draining LNs, upon topical application of allergen, while patients with psoriasis have impaired migration of LCs [32]. This suggests possible involvement of impaired LC function in the disease pathogenesis of psoriasis. Those reports together with our present results suggest the importance of LCs for preventing allergic and/or autoimmune cutaneous diseases.

It is believed that naïve lymphocytes circulate only through the secondary lymphoid tissues but do not enter the periphery. Many studies have shown that once the LCs migrate to the draining lymph nodes, they have undergone maturation process and are thus capable of stimulating naïve Tcells [33–35]. Based on this hypothesis, it appears to be difficult to conceive how LCs would exert their negative regulatory functions on naïve T cells. However, several possibilities account for the situation in which LCs negatively regulate naïve T cell activation. First, naive T cells may incidentally translocate into the skin from the circulation. As the skin is a large entrance of foreign substances due to facing and communicating with the outsides, those naïve Tcells may have a considerable chance to be activated by their proper Ag if the Ag is presented by professional APCs, which eventually results in frequent cutaneous inflammation. In this situation, LCs might negatively regulate the DC activation of naïve Tcells in the skin and form a powerful safety network for healthy skin. Second, immature LCs are reportedly accumulated in the LNs draining chronic skin alterations [36]. Dermatopathic lymphadenitis is a reactive condition characterized by enlarged LNs draining chronically inflamed skin lesions, including chronic eczema, acne and T cell lymphoma. In the LNs, large numbers of langerin$^+$CD68$^+$ immature LCs, but little langerin$^+$CD83$^+$ mature LCs, are localized in the T cell zone in close contact with Tcells [36], allowing us to speculate that the immature LCs might regulate T cell response during chronic inflammatory skin diseases. Thus, immature LCs might be important to regulate skin-associated immune/inflammatory response.

In this study we demonstrated that freshly isolated, highly purified murine LCs negatively regulate primary adaptive response. Our present findings might shed lights on new therapeutic regimen against atopic dermatitis and/or autoimmune skin diseases by enhancing regulatory actions of LCs and LC-mediated immune responses.

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Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system

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Abstract

Systemic administration of IL-18 induces polyclonal IgE responses by causing NKT cells to express CD40 ligand and to produce IL-4. Administration of IL-33 also induces IgE response, although the mechanism underlying IgE response is unclear. Here, we compared the effects of IL-18 and IL-33 on bone marrow-derived mast cells and basophils as well as non-polarized and T_h2-polarized $CD4^+$ T cells in vitro. Basophils, comprising IL-18Rα^+ cells (14.2%) and IL-33Rα^+ cells (34.6%), and mast cells, comprising IL-18Rα^+ cells (2.0%) and IL-33Rα^+ cells (95.6%), produce IL-4, IL-6, IL-13, granulocyte macrophage colony-stimulating factor (GM-CSF) and chemokines (RANTES, MIP-1a, MIP-1b and MCP-1), upon stimulation with IL-18 and/or IL-33 in the presence of IL-3. Only basophils strongly produce IL-4. Furthermore, compared with mast cells, basophils produce larger amounts of the above cytokines and chemokines in response to IL-33. Level of IL-33Rb-mRNA expression in basophils is higher than that in mast cells. Effect of IL-33 is dependent on ST2 binding, and its signal is transduced via MyD88 in vitro. We also found that IL-2 plus IL-18 or IL-33 alone stimulates non-polarized or T_h2-polarized $CD4^+$ T cells to produce IL-4 and IL-13 or IL-5 and IL-13, respectively. We finally showed that administration of IL-33 into mice ST2/MyD88 dependently induces airway hyperresponsiveness (AHR) and goblet cell hyperplasia by induction of IL-4, IL-5 and IL-13 in the lungs. Furthermore, same treatment of RAG-2$^{-/-}$ mice, lacking T and B cells, more strikingly induced AHR with marked goblet cell hyperplasia and eosinophilic infiltration in the lungs. Thus, IL-33 induces asthma-like symptom entirely independent of acquired immune system.

Introduction

We originally reported that basophils, mast cells, NK cells and NKT cells express IL-18Rα chain and produce T_h2 cytokines in response to IL-18 (1–3). Furthermore, we reported that systemic administration of IL-18 induces polyclonal IgE responses by activation of NKT cells to express CD40 ligand and to produce IL-4 (2, 4). Thus, IL-18 induces T_h2 cytokines/IgE responses without help from antigen. It is well-known evidence that basophils and mast cells produce T_h2 cytokines and various inflammatory mediators in response to cross-linking by allergens of the bound IgE on their cell surface (5). However, as we reported previously, basophils and mast cells express IL-18Rα chain markedly and modestly, respectively, and only basophils produce large amounts of IL-4 and IL-13, when stimulated with IL-3 and IL-18 (1), suggesting the possibility that degree of IL-18Rα chain determines IL-18 responsiveness.

Recently, IL-33 has been cloned as the ligand of ST2 (6). IL-33 is a member of the IL-1 family. Like IL-1b or IL-18, closely related IL-33 is also synthesized as a 31-kDa precursor form and becomes active after cleavage with caspase-1

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(6). In vivo treatment with IL-33 induces increases in expression of mRNAs for IL-4, IL-5 and IL-13 in spleen, liver and lungs and in serum levels of IL-5 and IL-13 (6). We and others previously reported that T_h1 and T_h2/mast cells preferentially express IL-18Rα chain (7, 8) and ST2 (9–12), respectively. IL-18Rα chain and ST2 are members of IL-1 receptor superfamily (13). Like functional IL-18R is composed of IL-18Rα and IL-18Rβ chain (13, 14), functional IL-33R is shown to consist of IL-33Rα (ST2) and IL-33Rβ (IL-1 receptor accessory protein; IL-1RAcP or IL-1Rβ chain) (15, 16). By using ST2-deficient (ST2 $^{-/-}$) mice, we revealed that the absence of this receptor does not affect T_h2 development (9). However, as reported by Schmitz et al. (6), IL-33 increases IL-5 and IL-13 production from T_h2 without enhancing their IL-4 production, suggesting that IL-33 is an important cytokine that augments T_h2 response.

Basophils and mast cells are important effector cells in allergic inflammation (17). Beside this function, basophils and mast cells are important in regulation of T_h2 response. Upon entry of invading pathogen, dendritic cells recognize pathogen-associated molecular patterns through Toll-like receptor and mature to express co-stimulatory molecules and to produce IL-12 and IL-18 (13, 18, 19), favoring the development of T_h1 response. In contrast, basophils are reported to be involved in induction of T_h2 by its unique function to produce primary IL-4 and thymic stromal lymphopoietin (TSLP) (20). Furthermore, basophils strongly produce IL-4 and IL-13 in response to IL-3 and IL-18 (2). For this reason, we regard it important to compare the effects of IL-18 and IL-33 on basophils, mast cells and T cells in vitro and in vivo.

In this study, we demonstrated that basophils and mast cells express IL-18R and IL-33R and only basophils strongly produce IL-4, IL-6 and IL-13 in response to IL-18 and/or IL-33. IL-33 acts on T_h2 to produce T_h2 cytokines, while IL-2 and IL-18 stimulate non-polarized $CD4^+$ T cells to produce T_h2 cytokines. We also show that IL-33 binding to ST2 leads to induction of T_h2 cytokines in a MyD88-dependent but TRIF-independent manner. In vivo IL-33 treatment induces goblet cell hyperplasia by induction of endogenous IL-13. We finally show that intra-nasal administration of IL-33 strongly induces airway hyperresponsiveness (AHR) and goblet cell hyperplasia even in the lungs of RAG-2 $^{-/-}$ mice lacking acquired immune system, suggesting the critical role of innate immune cells including basophils and mast cells in induction of IL-33-induced asthma-like symptom.

Methods

Mice

Specific pathogen-free (SPF) female BALB/c and C57BL/6 mice, 8 weeks of age, were purchased from Jackson Laboratory. C57BL/6 background MyD88 $^{-/-}$ and ST2 $^{-/-}$ mice were generated as described in our previous report (9, 21). Mice transgenic for αβ TCR recognizing ovalbumin peptide $(OVA)_{323–339}$ (DO11.10) were provided by Loh (Washington University, St Louis, MO, USA). C57BL/6 TRIF $^{-/-}$ mice (22) were kindly provided by Akira (Osaka University, Suita, Japan). C57BL/6 IL-13 $^{-/-}$ mice were generated by backcrossing B6X129 IL-13 $^{-/-}$ mice (23) with C57BL/6. BALB/c RAG-2 $^{-/-}$ mice were purchased from Taconic (Germantown, NY, USA). Mast cell-deficient WBB6F1-W/W^v mice (24) and littermate control WBB6F1$^{+/+}$ mice were purchased from Japan SLC (Hamamatsu, Japan), respectively. All mice were bred under SPF condition at the animal facilities of Hyogo College of Medicine (Nishinomiya, Japan) and were used at 8–12 weeks of age.

