KU812 cells.

We then examined the effects of co-cultured KU812 cells and hBE33 cells on IL-5 production. The supernatants of hBE33 cells induced IL-5 production by KU812 cells; however the co-culture of KU812 cells and hBE33 cells with or without HDM did not induce IL-5 production (Fig. 3). In addition, to evaluate the involvement of the interaction between cell-to-cell contact, we examined the co-culture of the KU812 cells and hBE33 cells by using the Transwell insert. The co-culture of the KU812 cells and hBE33 cells also did

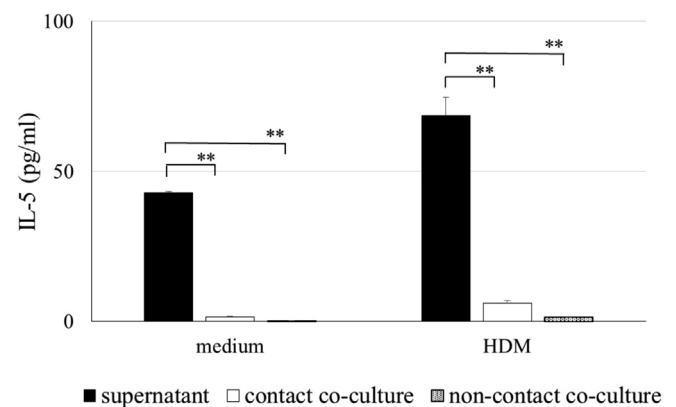


Fig. 3. The effect of the co-culture of hBE cells and KU812 cells on IL-5 production. The KU812 cells were stimulated with the supernatants of medium-treated or HDM-treated hBE33 cells collected at 1 h after the incubation for 48 h. The KU812 cells were also co-cultured with hBE33 cells in the Transwell insert with or without HDM for 48 h. ** $p < 0.01$, compared to medium-treated supernatants. (n = 5).

not induce IL-5 production (Fig. 3). These results indicated that some epithelial cell-derived factors may suppress IL-5 production by co-cultured KU812 cells.

Soluble ST2 suppressed IL-5 production by KU812 cells, which was induced by the supernatant of hBE33 cells

We hypothesized that sST2 may be a key factor to suppress the production of IL-5 in KU812 cells co-cultured with hBE33 cells. Figure 4a shows the time course secretion of sST2 by hBE33 cells. Soluble ST2 was not detected at 1 h after incubation. The sST2 secretion then increased in a time-dependent manner at 4 h and 16 h after incubation. HDM showed no effects on sST2 secretion from hBE33 cells.

We then examined the sST2 secretion in the HDM-treated KU812 cells co-cultured with hBE33 cells for 48 h, in KU812 cells incubated with HDM for 48 h, and in KU812 cells incubated with the supernatant of HDM-treated hBE33 for 48 h. Soluble ST2 was secreted by KU812 cells co-cultured with hBE33 cells, in KU812 cells incubated with only medium and in KU812 cells incubated with the supernatant of hBE33 cells. Soluble ST2 secretion was significantly elevated in KU812 cells co-cultured with hBE33 cells, compared to supernatant-treated KU812 cells and KU812 cells only. Treatment with HDM showed no effects on sST2 secretion (Fig. 4b). These results indicated that both KU812 and

