IL-33 promotes mouse keratinocyte-derived chemokine, an IL-8 homologue, expression in airway smooth muscle cells in ovalbumin-sensitized mice

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Summary

Background: Although it is recognized that IL-33 plays a key role in the onset of asthma, it is currently unclear whether IL-33 acts on any other target cells besides mast cells and Th2 cells in asthma. We investigated whether airway smooth muscle cells (ASMCs) could contribute to asthma via stimulation with IL-33.

Methods: To create a mouse model of acute asthma, murine ASMCs were isolated and cultured in vitro with IL-33. The ASMCs were divided into two groups, ASMCs from normal mice and ASMCs from ovalbumin-sensitized mice. The release of mouse KC was analyzed by PCR and ELISA. Immunocytochemical Staining of murine ASMCs for ST2 and IL-1RAcP was performed.

Results: IL-33 promoted KC expression, both in terms of mRNA and protein levels, in ASMCs from ovalbumin-sensitized mice. ST2 and IL-1RAcP were expressed in the membrane of ASMCs in ovalbumin-sensitized mice.

Conclusion: IL-33 may contribute to the inflammation in the airways by acting on airway smooth muscle cells. IL-33 and ST2 may play important roles in allergic bronchial asthma. (Asian Pac J Allergy Immunol 2014;32:337-44)

Keywords: IL-33, ST2, Bronchial asthma, ASMC, KC

Introduction

Asthma, which has increased significantly in prevalence worldwide over recent decades, has become a major public health problem that affects 300 million persons at least.1 It is a chronic inflammatory disease of the airways characterized mainly by bronchial hyper-responsiveness, airflow obstruction, and airway remodeling and correlates with Th2 lymphocyte-mediated immune responses. This Th2-biased inflammation is characterised by leukocyte recruitment and activation in the airways (mainly lymphocytes, eosinophils, and mast cells) and overproduction of cytokines.2,3 While airway smooth muscle cells (ASMCs) in asthma are thought to be crucial in the development of airway narrowing, their role goes far beyond this activity. They play an active role in airway inflammation through the voluntary release of inflammatory mediators, especially chemokines including IL-8, rather than being involved passively.4,5 Interleukin-8 (IL-8) is a major cytokine which attracts neutrophils to the inflamed area. Serum IL-8 is an indicator of disease severity and treatment effectiveness in bronchial asthma.6 Mouse keratinocyte-derived chemokine (mKC) is a functional homolog of human interleukin (IL) 8.7

IL-33 was identified by bioinformatic analysis of the human genome. It is now designated as a novel member of the IL-1 cytokine family, which is the ligand of ST2 that is expressed mainly in activated Th2 cells and mast cells.8,9 As a powerful inducer of Th2 immune responses, IL-33 has become a favorite with scientists and its role in asthma has been extensively explored.10 Reports of recent studies have indicated that the IL-33/ST2 axis plays an important role in allergic airway inflammation. Administration of IL-33 to mice produces eosinophilia, airway hyper-responsiveness, and goblet cell hyperplasia.11 More recently, the concentration of IL-33 has been found to be higher in human asthmatic subjects compared to controls by endobronchial biopsies. The IL-33 production is especially conspicuous in those with severe...
IL-33 is involved in both innate and adaptive immunity in an exclusive pattern via a variety of cells, including basophils, mast cells, eosinophils, innate lymphoid cells, NK and NKT cells, Th2 lymphocytes and a CD34 posprecursor cell population. It is reported that both endothelial and epithelial cells, but not fibroblasts or smooth muscle cells, are the IL-33–responsive cells among normal primary human lung tissue cells. ST2 mRNA are expressed in both endothelial and epithelial cells, but not in fibroblasts or smooth muscle cells. However, recent studies have shown that IL-33 and ST2 mRNA are present in the thoracic aorta of 18-wk-old C57BL/6 mice and in primary cultured human ECs and SMCs. It was also found that ST2 mRNA and proteins are also increased in the lung after ovalbumin (OVA) challenge and mouse lung fibroblasts can produce eosin upon stimulation with a combination of IL-33 and IL-13. However, it is controversial whether IL-33 acts on any other target cells besides mast cells and Th2 cells. Though it has been reported that ST2 mRNA is not expressed in fibroblasts or smooth muscle cells in humans, it has been proved that ST2 mRNA is present in the thoracic aorta of 18-wk-old C57BL/6 mice and in primary cultured human ECs and SMCs. Maybe ST2 expression is different between humans and mice. In our preliminary experiment, we found that Th2 cells from normal mice and asthmatic mice responded to IL-33 stimulation differently. We wondered how ST2L in mASMC was expressed and whether ASMCs could respond to IL-33 stimulation. We also aimed to explore the response difference between normal mice and asthmatic mice. We investigated the ST2L expression and chemokine expression of mASMC of different statuses in vitro.