Reagents

Recombinant mouse IL-3 and IL-18 were purchased from R&D Systems Inc. (Minneapolis, MN, USA) and MBL (Nagoya, Japan), respectively. Recombinant mouse IL-4 was purified in our laboratory as described before (8). Recombinant human IL-33 was made by Hokudo Co., Ltd (Sapporo, Japan). Briefly, IL-33 (mature form) was amplified from human lung cDNA (BioChain Institute) as a template and subcloned into pET28a vector (Novagen). BL21 (DE3) RIL was transformed and expressed recombinant protein was purified with Ni-NTA resin. Endotoxin was removed by filtration through Zetapor (Cuno). Purified antibodies [anti-mouse IL-4 (11B11), anti-mouse IL-12p40 (C17.8) and anti-mouse IFN-γ (R4-6A2)] were prepared in our laboratory. PE–anti-mouse CD4, FITC–anti-mouse CD62L and PE–anti-mouse c-Kit were purchased from BD Biosciences (San Diego, CA, USA). FITC–anti-mouse ST2 mAb (DJ8), biotin–anti-mouse FcεR1 (MAR-1), streptavidin–APC and streptavidin–FITC were purchased from eBioscience (San Diego, CA, USA). Anti-mouse IL-18Rα chain mAb (Y38) was provided by Hayashibara Biochemical Laboratories Inc.

Isolation of basophils and mast cells

Bone marrow cells cultured with IL-3 (10 U ml $^{-1}$) for 10 days in RPMI 1640 supplemented with 10% fetal bovine serum, 2-ME (50 mM), L-glutamine (2 mM), penicillin (100 U ml $^{-1}$) and streptomycin (100 mg ml $^{-1}$) were washed twice. Cells were first treated with 10 μg ml $^{-1}$ anti-FcγRII/III for 30 min at 4 °C followed by treatment with 5 μg ml $^{-1}$ biotin–anti-mouse FcεR1 for 1 h at 4 °C in staining buffer (PBS and 1% FCS). Cells were then washed twice and stained with streptavidin–FITC or streptavidin–APC and PE–anti-mouse c-Kit for 30 min. Samples were analyzed on a FACSCalibur (BD Biosciences) and separated into $FcεR^+$/c-Kit$^-$ cells (basophils) and $FcεR^+$/c-Kit$^+$ cells (mast cells) by fluorescence cell sorter (FACSAria; BD Biosciences). Purity of each population was >95%.

In vitro stimulation of basophils and mast cells

Sorted basophils and mast cells (10^5/0.2 ml per well) were washed and re-stimulated with medium alone, IL-18 (50 ng ml $^{-1}$) and/or IL-33 (1–100 ng ml $^{-1}$) in the presence of IL-3 (20 U ml $^{-1}$) for 24 h. After incubation, supernatants were collected and cytokine release was analyzed with ELISA Kits (R&D Systems Inc.). For some experiments, cytokine release was analyzed with the Bio-Plex Mouse Cytokine 23-Plex Panel (Bio-Rad, Hercules, CA, USA) using beads specific for IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, RANTES, eotaxin, MCP-1, MIP-1a, MIP-1b, tumor necrosis factor-α, IFN-γ, and granulocyte macrophage colony-stimulating factor (GM-CSF), according to the manufacturer's instructions. The broad assay range was from 0.2 to 5000 pg ml $^{-1}$.

In vitro stimulation of CD4+ T cells

Purified splenic CD4+ T cells from BALB/c mice by MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) (10^5/0.2 ml per well) were cultured with medium alone or various combinations of IL-2 (200 pM), IL-18 (50 ng ml $^{-1}$) and IL-33 (100 ng ml $^{-1}$) for 4 days. For generation of T_h2, sorted splenic CD4+CD62L+ T cells (1 3 10^5 ml $^{-1}$) from DO11.10 mice were stimulated with IL-4 (1000 U ml $^{-1}$), anti-IL-12p40 (20 lg ml $^{-1}$), anti-IFN-c (20 lg ml $^{-1}$), IL-2 (100 pM) and $OVA_{323-339}$ (1 lM) in the presence of irradiated T cell-depleted BALB/c splenocytes (1 3 10^6 ml $^{-1}$) in 24-well plate in a total 1-ml volume of medium for 7 days as described previously (25). Polarized T_h2 (1 3 10^5/0.2 ml per well) were re-cultured with IL-2 (100 pM) and $OVA_{323-339}$ (1 lM) and irradiated T cell-depleted BALB/c splenocytes (1 3 10^5/0.2 ml per well) in the presence of IL-18 (50 ng ml $^{-1}$) or IL-33 (100 ng ml $^{-1}$) for 48 h. After incubation, supernatants were harvested and tested for IL-4 and IL-13 contents by ELISA.

In vivo treatment of mice

Mice were daily injected intra-peritoneally with PBS alone or with IL-33 (4 lg day $^{-1}$) for 4 days. In some experiments, mice were daily exposed intra-nasally to IL-33 (1 lg day $^{-1}$) in 50 ll of PBS for 4 days. Control mice were exposed to PBS alone. Twenty-four hours after the final treatment with PBS alone or IL-33, lungs were removed for histological examination. To deplete CD4+ T cells, WBB6F1-W/W^v mice were intra-peritoneally injected four times (14, 10, 7 and 4 days before IL-33 treatment) with mAb to CD4 (clone, GK1.5; 0.5 mg day $^{-1}$) as described previously (26). To deplete NK cells, RAG-2 $^{-/-}$ mice were intravenously injected two times (7 and 4 days before IL-33 treatment) with anti-asialo GM1 (1 mg day $^{-1}$) as described (27).

Measurement of AHR

We measured AHR to b-methacholine (Mch) inhalation in mice by using Pulmos-I (MIPS, Osaka, Japan) hardware and software as described in our previous report (7). We placed a mouse in a chamber and exposed it to aerosols of saline (baseline) first and then to increased concentrations of b-Mch (5 and 10 mg ml $^{-1}$). After each 2-min exposure, we measured enhanced pause, a dimensionless index that reflects changes in amplitude of pressure waveform and expiratory time, for 3 min.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed with three aliquots of 1.0 ml of PBS per mouse. Total cell counts were performed. Cytospin preparations of bronchoalveolar lavage fluid (BALF) were stained with Diff-Quik (Baxter Healthcare Corporation, Miami, FL, USA), and differentials were performed based on morphology and staining characteristics.

Histological examination

Lungs were prepared for histology by perfusion of the animal via the right ventricle with 10 ml of PBS. Tissues were fixed in 10% buffered formalin, cut into 3-1m sections and stained with periodic acid Schiff.

Electron microscopy

Sorted basophils and mast cells were fixed with 2% PFA and 1.25% glutaraldehyde, post-fixed with 1% OsO4 and embedded in EPON. Ultrathin sections were double stained with uranyl acetate and lead citrate and examined with a JEM-1220 transmission electron microscopy (JEOL, Tokyo, Japan).

Flow cytometry

For staining of IL-18Ra chain and ST2 (IL-33Ra chain), sorted basophils and mast cells were further incubated with rat anti-mouse IL-18Ra chain mAb plus FITC–anti-rat IgG1 mAb or FITC–anti-mouse ST2 mAb for 30 min at 4 C in staining buffer (PBS and 1% FCS). For staining of IL-33Ra chain on T cells, freshly isolated splenic CD4+ T cells and polarized T_h2 were first treated with 10 lg ml $^{-1}$ anti-FccRII/III for 30 min at 4 C followed by treatment with PE–anti-CD4 and FITC–anti-mouse ST2 mAb for 30 min at 4 C in staining buffer (PBS and 1% FCS). Samples were analyzed on a FACSCalibur. For preparation of CD4+CD62L+ resting T cells, splenic CD4+ T cells from DO11.10 mice were purified by MicroBeads (anti-mouse CD4; clone RM4-5). The enriched CD4+ T cells were first treated with 10 lg ml $^{-1}$ anti-FccRII/III for 30 min at 4 C followed by treatment with PE–anti-CD4 and FITC–anti-CD62L for 30 min at 4 C in staining buffer (PBS and 1% FCS). Stained samples were separated into CD4+CD62L+ T cells by FACSAria (Becton Dickinson). Purity of sorted cells was >98.5% after re-analysis.