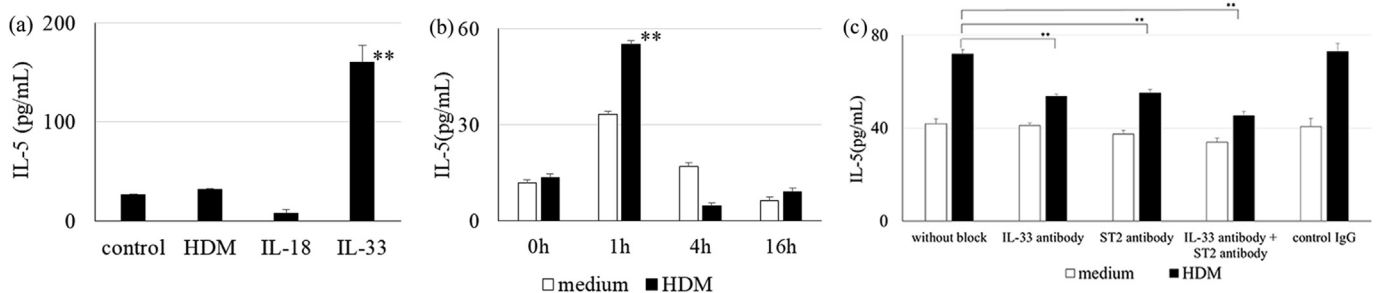


Fig. 2. KU812 cell produce IL-5 in response to IL-33 and to the supernatant of HDM-treated hBE33 cells. The IL-5 concentration in the cell-free supernatants was measured using ELISA (n = 6). (a) KU812 cells incubated with HDM (100 μ g/mL), IL-18 (100 ng/mL) or IL-33 (100 ng/mL) for 48 h. ** $p < 0.01$, compared to supernatants with medium alone. (b) The hBE33 cells were stimulated with medium or with HDM (100 μ g/mL) for 0 h, 1 h, 4 h, and 16 h. The KU812 cells were incubated with the supernatant of HDM-treated hBE33 cells for 48 h. ** $p < 0.01$, compared to supernatants with medium-treated hBE33 cells. (c) The supernatants of medium-treated or HDM (100 μ g/mL)-treated hBE33 cells collected at 1 h after incubation were preincubated with IL-33 antibody (1.2 μ g/mL) for 1 h, and/or KU812 cells were preincubated with ST2/IL-1 R4 antibody (2.4 μ g/mL) for 1 h. The KU812 cells were then stimulated by the supernatants for 48 h. The KU812 cells were stimulated by normal goat IgG (2.4 μ g/mL) as the negative control. ** $p < 0.01$, compared to the HDM-treated supernatants of epithelial cells.

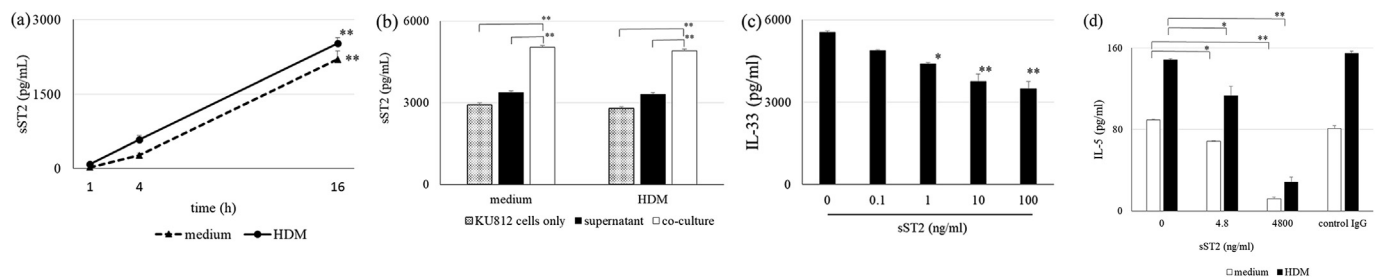


Fig. 4. Soluble ST2 (sST2) regulated the bioactivity of IL-33 and suppressed IL-5 production from KU812 cells. **(a)** The hBE33 cells were incubated with medium or with HDM (100 μ g/mL) for up to 16 h. The sST2 concentration in cell-free supernatants was measured by ELISA. ** $p < 0.01$, compared to supernatants collected at 1 h after incubation ($n = 6$). **(b)** The KU812 cells were incubated with medium or with HDM (100 μ g/mL) for 48 h. The KU812 cells were also incubated with the supernatants of medium-treated or HDM (100 μ g/mL)-treated hBE33 cells collected at 1 h for 48 h. The KU812 cells were also co-cultured with hBE33 cells with or without HDM for 48 h. sST2 concentration in cell-free supernatants was measured by ELISA. ** $p < 0.01$ ($n = 6$). **(c)** Recombinant human IL-33 (10 ng/mL) were pretreated with 0.1–100 ng/mL of sST2 for 2 h in RPMI medium at 37 $^{\circ}$ C in 5% carbon dioxide. The IL-33 concentration was measured using ELISA. * $p < 0.05$ and ** $p < 0.01$, compared to without sST2 ($n = 6$). **(d)** The KU812 cells were incubated with the supernatants of medium or HDM-treated hBE33 cells for 48 h, which were pretreated with 4.8 ng/mL or 4800 ng/mL of the ST2/IL-1 R4 Fc chimera (i.e., sST2) for 1 h. Human FC fusion protein (4800 ng/mL) was used as the control for sST2. The IL-5 concentration in cell-free supernatants was measured using ELISA. * $p < 0.05$ and ** $p < 0.01$ ($n = 6$).