Methods

Animals

Six-week-old Female BALB/c mice were obtained from the Disease Control and Prevention Center of Hubei province (CDC). All animals were maintained in filter-top cages under specific pathogen-free conditions at the animal facility of the Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China.

Reagents

Ovalbumin (OVA, Sigma, USA), Al(OH)₃ (Sigma), Recombinant Murine IL-33 (Peprotech), Dulbecco's modified Eagle's medium (DMEM, GIBICO), Fetal bovine serum (FBS, GIBICO), ELISA KIT (R&D Systems), RIZOL reagent (TaKaRa), PrimeScript® RT Master Mix (TaKaRa), SYBR® Premix Ex Taq™ (TaKaRa), ST2, Ab (Enzo), and IL-1RAcP, Ab (abcam).

Antigen Sensitization and challenge

Female BALB/c mice (from Hubei province Center for Disease Control and Prevention), 4-6 weeks old, with a body weight of 15.0-18.7g, were divided into two groups (six mice/group): NC (negative control), mice sensitized and nebulized with phosphate buffered saline (PBS); AM (asthmatic mice), mice sensitized and nebulized with OVA. Mice were sensitized with 10mg 50 mL-1 OVA adsorbed in 1.125g of 50 mL-1 aluminum hydroxide gel adjuvant by i.p. injection on days 0 and 14. Mice were then challenged with nebulised 5% OVA in PBS for 30 min once a day for 7 days, from day 21 to day 27. Control mice were sensitized and challenged with PBS. All mice were killed on day 28.

Bronchoalveolar lavage fluid (BALF)

Immediately after sacrifice, the thoracic cavities of the mice were carefully opened. The tracheas were exposed and BAL fluid was collected by cannullating the upper part of the trachea and lavaging twice with 1 mL and then 0.8 mL PBS (85–90% of the lavage volume was recovered). Lavaged samples from each mouse were kept on ice, and were centrifuged at 1000 rpm for 5 min at 4°C. After centrifugation, lavage supernatants were collected and stored at -80°C.

Lung tissue histology

Lungs were perfused with 5 mL PBS via the right ventricle to wash out blood. Left lung tissues were removed from the chest cavity, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 3 mm. Tissue sections were subjected to haematoxylin and eosin staining to examine changes in general histology.

Murine ASMC isolation and culture

Airway tissue was obtained from the lungs of sacrificed mice in the two groups (NC group, negative control; AM group, asthmatic mice). Murine airway smooth muscle was dissected out and ASMCs were established in culture as follows. Briefly, the trachea and bronchi were dissected free from the surrounding tissue, the epithelium was removed and the underlying bands of smooth muscle gently separated from the connective tissue and cut into 1–2-mm cubes (smooth muscle cell explants) and placed on culture dishes with a
minimal volume of DMEM supplemented with 15% FCS supplemented with penicillin (100U ml-1) and streptomycin (100 μg ml-1). In an incubator at 37°C in a humidified atmosphere (5% CO2-balanced air), ASMCs migrated from the tissue explants during the following days. When cells were approaching confluence in some parts of the dish at 7–10 days after being explanted, the explants were removed and the ASMCs were passaged with 0.25% trypsin and 0.02% EDTA. For the experiments, ASMCs in passages 2–3 were grown to confluence in DMEM with 10% FCS and antibiotic-antimycotic reagents and then changed to serum-free DMEM containing antibiotics for 24hs before addition of IL-33.