Quantitative reverse transcription–PCR

Total RNA was extracted from sorted basophils, mast cells and total lung with RNeasy Plus Mini Kit (QIAGEN) and the cDNA was synthesized using SuperScript III RNase H Reverse Transcriptase (Invitrogen). The expression of the gene was quantified by real-time PCR with TaqMan Gene Expression Assays (Applied Biosystems). The results were showed as relative expression standardized with the expression of the gene-encoding eukaryotic 18S rRNA (18S). Specific primers used for quantitative RT-PCR were as follows: IL-33Ra chain (assay ID: Mm01233982_m1), IL-33Rb chain (assay ID: Mm00492638_m1), IL-4 (Il4) (assay ID: Mm00445259_m1), IL-5 (Il5) (assay ID: Mm00439646_m1), IL-13 (Il13) (assay ID: Mm00434204_m1) and 18S rRNA (18S) (assay ID: Hs99999901_s1).

Results

IL-33 stimulates basophils to produce T_h2 cytokines in vitro

To compare the effects of IL-18 and IL-33 on mast cells and basophils, we first developed mast cells and basophils by culturing bone marrow cells with IL-3 (10 U ml $^{-1}$) for 10 days. We examined the proportions of FceRI+/c-Kit cells and FceRI+/c-Kit+ cells (1), and then highly purified both populations by FACS (Fig. 1A). Light and electron microscopical examination revealed that resultant FceRI+/c-Kit cells and FceRI+/c-Kit+ cells are basophils and mast cells, respectively (Fig. 1B). Next, we examined the proportions of cells positive for IL-18Ra chain or IL-33Ra chain in each population (Fig. 1C and D). Basophils were composed of IL-18Ra+ cells

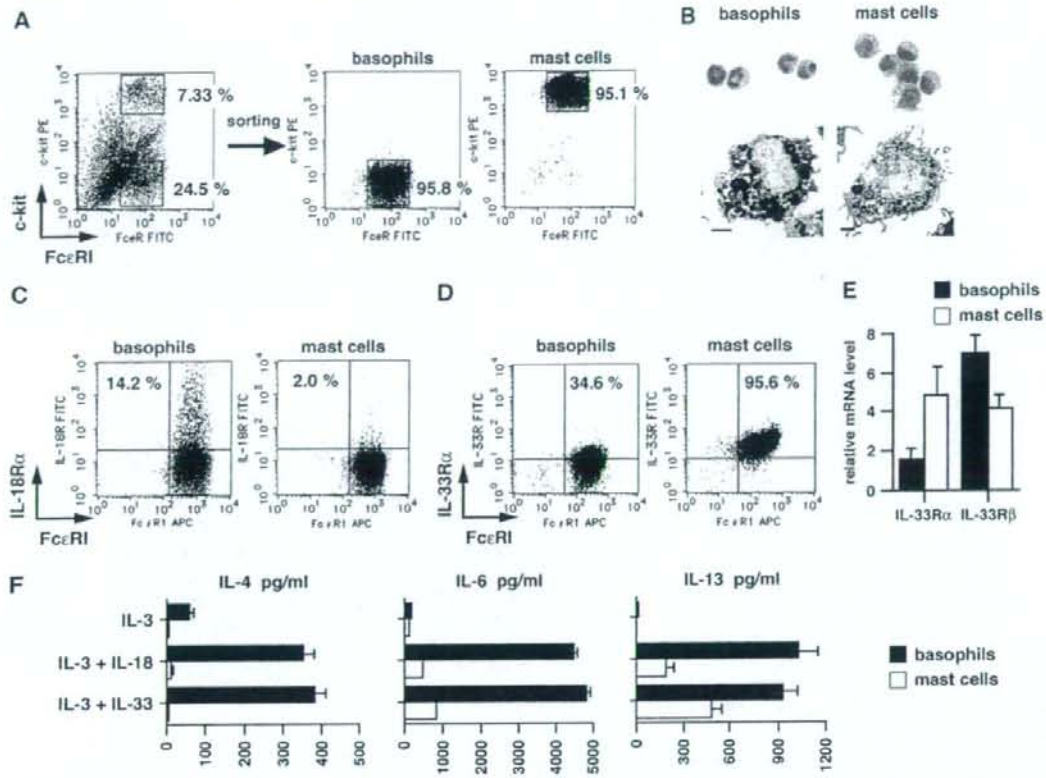

Fig. 1. IL-3 plus IL-33-induced T_h2 cytokine production from bone marrow-derived basophils and mast cells. (A) Bone marrow cells from BALB/c mice cultured with IL-3 (10 U ml 1) for 10 days were analyzed for expression of FcεRI and c-Kit by flow cytometry and then sorted into FcεRI$^+$/c-Kit (basophils) or FcεRI$^+$/c-Kit$^+$ (mast cells) cell populations by fluorescence cell sorter. Percentage of cells in selected populations are indicated. (B) Sorted FcεRI$^+$/c-Kit (basophils) or FcεRI$^+$/c-Kit$^+$ (mast cells) cell populations were stained for Wright–Giemsa staining (3100) (upper) and subjected to electron microscopic examination (lower). Scale bar, 1 I M. (C and D) Surface expression of IL-18Rα chain (C) or IL-33Rα chain (D) on basophils and mast cells by flow cytometry. The percentages shown represent the population of IL-18Rα chain$^+$ (C) or IL-33Rα chain$^+$ (D) cells among FcεRI$^+$ cells. (E) The relative mRNA expression levels of IL-33Rα and IL-33Rb chains in basophils and mast cells were determined by real-time PCR. (F) The sorted basophils and mast cells (each 10^5/0.2 ml per well) were re-stimulated with IL-3 (20 U ml $^{-1}$) plus IL-18 (50 ng ml $^{-1}$) or IL-33 (100 ng ml $^{-1}$). After 24 h of culture, supernatants were harvested and tested for production of IL-4, IL-6 and IL-13 by ELISA. Results are geometric means + SEM. Results are representative of four independent experiments.

(14.2%) and IL-33Rα$^+$ cells (34.6%), and mast cells were composed of IL-18Rα$^+$ cells (2.0%) and IL-33Rα$^+$ cells (95.6%). We simultaneously examined the expression of mRNAs for IL-33R components in basophils and mast cells (Fig. 1E). As expected from the results of Fig. 1(D), level of IL-33Rα-mRNA in basophils is less than that in mast cells. However, level of IL-33Rb-mRNA in basophils is comparable to or rather higher than that in mast cells, suggesting the possibility that both mast cells and basophils are highly responsive to IL-33.

We next compared IL-18 or IL-33 responsiveness of basophils and mast cells (Fig. 1F). Since IL-3 is essential for the survival of basophils and mast cells in vitro, we stimulated them in the presence of IL-3. Basophils strongly produced IL-4, IL-6 and IL-13 when stimulated with IL-3 and IL-18, while mast cells produced IL-6 and IL-13 at relatively low level, revealing that only basophils are highly responsive to IL-18. We simultaneously stimulated basophils and mast cells with IL-3 and IL-33. Again, only basophils strongly produced IL-4, IL-6 and IL-13 in response to IL-3 and IL-33. In contrast, mast cells could not produce IL-4, although they could produce substantial amounts of IL-6 and IL-13 in response to IL-3 and IL-33, suggesting that levels of IL-33Rα chain and IL-33Rb chain as well as the nature of responding cells determine quality and quantity of final response.