hBE33 cells produce sST2, and that sST2 concentration is higher in co-cultured cells.

Figure 4c shows the inhibitory effect of sST2 on recombinant IL-33 protein. IL-33 protein pretreated with sST2 was inhibited in dose-dependent manner. These results indicated that sST2 is one factors that suppresses IL-5 production by KU812 cells through inactivated IL-33.

Figure 4d shows the effects of the ST2/IL-1 R4 Fc chimera (i.e., sST2) on the KU812 cell production of IL-5, which was induced by the supernatant of HDM-treated hBE33 cells. For 1 h, the supernatants of HDM-treated hBE33 cells were pretreated with low concentration of sST2 (4.8 ng/mL) or high concentration of sST2 (4800 ng/mL). They were then incubated with KU812 cells for 48 h. Human FC fusion protein (4800 ng/mL) was used as the negative control for sST2. Both concentrations of sST2 significantly suppressed the KU812 cell production of IL-5, which was induced by the supernatants of HDM-treated or HDM-untreated hBE33 cells. The FC fusion protein did not suppress IL-5 production by KU812 cells. These results indicated that sST2 in the co-culture of KU812 cells and hBE33 cells suppressed IL-5 production by KU812 cells.

Discussion

In the present study, HDM stimulated the rapid release of IL-33 from cultured epithelial (i.e., hBE33) cells. IL-33 and the supernatant of HDM-treated hBE33 cells stimulated IL-5 production by KU812 cells, a human basophilic cell line. Anti-IL-33 antibody and anti-ST2 antibody suppressed IL-5 production by KU812 cells, which was induced by the supernatant of HDM-treated hBE33 cells. This is a first report demonstrating that epithelial cell-derived IL-33 after stimulation with HDM promotes IL-5 production by basophilic KU812 cells, and that the subsequently produced sST2 has important roles in controlling IL-5 production in vitro.

Derp1 and Derp2 are major HDM allergens with cysteine protease activity, which induces IL-33 release from epithelial cells. Epithelial cell-derived IL-33 is crucial in the initiation and development of airway allergic inflammation by activating the immune cells such as basophils, eosinophils, group 2 innate lymphoid cells (ILC2s), mast cells, and Th2 cells. IL-33 was over-expressed in the epithelium of allergic airway diseases such as bronchial asthma²⁹ and allergic rhinitis.³⁰ Protease allergens such as HDM, the fungi genus *Alternaria*, and papain cause asthmatic airway responses in the absence of Th2 cells by stimulating the release of IL-33, IL-25, and TSLP from epithelial cells. These epithelial cell-derived cytokines activate innate immune cells

(e.g., basophils, eosinophils and ILC2s) to produce Th2 cytokines.³¹ It is interesting that in one study³² basophil-deficient mice failed to show papain-induced eosinophil infiltration, mucus hypersecretion, and airway hyperresponsiveness; this finding suggests that basophils have critical roles in protease allergen-induced innate allergic inflammation. Basophils are also important as antigen-presenting cells, which are necessary and sufficient for Th2 priming.^{33,34}