**Stimulation of ASMC**

ASMC were divided into two groups, NC group (negative control) and AM group (asthmatic mice). All ASMCs were seeded onto six-cell plates with 10% FBS-DMEM and then changed to serum free DMEM for 24 hours synchronization when nearly 70% confluent. Finally, ASMCs from groups NC and AM were stimulated with different concentrations of IL-33 (0ng/ml, 10ng/ml, 100ng/ml) for different times. After stimulation, both medium and cells were collected.

**RNA preparation and real-time PCR**

ASMCs were plated at a seeding density of 1x10⁴ cells/cm² in six-well plates. The 24h serum-deprived cells were harvested. Total RNA was isolated using TRIZOL reagent (TaKaRa) according to the manufacture’s instructions. Subsequently, the cDNAs were synthesized using the PrimeScript® RT Master Mix. Primers were designed for mouse KC: RT-PCR was performed using SYBR® Premix Ex Taq™ and normalized to Gapdh or Actb using the ΔΔCT method. The PCR primers were as follows (5’-3‘): MUSKC(175bp):f-TGCACCCAAA CCGAAAGTC; r-GTCAGAAGCCAGGTTCACC.  

**Immunocytochemical Staining for ST2 and IL-1RAcP**

Six days after primary culture, the cells were plated onto glass coverslips coated in polyornithine (Sigma) in a medium with 10% FBS in individual wells of a 6-well culture plate. Cells were fed with FBS-supplemented medium every 2 days and coverslips were processed 7 days after plating using immunocytochemistry. Immunocytochemistry was performed as follow: each group of cells were fixed with 4% paraformaldehyde and divided in two. One was stained with antibodies against ST2 and the other with antibodies against IL-1RAcP(III-1R accessory protein). Appropriate secondary antibodies (ChemMate TMEh Visio+/HRP) were used.

**ELISAs**

Measurement of KC in culture supernatants was performed using ELISA DuoSets according to the manufactures’ instructions (R&D Systems). Supernatants were stored at -80°C prior to assay.

**Data and Statistical Analysis**

Data are presented as mean ± SEM. Data were compared using one-way analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc t-test to determine statistical differences after multiple comparisons using Prism software. A probability value of less than 0.05 was considered significant.

**Ethical considerations**

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Huazhong University of Science and Technology in accordance with the guidelines of Institutional Animal Care and Use Committee.

**Results**

**Pathomorphological changes of airways in ovalbumin-sensitized mice**

To evaluate the acute asthma mouse model, histopathological studies were performed. In OVA-challenged mice, inflammatory cell infiltration in the peribronchial areas was observed; the mucosal fold membranes of bronchiole were increased and broken, the thickness of the airway wall and bronchial smooth muscle were significantly increased, while the bronchial lumen was narrowed (Figure 1).

![Eosin staining of the lung tissue slices in mice. A: Control mice at original magnification × 200; B: ovalbumin-sensitized mice at × 200.](image-url)
Changes in inflammatory cells in BALF of ovalbumin-sensitized mice

The inflammatory cell levels (i.e., total cells, eosinophils and neutrophils) in the bronchoalvelar lavage fluid were significantly elevated in OVA-challenged mice versus control mice. The eosinophils were notably increased in OVA-challenged mice, while they were inconspicuous in control mice (Figure 2).

Immunocytochemical Staining for ST2 and IL-1RacP in ASMCs

When ASMC coverslips were ready, immunocytochemistry was performed. ST2 and IL-1RacP were both expressed in the membrane of airway smooth muscle cells in ovalbumin-sensitized mice. It was found that ST2 was slightly positively distributed in the membranes of ASMCs in ovalbumin-sensitized mice, while ST2 was almost not expressed in the membranes of ASMCs in the group of negative control mice. IL-1RacP was predominately positively distributed on the membrane of ASMCs in the two groups. (Figure 3).

KC expression in ASMC induced by IL-33

ASMCs were divided into two groups, NC group (negative control) and AM group, (asthmatic mice). Then they were stimulated with different concentrations of IL-33 (0ng/ml, 10ng/ml and 100ng/ml, respectively) for 24 hours. After stimulation with IL-33 (100ng/ml) for 24hs, the KC expression of ASMCs in negative control mice showed no significant change, while KC expression in ASMCs in ovalbumin-sensitized mice was significantly increased. In the AM group, the ASMCs after stimulation with IL-33 (10ng/ml) also showed a rising level of KC expression, but there was no statistically significant difference. Correspondingly, we showed that in the group with IL-33 (100ng/ml), the expression of KC mRNA increased too (Figure 4).