We simultaneously examined the capacity of basophils and mast cells to increase production of IL-4, IL-6, IL-13 and other cytokines (IL-5, IL-9, IL-17, IFN-c and GM-CSF) and chemokines (RANTES, MIP-1a, MIP-1b and MCP-1) in response to IL-18 and/or IL-33 (Fig. 2). Basophils dose responsively increased their productions of IL-4, IL-6, IL-9, IL-13, GM-CSF and chemokines. However, basophils did not produce IL-5, IL-17 and IFN-c. In contrast, mast cells are generally poor producers of cytokines and only produced IL-6 and IL-13 at the lower level. However, mast cells dose dependently increased their production of some

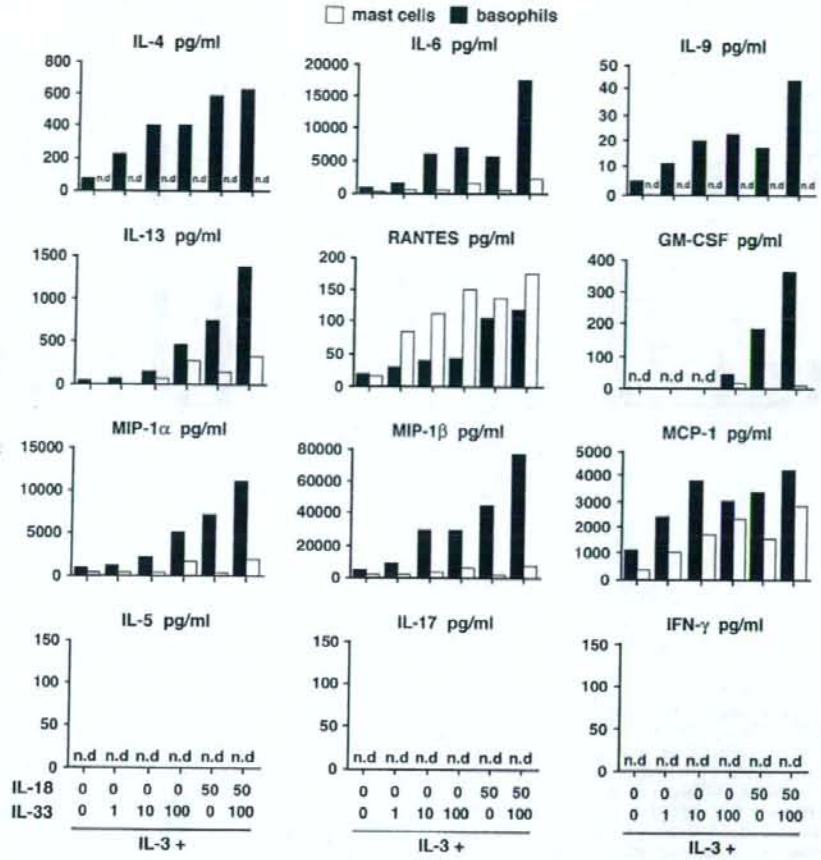

Fig. 2. IL-3 plus IL-33-induced cytokines and chemokines production from bone marrow-derived basophils and mast cells. The sorted basophils and mast cells (each 10^5/0.2 ml per well) as shown in Fig. 1 were re-stimulated with IL-18 (50 ng ml $^{-1}$), IL-33 (1–100 ng ml $^{-1}$) or IL-18 (50 ng ml $^{-1}$) plus IL-33 (100 ng ml $^{-1}$) in the presence of IL-3 (20 U ml $^{-1}$). After 24 h of culture, supernatants were harvested and tested for production of cytokine and chemokines by Bio-Plex. Results are representative of three independent experiments.

chemokines (RANTES and MCP-1), although productions of other chemokines (MIP-1a and MIP-1b) are relatively low. Co-stimulation with IL-18 and IL-33 showed somewhat additional effects on productions of some cytokines or chemokines from basophils or mast cells.

IL-18 or IL-33 stimulates basophils to produce T_h2 cytokines via MyD88

We wished to determine the pathway involved in IL-18- or IL-33-induced production of T_h2 cytokines by basophils or mast cells in vitro. Since basophils are good producers of T_h2 cytokines, we preferentially examined their responsiveness to IL-18 and/or IL-33. MyD88 is a common adapter molecule essential for signaling through IL-18R and IL-33R (13, 19, 21, 28). Thus, we examined the responsiveness of basophils from MyD88 $^{-/-}$ mice to IL-18 and/or IL-33. We simultaneously examined the responsiveness of basophils from TRIF $^{-/-}$ mice (22) to determine that IL-18 or IL-33 signal is transduced entirely through MyD88 pathway. We also stimulated basophils from ST2 $^{-/-}$ mice with IL-18 and/or IL-33 to show the specificity of the action of IL-33-induced responses. We found that basophils derived from ST2 $^{-/-}$ mice or MyD88 $^{-/-}$ mice failed to produce T_h2 cytokines in response to IL-33 (Fig. 3), revealing that IL-33 stimulates basophils in an ST2/MyD88-dependent manner. Interestingly, ST2 $^{-/-}$ basophils only modestly increased IL-4 and IL-13 production in response to IL-18, although they markedly increased IL-6 production, suggesting that ST2 might be involved in the regulation of IL-4 and IL-13 production by basophils. Interestingly, TRIF $^{-/-}$ basophils produced larger amounts of IL-6 and IL-13 than basophils from wild-type mice (Fig. 3), suggesting some cross-talk between TRIF and MyD88 in production of these cytokines. These results taken together indicate that IL-3 either with IL-18 or with IL-33 can stimulate basophils to secrete T_h2 cytokines via respective receptor and that MyD88 is an essential adapter molecule required for IL-18Rα or IL-33Rα chain-mediated T_h2 cytokine production from basophils.

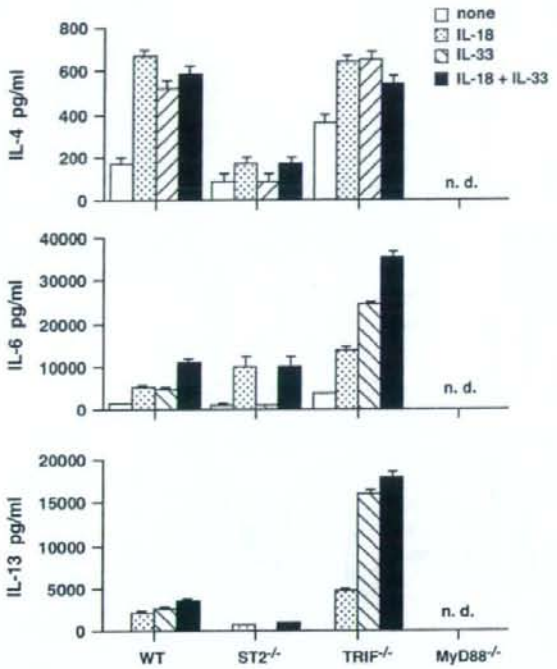

Fig. 3. ST2- and MyD88-dependent and TRIF-independent T_h2 cytokine production from IL-33 stimulated basophils. Bone marrow cells from C57BL/6 (WT), ST2$^{-/-}$, TRIF$^{-/-}$ and MyD88$^{-/-}$ mice cultured with IL-3 (10 U ml^{-1}) for 10 days were sorted into FcεRI$^+$/c-Kit$^+$ (basophils) cell populations by fluorescence cell sorter. The sorted basophils (10^5/0.2 ml per well) were re-stimulated with IL-18 (50 ng ml^{-1}), IL-33 (100 ng ml^{-1}) or IL-18 (50 ng ml^{-1}) plus IL-33 (100 ng ml^{-1}) in the presence of IL-3 (20 U ml^{-1}). After 24 h of culture, supernatants were harvested and tested for production of IL-4, IL-6 and IL-13 by ELISA. Results are geometric means + SEM. Results are representative of three independent experiments.

IL-33 induces T_h2 cytokine production from basophils and T_h2 in vitro

We next compared the effects of IL-33 or IL-18 on freshly prepared non-polarized CD4$^+$ T cells, composed of conventional CD4$^+$ T cells and NK1.1$^+$ CD4$^+$ T (NKT) cells (29), and on in vitro T_h2-polarized CD4$^+$ T cells. As we reported previously (2), a combination of IL-2 and IL-18 strongly induced IL-4 and IL-13 production from non-polarized CD4$^+$ T cells particularly from NKT cells without TCR engagement (Fig. 4A). In contrast, IL-33 by itself or even with IL-2 could not induce non-polarized CD4$^+$ T cells to produce T_h2 cytokines. Therefore, non-polarized CD4$^+$ T cells showed completely different responsiveness to IL-18 or IL-33. However, non-polarized CD4$^+$ T cells become responsive to IL-33 after their development into T_h2 (Fig. 4B), which express IL-33Rα (Fig. 4C). Indeed, IL-33 dose dependently increased IL-5 and IL-13 production from T_h2 without affecting their IL-4 production (Fig. 4B). In contrast, as we reported previously (7), IL-18 showed modest enhancing effect on T_h2. These results clearly indicated that the effects of IL-18 and IL-33 on non-polarized CD4$^+$ T cells and T_h2 are entirely different.