The present study revealed that IL-33 was rapidly released from epithelial (i.e., hBE33) cells at 15 min, and that IL-33 levels had declined to the baseline level by 2 h after HDM stimulation (Fig. 1). IL-33 protein was partially inhibited by sST2. The present study revealed that sST2 is one factors that inactivates IL-33 in the supernatant of epithelial cells after stimulation with HDM. IL-33 activity is regulated by several mechanisms such as sequestration by sST2, nuclear localization or sequestration, regulation by proteases, or inactivation by oxidation of cysteine residues.³⁵ Several models have demonstrated that IL-33 accumulates for 2 h after release and that it is generally no longer detectable after 6 h.³⁵ These mechanisms may also have been involved in the decreased the concentration of IL-33 in the supernatant of hBE33 cells. The supernatant of HDM-treated epithelial cells collected at 1 h significantly stimulated IL-5 production by KU812 cells; this production was inhibited by anti-IL-33 antibody and anti-ST2 antibody. However, the supernatants collected at 4 h and 16 h, at which points the IL-33 concentration was decreased, showed no effect on IL-5 production (Fig. 2b). These results indicated that IL-33 is important in the innate phase of IL-5 production after protease allergen exposure, and that an innate cell network (i.e., the epithelial cell-IL-33-basophil-IL-5 axis) have important roles in the initiation and development of Th2 immune responses.

Soluble ST2 is a decoy receptor for IL-33 and regulates IL-33 activity during inflammatory responses. Soluble ST2 directly binds to IL-33 and inhibits the IL-33-mediated activation of the nuclear factor κ B (NF- κ B) pathway and production of Th2 cytokines, which suggests that the complex of sST2 and IL-33 fails to bind to ST2L.³⁶ Elevated sST2 concentrations have been reported in nasal secretion after allergen challenge in patients with pollen allergy and in the serum of asthmatic patients.^{21,37} Proinflammatory cytokines (e.g., IL-1 β and tumor necrosis factor- α) stimulated sST2 production by human lung epithelial cells, and sST2 is also produced by Th2 cells, mast cells, and basophils.³⁸ Dysregulation of IL-33/ST2 signaling and sST2 production have been implicated in a variety of inflammatory diseases such as cardiac disease, inflammatory bowel

disease, graft-versus-host disease, small bowel transplant rejection, and type 2 diabetes.³⁸

In the present study, HDM did not induce IL-5 production in co-cultured KU812 cells and hBE33 cells, whereas the supernatant of HDM-treated hBE33 cells significantly stimulated IL-5 production by KU-812 cells (Fig. 3). Therefore, we hypothesized that sST2 may be involved in the suppression of IL-5 production in KU812 cells co-cultured with hBE33 cells.

We found that sST2 was produced by hBE33 cells in a time-dependent manner at 4 h and 16 h after incubation, and that HDM showed no effects on sST2 production. Soluble ST2 suppressed the KU812 cell production of IL-5, which was induced by the supernatant of HDM-treated hBE33 cells collected at 1 h. Soluble ST2 concentration was significantly higher in KU812 cells co-cultured with hBE33 cells, compared to supernatant-stimulated KU812 cells (Fig. 4a, b). These results indicated that sST2 is one factor that suppressed IL-5 production by KU812 cells. Previous reports have shown that treatment with recombinant sST2 to block IL-33 at the time of sensitization completely abolished eosinophilic airway inflammation and Th2-cell-associated cytokine production, when administered during the period of maximal lung alveolarization in vivo. Moreover, decoy sST2 had a strong preventive effect on HDM-sensitized asthma in the neonatal period, but less so in adulthood.³⁹ Therefore, our study may reflect in innate sensitization phase during Th2 type inflammation in vivo. However, the present study showed that cell-to-cell contact was not the important factor in suppressing IL-5 production by KU812 cells. Epithelial cell-derived factor, except for sST2, may be involved in suppressing IL-5 production by KU812 cells co-cultured with hBE33 cells. Future studies will be required to examine the mechanism of the interaction between epithelial cells and basophils in immune responses.

In conclusion, HDM induces a rapid and temporary release of IL-33 from airway epithelial cells. After the allergen exposure, rapidly produced IL-33 promotes IL-5 production by human basophilic KU812 cells. The subsequently produced sST2 has important roles in the suppression of excess innate IL-33-mediated IL-5 production after allergen exposure. A better understanding of sST2 may provide potential novel therapeutic approaches for the treatment of allergic airway inflammation.

Acknowledgements

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

KM and HKo designed the study, contributed to data collection and wrote the manuscript. HKi, TK, IT, and SS designed the study. TS interpreted the results. All authors approved the final manuscript.

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