Discussion

In this study, using a murine model of allergic asthma, we demonstrated that ST2 and IL-1RacP are both expressed in the membranes of airway smooth muscle cells in ovalbumin-sensitized mice. However, in the group of negative control mice, ST2 was almost not expressed in the membranes of airway smooth muscle cells, while IL-1RacP was positively distributed in the membranes of ASMCs. ST2 is a member of the IL1R/TLR superfamily. There are three isoforms of ST2 in humans, which are produced by variant splicing of a single transcript: sST2, ST2L, and ST2V. The membrane-anchored long form, ST2L, is expressed mainly, but
not exclusively, on immune cells associated with allergic inflammation.\textsuperscript{19} The results of some studies have shown that ST2 is restricted to the surface of Th2 cells, mast cells, basophils, eosinophils, myeloid DC and NKT cells.\textsuperscript{20-22} ST2 is the specific IL-33 receptor. The bioactive receptor complex consists of IL-33-bound ST2 and a ubiquitously expressed, shared co-receptor termed IL-1R accessory protein (AcP).\textsuperscript{19} IL-1RAcP, a signal transduction receptor subunit, is also an indispensable member of the IL-1R complex. It is necessary for IL-33-induced in vivo effects. IL-33-mediated signaling can be blocked by dominant-negative IL-1RAcP.\textsuperscript{23} The IL-33/ST2 axis seems to play a key role in several inflammatory disorders, including asthma, anaphylactic shock and rheumatoid arthritis.\textsuperscript{24} Treatment with anti-IL-33 significantly reduces serum IgE levels, the numbers of eosinophils and lymphocytes, and concentrations of several cytokines in bronchoalveolar lavage fluid, as compared with administration of a control antibody.\textsuperscript{25} While airway smooth muscle cells in asthma are thought to be crucial for airway narrowing, their role goes beyond this activity. They seem to play an active role in airway inflammation through the voluntary release of inflammatory

\textbf{Figure 4.} KC protein and mRNA expression in ASMCs induced by IL-33. A. KC protein levels in culture supernatants in three groups of different concentrations of IL-33 (0ng/ml, 10ng/ml, 100ng/ml) in the ASMCs in negative control mice (NC) by EILSA. There were no statistical differences between the three groups. B. KC protein levels in culture supernatants in three groups of different concentrations of IL-33 in the ASMCs in ovalbumin-sensitized mice (AM) by EILSA. The group with IL-33 (100ng/ml) showed rising level of KC expression (*, p<0.05), compared with the CM group (IL-33 0ng/ml). The group with IL-33 (10ng/ml) also showed rising levels of KC expression, compared with the CM group (IL-33 0ng/ml), but there was no statistically significant difference between them. C. KC mRNA levels in the ASMCs in the three groups with different concentrations of IL-33 in the negative control mice (NC) by EILSA. There was no statistically significant difference between the three groups. E. KC mRNA levels in the ASMCs in three groups with different concentrations of IL-33 in the ovalbumin-sensitized mice (AM) by EILSA. The group with IL-33 (100ng/ml) showed obviously increased levels of KC mRNA expression (*, p<0.05).
mediators, especially chemokines, rather than acting passively.\(^4,5\) It is reported that ASMCs secrete large amounts of immunomodulatory factors (IMFs), many of which are emblematically ascribed to trafficking leukocytes (e.g., GM-CSF, IL-6, IL-13, and eotaxin), and may be indicative of an immunomodulatory role of disease activity and treatment efficacy, such as in asthma and COPD.\(^26\) While Miller et al. reported that ST2 mRNA was expressed in epithelial and endothelial cells, but not in normal human lung fibroblasts and ASMCs.\(^15\) Kurokawa et al. reported that the ST2 gene was originally identified as that induced by fibroblasts and their results showed that ST2 expression is different in fibroblasts cell types.\(^16\) Recent studies have shown that IL-33 and ST2 mRNA are present in the thoracic aorta of 18-wk-old C57BL/6 mice and in primary cultures of human ECs and SMCs.\(^14\) Our results also demonstrated that ST2 was expressed on the membranes of ASMCs in ovalbumin-sensitized mice and IL-1RAcP expression was more obvious than ST2, both on the membranes of ASMCs in negative control mice, and of ovalbumin-sensitized mice because IL-1RAcP is the co-receptor for the IL-1 families.\(^23\)