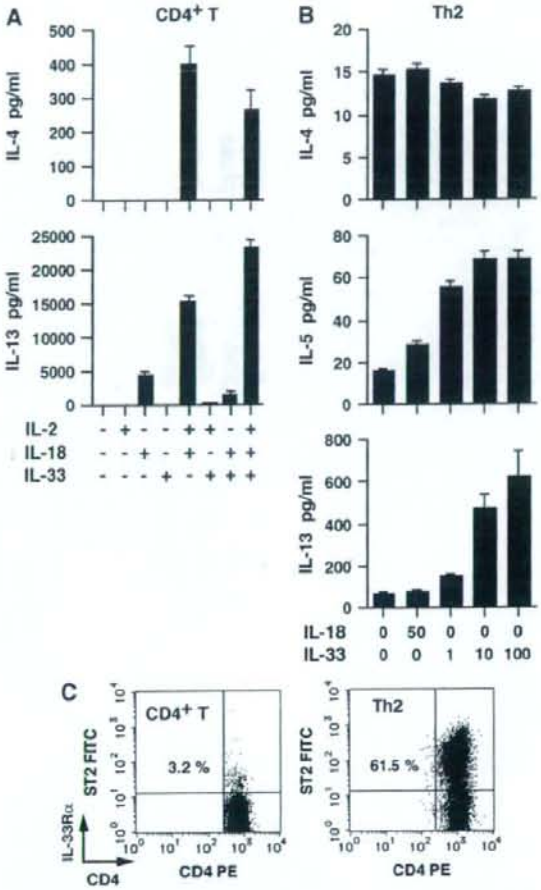

Fig. 4. T_h2 cytokine production from T cells cultured with IL-33. (A) Splenic CD4$^+$ T cells (10^5/0.2 ml per well) from BALB/c mice were cultured with medium alone or various combinations of IL-2 (200 pM), IL-18 (50 ng ml^{-1}) and IL-33 (100 ng ml^{-1}). After 4 days of culture, supernatants were harvested and tested for production of IL-4 and IL-13 by ELISA. (B) Naive splenic CD4$^+$CD62L$^+$T cells (10^5 ml^{-1}) from DO11.10 Tg mice were cultured with IL-2 (100 pM), $OVA_{323-339}$(1 1M) and 1 3 10^6 ml^{-1} irradiated T cell-depleted BALB/c splenocytes in T_h2 condition [IL-4 (1000 U ml^{-1}), anti-IL-12p40 (20 1g ml^{-1}) and anti-IFN-c (20 1g ml^{-1})]. After initial two rounds of priming of T_h2 condition, cells (10^5/0.2 ml per well) were washed and re-cultured with IL-2 (100 pM), $OVA_{323-339}$ (1 1M) and irradiated T cell-depleted BALB/c splenocytes (10^5/0.2 ml per well) in the presence of IL-18 (50 ng ml^{-1}) or IL-33 (0–100 ng ml^{-1}). After 48 h of culture, supernatants were harvested and tested for production of IL-4, IL-5 and IL-13 by ELISA. Results are geometric means + SEM. Results are representative of three independent experiments. (C) Surface expression of IL-33Rα chain on non-polarized CD4$^+$ T cells and T_h2 by flow cytometry. The percentages shown represent the population of IL-33Rα chain$^+$ cells among CD4$^+$ cells.

Thus, we assumed the possibility that non-polarized CD4$^+$ T cells and T_h2 are target cells of IL-18 and IL-33 in vivo and produced T_h2 cytokines when they are stimulated with IL-18 or IL-33, respectively.

IL-33 stimulates goblet cells to produce mucin in vivo via endogenous IL-13

We next examined whether intra-peritoneal IL-33 injection induces T_h2 cytokine response in vivo in an ST2/MyD88-dependent manner. Thus, we injected IL-33 (4 1 g) once a day for consecutive 5 days into C57BL/6 wild-type or C57BL/6 background ST2 $^{/}$, MyD88 $^{/}$ or TRIF $^{/}$ mice. Wild-type and TRIF $^{/}$ mice markedly developed goblet cell hyperplasia in their lungs in response to IL-33, while ST2 $^{/}$ and MyD88 $^{/}$ mice failed to do so (Fig. 5A), indicating that IL-33 induced mucin production in the lung in an ST2/MyD88-dependent manner. Then, to examine the possibility that administration of IL-33 induces goblet cell hyperplasia via endogenous IL-13, we injected IL-33 into C57BL/6 wild-type, C57BL/6 background IL-13 $^{/}$ or STAT6 $^{/}$ mice. As we expected, daily intra-peritoneal injection of IL-33 induced goblet cell hyperplasia in the airways of wild-type mice but not in those of IL-13 $^{/}$ or STAT6 $^{/}$ mice, suggesting that IL-33 induces these responses by induction of endogenous IL-13 (Fig. 5A).

As basophils, mast cells or T_h2 produce IL-13 in response to IL-33 (Figs 1F, 2 and 4B), we examined the possibility that administration of IL-33 induces goblet cell hyperplasia in the absence of acquired immune system in vivo. Thus, we daily injected IL-33 for 5 days into BALB/c background RAG-2 $^{/}$ mice, lacking T cells and B cells. As we expected, these mice normally developed goblet cell hyperplasia (Fig. 5B). Since mast cells produce IL-13 in response to IL-33, we injected IL-33 into CD4^{+} T cell-depleted WBB6F1-W/W^{v} mice, lacking CD4^{+} T cells and mast cells (24). We found that these mice normally developed goblet cell hyperplasia in their lungs (Fig. 5C). Instead of intra-peritoneal administration of IL-33 into mice, we intra-nasally administered IL-33 into RAG-2 $^{/}$ mice or NK cell-depleted RAG-2 $^{/}$ mice. As shown in Fig. 5(D), this treatment strongly induced goblet cell hyperplasia in the lungs of mice lacking T cells and B cells or T cells, B cells and NK cells. However, as expected from the result of Fig. 5(A), intra-nasal administration of IL-33 did not induce goblet cell hyperplasia in the lungs of IL-13 $^{/}$ or STAT6 $^{/}$ mice (data not shown). Since IL-33 induces goblet cell hyperplasia by induction of endogenous IL-13, we next examined whether administration of IL-33 into mice lacking T cells, B cells and basophils induces endogenous IL-13. We injected anti-mouse FcεR1 antibody (MAR-1), which is shown to specifically deplete basophils in vivo (20), into RAG-2 $^{/}$ mice. We found that these mice still have the capacity to promptly express IL-13-mRNA in their spleens or other organs following intra-peritoneal administration of IL-33, suggesting the presence of innate type IL-13-producing cells other than basophils in IL-33-treated RAG-2 $^{/}$ mice.

IL-33 administration induces AHR in vivo via endogenous IL-13

We finally examined whether daily intra-nasal administration of IL-33 for consecutive 4 days induces asthma-like symptom in RAG-2 $^{/}$ mice. As shown in Fig. 6(A), wild-type and RAG-2 $^{/}$ mice developed AHR following intra-nasal

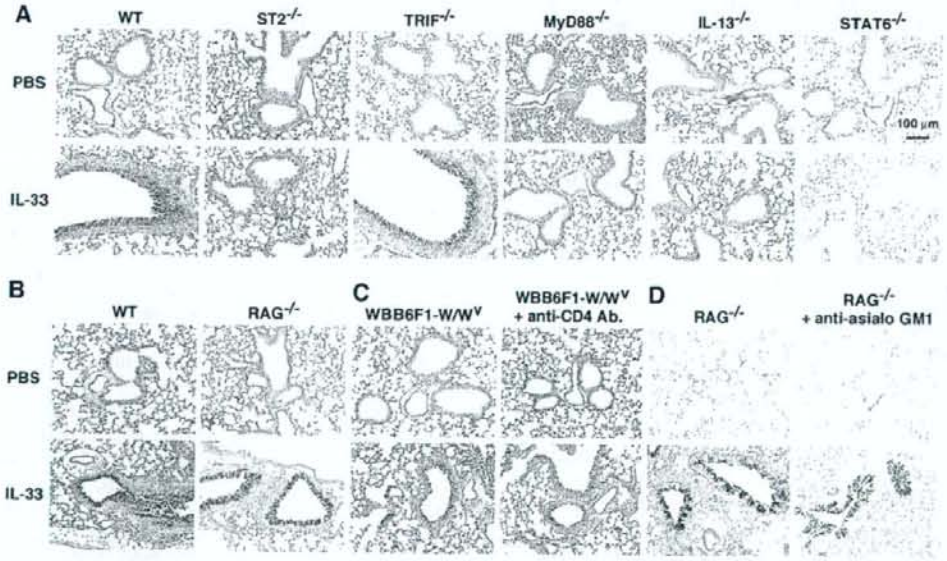

Fig. 5. IL-33-induced IL-13-dependent goblet cell hyperplasia. C57BL/6 (WT), ST2 $^{/}$, TRIF $^{/}$, MyD88 $^{/}$, IL-13 $^{/}$ and STAT6 $^{/}$ mice (A), BALB/c (WT) and RAG-2 $^{/}$ (RAG $^{/}$) (B) and WBB6F1-W/W^{v} mice and WBB6F1-W/W^{v} mice depleted of CD4^{+} T cells (C) were daily injected intra-peritoneally with IL-33 (4 1 g day ^{1}) for 5 days. (D) RAG $^{/}$ and RAG $^{/}$ depleted of NK cells mice were daily exposed intra-nasally to IL-33 (1 1 g day ^{1}) for 4 days. Twenty-four hours after the final treatment of IL-33, lungs were isolated and stained with predigested periodic acid Schiff. To deplete CD4^{+} T cells, WBB6F1-W/W^{v} mice received anti-CD4 (GK1.5; 0.5 mg day ^{1}) antibody at 4, 7, 10 and 14 days before initial IL-33 treatment. To deplete NK cells, RAG $^{/}$ mice received ant-asialo GM1 (1 mg day ^{1}) antibody at 4 and 7 days before initial IL-33 treatment. Representative results of four to six animals were shown.

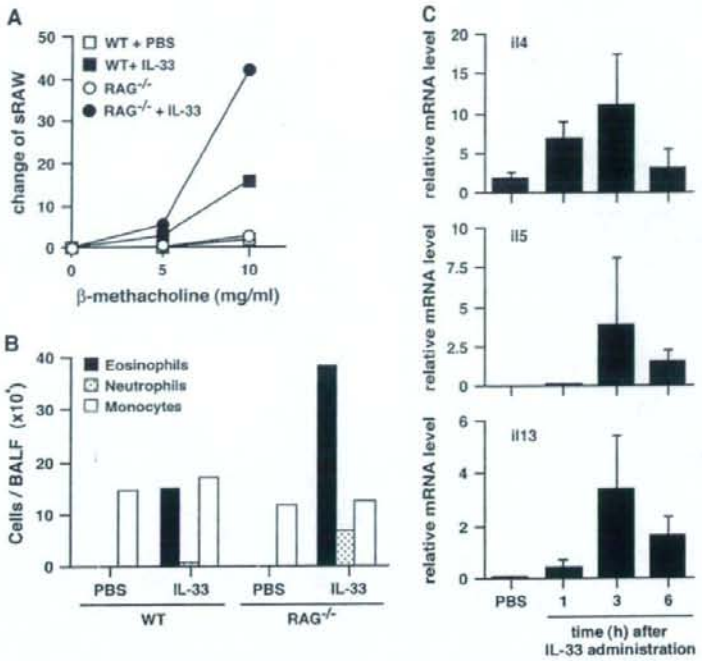

Fig. 6. IL-33-induced AHR and airway inflammation. (A and B) BALB/c (WT) and RAG $^{-/-}$ mice (four to six mice per group) were daily exposed intra-nasally to PBS alone or IL-33 (1 1g day $^{-1}$) in 50 1l of PBS for 4 days. (A) AHR in response to increased concentrations of inhaled b-Mch was measured in a whole-body plethysmograph. (B) Inflammatory cell composition of BALF from mice was determined by light microscopic evaluation of cytospin preparation. Data are expressed as absolute numbers of cells. Representative results of four animals were shown. (C) BALB/c mice were exposed intra-nasally to IL-33 (1 1g day $^{-1}$) at once. IL-33 (1 1g) was administered intra-nasally into BALB/c mice. Lungs were removed at 1, 3 and 6 h after IL-33 administration and total RNA was extracted. The relative mRNA expression levels of IL-4, IL-5 and IL-13 in lungs were determined by real-time PCR.

administration of IL-33. To our surprise, RAG-2 $^{-/-}$ mice developed severer AHR, suggesting that absence of acquired immune system rather augments IL-33-induced AHR development. BALF examination revealed that administration of IL-33 induced increases in the numbers of eosinophils and neutrophils both in wild-type and in RAG-2 $^{-/-}$ mice (Fig. 6B). Therefore, RAG-2 $^{-/-}$ mice developed severer AHR and airway inflammation in response to intra-nasal administration of IL-33 even in the absence of T cells and B cells.

We simultaneously examined whether intra-nasal IL-33 administration induces IL-13 production in the lungs. Thus, we examined the expression of mRNAs for IL-4, IL-5 and IL-13 in the lungs at 1, 3 and 6 h after intra-nasal administration (Fig. 6C). This treatment very rapidly induced increases in the levels of these messages in the lungs of wild-type mice. Induction of goblet cell hyperplasia is entirely dependent on endogenous IL-13 (Fig. 5A). Furthermore, BALF examination revealed that administration of IL-33 only marginally induced increases in the numbers of eosinophils and neutrophils in IL-13 $^{-/-}$ mice (data not shown). These results taken together indicate that intra-nasal administration of IL-33 induces asthma-like symptom by induction of endogenous IL-13 even in the lungs of mice lacking an acquired immune system.

Discussion

Like systemic administration of IL-18 (2, 4), systemic injection of IL-33 induces polyclonal IgE response and goblet cell hyperplasia in the lungs (6). However, there is striking difference in the process of these responses in vivo. IL-18 demonstrates these effects in vivo only in the presence of $CD4^+$ T cells (2, 4). IL-18 stimulates NKT cells in non-polarized $CD4^+$ T cells to produce IL-4 and to express CD40 ligand (2, 4). In contrast, IL-33 fails to act on non-polarized $CD4^+$ T cells (Fig. 4A). As we noted here, non-polarized $CD4^+$ T cells acquire IL-33 responsiveness after their development into T_h2, which express IL-33Ra chain and produce IL-5 and IL-13 but not IL-4 in response to IL-33 (Fig. 4B and C). Thus, freshly prepared $CD4^+$ T cells and T_h2 showed completely different responsive pattern to IL-18 and IL-33.

In this report, we have also demonstrated that intra-peritoneal injection of IL-33 induces airway goblet cell hyperplasia even in RAG-2 $^{-/-}$ mice (Fig. 5B), lacking both T cells and B cells. In addition, daily peritoneal or intra-nasal administration of IL-33 into IL-13 $^{-/-}$ mice did not induce goblet cell hyperplasia, revealing that IL-33-induced goblet cell hyperplasia is entirely dependent on the action of endogenous IL-13 derived from acquired immune cells and/or innate immune cells. Furthermore, we demonstrated that intra-nasal

administration of IL-33 into RAG-2 $^{/}$ mice induces AHR and airway inflammation in the absence of acquired immune system (Fig. 6A and B), clearly indicating that IL-33 has potential to induce AHR without help from CD4^{+} T cells. The evidence, that RAG-2 $^{/}$ mice developed severer AHR than wild-type mice following intra-nasal administration of IL-33 (Fig. 6A), might suggest contribution of regulatory effect of T cells onto IL-33-induced AHR. Thus, intra-nasal administration of IL-33 induces asthma-like symptom in the absence of acquired immune system, contrasting to IL-18-induced AHR in which CD4^{+} T cells play a critical role (30).

In this study, we first compared the biological effects of IL-18 and IL-33 on basophils, mast cells, non-polarized or T_h2-polarized CD4^{+} T cells in vitro. As shown in Fig. 1(F), responsiveness of basophils to IL-18 or IL-33 is much higher than that of mast cells to IL-18 or IL-33, respectively. Since there are more IL-18Rα^{+} cells (14.2%) in basophils comparing with that (2.0%) in mast cells (Fig. 1C), we can speculate that basophils are more responsive to IL-18, and we have shown it is the case (Fig. 1F). In spite of the fact that there are more IL-33Rα^{+} cells (95.6%) in mast cells than that (34.6%) in basophils (Fig. 1D), only basophils strongly produce IL-4, IL-6 and IL-13 (Fig. 1F). To understand this discrepancy, we simultaneously examined the level of IL-33Rb-mRNA expression in basophils and found that they have higher level expression of this message, possibly allowing basophils to be highly responsive to IL-33.