We also showed that the KC expression in ASMCs in asthmatic mice was significantly increased after the stimulation with IL-33 (100ng/ml), as compared with that for negative control mice. Interleukin-8 (IL-8) is a major cytokine responsible for attracting neutrophils inflamed areas. Serum IL-8 is an indicator of disease severity and treatment effectiveness in bronchial asthma.\(^6\) Previous studies have shown that levels of mRNA for KC, the murine functional equivalent of IL-8, are elevated in severe asthma.\(^27\) Similarly, it has been reported that higher levels of IL-6 and KC were detected both in acute and chronic OVA-alum models, as compared with vehicle-treated animals.\(^28\) It was found that the serum levels of ECP, IL-5, -6, -8 and -10, G-CSF, MCP-1, and IP-10 are significantly elevated in acute, as compared with stable asthma.\(^29\) ST2 and AcP recruit the adaptor molecules, MyD88 and IRAK, to stimulate activation of the transcription factor NF-κB, as well as MAP kinases, such as p38 and JNK.\(^19\) Induction of IL-33 expression is also a cascade reaction. IL-33/ST2/IL-1R-associated kinase (IRAK) accessory protein coupling invokes the MyD88/IRAK1/IRAK4 complex, which then activates the mitogen-activated protein kinase kinase (MAPKK), extracellular signal-regulated kinase (ERK), p38 and JUN N-terminal kinase (JNK), creating increased expression of IL-5, IL-13, CCL5, CCL17 and CCL24. Down stream of the MyD88 complex, IL-33 also invokes the PLD/SpHK (phospholipase D/sphingosine kinase) complex, bringing about calcium influx and the degradation of the inhibitor of kappa B (IKB), and then the sensitization of nuclear factor, kappa-light-chain-enhancer of activated B cells (NFkB) and the secretion of IL-1β, IL-3, IL-6, TNFα, CXCL2, CCL2, CCL3, prostaglandin D (PGD) and leukotriene B (LTB).\(^9\) Studies with mouse cells show that expression of ST2 on Th2 cells is subsequent to IL-4 expression.\(^19\) IL-13 and IL-4 share many functional properties, arising from the fact that they share the subunit of the IL-4R. IL-4 or IL-13 stimulation causes activation of multiple signal transduction pathways via IL-4R, one of which involves a transcription factor, STAT6. Transfection of STAT6 siRNA markedly reduces the IL-4-dependent upregulation of both ST2L mRNA and sST2 mRNA, suggesting that STAT6 is necessary for increased expression of ST2 genes with IL-4 stimulation. Th2 cytokines, such as IL-4 and IL-13, significantly enhance ST2 expression and function in both endothelial and epithelial cells.\(^15\) As epigenetics becomes a hotspot in scientific research, gene-environmental interaction has received much attention. It is reported that dynamic DNA methylation changes within repetitive sequences or transposons can regulate neighboring genes in response to SA stress. In the OVA-challenged acute mouse model, ASMCs seem to be more sensitive to IL-33 stimulation, despite the fact that another study \(^13\) reported that fibroblasts or smooth muscle cells were not the IL-33–responsive cells in normal primary human lung tissue cells. This discrepancy can only be resolved by further research into epigenetics of asthma.\(^30\)

**Conclusions**

In this study, we showed that ST2 and IL-1RAcP are both expressed on the membranes of ASMCs in ovalbumin-sensitized mice. We suggest that IL-33 can induce increased KC expression in the ASMCs of asthmatic mice, therefore enhancing eosinophilia in the tissues. These results may indicate a key role for IL-33 and ST2 in allergic bronchial asthma.

**Conflict of interest**

The authors declare that no financial or other conflict of interest exists in relation to the content of this article.
IL-33 promotes KC expression in mouse airway smooth muscle cells

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References
25. Liu X, Li M, Wu Y, Zhou Y, Zeng L, Huang T. Anti-IL-33 antibody treatment inhibits airway inflammation in a murine...