We also demonstrated that IL-33 induced IL-13 production in vitro (Fig. 3) and goblet cell hyperplasia in vivo (Fig. 5A) in an ST2- and MyD88-dependent manner. We have examined the mechanism underlying IL-33-induced goblet cell hyperplasia in the lungs. As shown in Fig. 5(A), IL-33 induced goblet cell hyperplasia in the lungs in an endogenous IL-13-dependent manner. Indeed, this treatment promptly induced IL-13-mRNA in the lungs (Fig. 6C). IL-18 or IL-33 with IL-3 strongly induces IL-4 and IL-13 production from basophils (Figs 1 and 2). Mast cells also produce a considerable amount of IL-13 in response to IL-33 in vitro (Figs 1F and 2). These results strongly suggest that both basophils and mast cells might become important IL-13-producing innate immune cells in IL-33-treated mice.

Since T_h2 also produce IL-13 in response to IL-33 in vitro (Fig. 4B), we examined the capacity of CD4^{+} T cell-depleted WBB6F1-W/W^{v} mice, having basophils but lacking mast cells and T_h2, to develop goblet cell hyperplasia following administration of IL-33. We found that they normally developed goblet cell hyperplasia, suggesting that innate immune cells other than mast cells produce IL-13 in response to IL-33. Most surprisingly, intra-peritoneal injection of IL-33 into RAG-2 $^{/}$ mice induces goblet cell hyperplasia in the lungs (Fig. 5B). Furthermore, intra-nasal administration of IL-33 induces goblet cell hyperplasia (Fig. 5D), AHR (Fig. 6A) and eosinophilic infiltration (Fig. 6B) even in the lungs of RAG-2 $^{/}$ mice, suggesting that administration of IL-33 induces asthma-like phenotype entirely independent of acquired immune system.

Next, we wished to determine the nature of innate immune cells that produce IL-13 in response to IL-33 in RAG-2 $^{/}$ mice. We found that there are a substantial number of basophils in the spleen and peripheral blood of RAG-2 $^{/}$ mice (data not shown). Thus, we depleted basophils in RAG-2 $^{/}$ mice by injection of MAR-1, mAb against FcεR1 (20). We found that this treatment very efficiently depleted basophils in RAG-2 $^{/}$ mice (data not shown). However, administration of IL-33 normally induced IL-13-mRNA expression in the spleen of basophil-depleted RAG-2 $^{/}$ mice, suggesting that there are still other types of innate immune cells that produce IL-13 in response to IL-33 in vivo. We found that administration of IL-33 did not induce AHR and goblet cell hyperplasia in cc $^{/}$ RAG-2 $^{/}$ mice, lacking T cells, B cells and NK cells (data not shown). But, we could detect a substantial number of basophils in their spleen and peripheral blood (data not shown), suggesting the possibility that their basophils might be functionally unresponsive to IL-33. Examination of the capacity of bone marrow-derived basophils from cc $^{/}$ RAG-2 $^{/}$ mice to produce IL-13 in response to IL-3 plus IL-33 in vitro is eagerly needed.

Thus, at present time, we could not determine what types of innate immune cells become IL-13-producing cells in response to IL-33 in vivo. In another word, the absence of one type of innate immune cell or acquired immune cell does not affect IL-33-induced goblet cell hyperplasia.

Basophils are unique IL-4-producing cells, characterized by their striking capacity to produce IL-4 and IL-13 in response to IL-18 (2), IL-33 or allergens with enzymatic activity (20), and are important effector cells in allergic inflammation. Helminth infection induces an increase in the number of basophils in the spleen and liver (31), suggesting their role in induction or augmentation of T_h2 response. Very recent study further suggests their involvement in induction of T_h2 by its unique function to produce IL-4 and TSLP in response to cysteine proteases papain and bromelain (20). It is well known that allergic inflammatory responses are often enhanced by some infectious agents, such as parasite, bacteria or virus (32–34). If IL-18 and/or IL-33 are produced by epithelial cells in the respiratory tract or gastrointestinal tract, these cytokines either collaboratively or separately stimulate basophils to produce T_h2 cytokines and TSLP, resulting in induction of T_h2, mastocytosis and goblet cell hyperplasia in those organs. As IL-33 induces endogenous IL-13 even in the absence of adaptive immune cells, local induction of IL-33 may induce T cell/B cell-independent allergic inflammation, which we would like to call innate immune cell-dependent type 2 responses.

In summary, only basophils produce IL-4 and IL-13 in response to IL-18 or IL-33 with IL-3. In spite of outstanding expression of IL-33Rα chain by mast cells, they only produce IL-13 when stimulated with IL-3 and IL-33. Although we need further study, IL-33-stimulated innate immune cells including basophils and mast cells might be important for induction of type 2 responses by production of IL-4 and IL-13 in the absence of allergen and IgE.

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Abbreviations

AHR	airway hyperresponsiveness
BAL	bronchoalveolar lavage

BALF	bronchoalveolar lavage fluid
GM-CSF	granulocyte macrophage colony-stimulating factor
Mch	methacholine
OVA	ovalbumin peptide
SPF	specific pathogen free
ST2 $^{-/-}$	ST2 deficient
TSLP	thymic stromal lymphopoietin

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Association of serum interleukin-33 level and the interleukin-33 genetic variant with Japanese cedar pollinosis

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Summary

Background IL-33, an IL-1-like cytokine, is a ligand for ILI RLI, which is an important effector molecule of type 2 T helper responses. Although IL-33/ILI RLI interaction has been suggested to be important in induction of allergic airway inflammation, serum levels of IL-33 and the genetic influences of the polymorphisms of IL-33 in human allergic diseases are unclear.

Objective The aim of this study was to examine whether the serum IL-33 level and polymorphisms in IL-33 are associated with Japanese cedar (JC) pollinosis, the most common form of allergic rhinitis, and a major public health problem, in Japan.

Methods We performed linkage disequilibrium (LD) mapping of the gene using the HapMap database, and two selected tag single nucleotide polymorphisms were genotyped. We conducted an association study of IL-33 (JC pollinosis, n = 170; normal controls, n = 100) and measured the IL-33 levels in sera of the 270 subjects by ELISA.

Results Serum levels of IL-33 were significantly higher in patients with JC pollinosis ($P = 0.0018$) than in controls. In genetic association analysis, we found a positive association between the polymorphism and JC pollinosis ($P = 0.048$).

Conclusion Our results support a role for IL-33 in the pathogenesis of JC pollinosis.

Keywords association, IL-33, JC pollinosis, polymorphism, serum level
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Introduction

Allergic diseases are increasing world-wide, and Japanese cedar (JC) pollinosis, which is a disease of allergic rhinitis and allergic conjunctivitis caused by exposure to JC pollen, is one of the most common allergic diseases in Japan [1]. The increase of JC pollinosis in Japan has become a social problem, with a prevalence of 4·16% [1]. Allergic rhinitis is the result of an inflammatory reaction triggered by type 2 T helper (Th2) cell-mediated immune responses against allergens [2]. Th2 cytokines induce B cells to produce high amounts of IgG4 and IgE in humans, and promote the growth and differentiation of mast cells and eosinophils [3, 4]. ILI RLI belongs to the IL-1 receptor family and functions as an important effector molecule of Th2 responses [5–8]. IL-33, an IL-1-like cytokine, has been identified as a ligand for ILI RLI, and can be detected in epithelial cells from the bronchi and small airways, which indicates a possible role in the regulation of mucosal function [9]. It activates NF-kB and mitogen-activated protein kinases, and drives production of Th2-associated cytokines from in vitro polarized Th2 cells via ILI RLI [9]. In vivo analysis has demonstrated that IL-33 strongly induces gene expression of Th2-associated cytokines such as IL-4, IL-5 and IL-13, and IL-33-treated mice have significantly higher serum levels of IgE. In addition, IL-33 induces pathological changes in mucosal organs such as the lung, resulting in hypertrophied epithelial lining of the airways with large amount of mucus,

and those changes are primarily restricted to the bronchi and larger bronchioles [9].

Although very little work has been done with IL-33, intensive studies of IL-33 receptor ILI RLI have shown its regulatory functions in the development and effector phases of Th2 responses [10]. The ILI RLI gene encodes a soluble-secreted protein, ILI RLI, and a transmembrane protein, ST2L [11]. In murine models of allergic airway inflammation, increases in endogenous ILI RLI protein after allergen exposure modulate Th2-mediated airway inflammation [12], and blockade of the binding of the ligand for ST2L using a recombinant IgG fusion protein inhibits allergic inflammation [6, 7]. Other studies have reported that ST2L is a reliable selective marker of both murine and human Th2 lymphocytes in allergic airway inflammation [7, 13]. Moreover, a study has revealed that soluble ILI RLI acts as a negative regulator of Th2 cytokine production via IL-33 signalling in allergic airway inflammation. In asthmatic patients, serum levels of soluble ILI RLI are markedly elevated during acute attacks and the magnitude of the elevation correlates with the reduction of pulmonary functions and increased levels of serum IL-5 [14]. These findings imply that IL-33 is a good candidate for involvement in JC pollinosis, an allergen-induced upper airway inflammation.

A large number of association studies using polymorphic markers have been performed to discover genetic components in the pathogenesis of allergic diseases [15–17]. Recently, we have reported that functional single nucleotide polymorphisms (SNPs) in the ILI RLI distal promoter region are associated with atopic dermatitis. The genetic variants regulate ILI RLI expression, and immunohistochemical staining of a skin biopsy specimen from an atopic dermatitis patient showed ILI RLI staining in keratinocytes as well as in cells infiltrating the dermal layer [18]. However, there have been no genetic association studies with IL-33.

In this study, to test whether genetic variations of IL-33 contribute to susceptibility to JC pollinosis, we first selected a genetic polymorphism of IL-33 using HapMap linkage disequilibrium (LD) data and conducted association studies. In addition, we examined the associations between serum IL-33 levels and JC pollinosis and serum total IgE levels.

Methods

Study subjects

All subjects were recruited from residents of Eiheiji-cho, in Fukui prefecture, in the central area of Japan between May and June 2006. Because these participants were workers of the Fukui University hospital and students of nursing and medical colleges in Fukui, the number of females was higher than that of males. Specific IgE to seven aeroallergens, Cryptomeria japonica, Dermatophagoides pteronyssinus, Dermatophagoides farinae, Candida albicans, Aspergillus fumigatus, Dactylis glomerata and Ambrosia, were measured with a Pharmacia CAP System (Pharmacia CAP, Uppsala, Sweden) (Table 1). Positive sensitization refers to an allergen-specific serum IgE level ≥0.7 (CAP RAST score of 2). Diagnosis of JC pollinosis was confirmed by symptoms of allergic rhinoconjunctivitis during the JC pollinosis season and positive serum-specific IgE towards JC pollinosis. A total of 170 patients with JC pollinosis were recruited (Table 1). One hundred healthy subjects who had never had symptoms of allergic rhinitis and showed no sensitization to any of the seven aeroallergens were recruited as controls (Table 1). We recruited 29 subjects with infectious rhinitis who were diagnosed by otolaryngologists and showed no sensitization to any of the seven aeroallergens. All individuals were unrelated Japanese and gave written informed consent to participate in the study according to the rules of the process committees at the School of Medicine, University of Fukui, the Nippon Medical School and The Institute of Physical and Chemical Research.

Selection of polymorphisms for genotyping

Genomic DNA was prepared from peripheral blood samples, using standard protocols. There were 22 SNPs in the IL-33 gene with a minor allele frequency (MAF) of ≥10% in the HapMap Japanese data set (URL: http://www.hapmap.org/index.html.en) (Table 2). Pairwise LD was calculated as r^2 by using the Haploview 3.2 program (http://www.broad.mit.edu/mpg/haploview/). Genotyping of SNPs was performed by the TaqMan™ allele-specific amplification (TaqMan-ASA) method (Applied Biosystems, Foster City, CA, USA). rs1929992 was genotyped by Custom TaqMan® SNP Genotyping Assay Service with primers 5′-GGAAAAAAACACATTTTCCCCCCAA-3′ and 5′-AAACCATCTTAACTACTACTTAAAATGTATAAAGTGT TAGAATTAT-3′. The probes used were VIC-TCATGGT CAAAATATTGAAAT and FAM-ATGGTCAAAATGTTGAA AT. rs10975519 was genotyped by TaqMan(R) Pre-Designed SNP Genotyping Assays, C___2762153_10.

Reagents for human interleukin-33

Recombinant human IL-33 (rhIL-33) and a rabbit-neutralizing anti-hIL-33 IgG antibody were made by Hokudo Co., Ltd. (Sapporo, Japan). Briefly, rhIL-33 (mature form) was amplified from human lung cDNA (BioChain Institute, Hayward, CA, USA) as a template, and subcloned into pET28a vector (Novagen, Madison, WI, USA). BL21 (DE3) RIL was transformed and the expressed recombinant protein was purified with Ni-NTA resin. Endotoxin was removed by filtration through Zetapor (Cuno, Meriden, CT, USA). For establishment of a polyclonal antibody to hIL-33, rabbits

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were immunized with rhIL-33 (200 mg/body) with CFA, and boosted with rhIL-33 (200 mg/body) with IFA three times every 2 weeks. Seven weeks later, serum was collected and the antibody was purified using a Protein-A sepharose column. This IgG antibody (R2) was further purified with an rhIL-33 sepharose column and was biotinylated with NHS-biotin (Sigma, St Louis, MO, USA) in our laboratory. This purified anti-hIL-33 antibody could completely neutralize 50 ng/mL of IL-33 at the concentration of 10 mg/mL in vitro.

Table 1. Characteristics of the patients with Japanese cedar (JC) pollinosis and controls

Characteristics	Case	Control
Age (year, median with range)	30 (20–49)	32.5 (20–49)
Total subjects and sex (% male subjects)	170 (14)	100 (9.0)
Serum total IgE (IU/mL, mean SEM)	280.2 879.2	42.9 51.5
Atopic sensitization (RAST) (number (%))		
Japanese cedar pollen positive	170 (100)	0 (0)
Dermatophagoides pteronyssinus positive	80 (47.1)	0 (0)
Dermatophagoides farinae positive	78 (45.9)	0 (0)
Candida albicans positive	10 (5.9)	0 (0)
Aspergillus positive	3 (1.8)	0 (0)
Dactylis glomerata positive	61 (35.9)	0 (0)
Ambrosia positive	23 (13.5)	0 (0)

Enzyme-linked immunosorbent assay of serum levels of interleulun-33

To elucidate the biological roles of the IL-33 gene, we constructed an ELISA system to quantify human IL-33 protein in sera of subjects with JC pollinosis and controls. A 96-well plate (Costar, Cambridge, MA, USA) was coated with the anti-hIL-33 IgG antibody (R2) and blocked with StartingBlock™ blocking buffer (PIERCE, Rockford, IL, USA). Human IL-33 was detected with the biotinylated-anti-IL-33 antibody and streptavidin-HRP. The ELISA system was specific for hIL-33 and did not cross-react with other cytokines tested, which included IL-1b, IL-2, IL-4, IL-12, IL-18, TNF-a, IFN-g and GM-CSF. Serum samples were collected, and then they were stored at 80°C until measurement. IL-33 was assayed by ELISA with reference standard curves using known amounts of hIL-33. The lower limit of ELISA sensitivity for serum IL-33 was 30 pg/mL. A value of 0 was assigned to results that were below the assay's lower limit of detection for non-parametric statistical calculations in Fig. 2.

Statistical analysis

We calculated allele frequencies and tested agreement with Hardy–Weinberg equilibrium using a w^2 goodness-of-fit

Table 2. Locations and allele frequencies of polymorphisms in IL-33 based on the HapMap JPT data set

SNP	Location	Amino acid	MAF (%)[z]	NCBI[y]
5345 G/A	5′-Flanking region		0.477	rs9284 14
5194 T/G	5′-Flanking region		0.477	rs4237164
4432 G/A	5′-Flanking region		0.477	rs10975509
1611 C/T	5′-Flanking region		0.466	rs7025417
1037 T/C	Intron 1		0.467	rs10975511
1256 C/T	Intron 1		0.455	rs4742170
2241 C/G	Intron 1		0.455	rs7019575
4450 G/A	Intron 1		0.455	rs10975514
5999 G/A	Intron 1		0.443	rs10975516
9318 C/A	Intron 2		0.443	rs1317230
9813 G/T	Intron 3		0.455	rs1330383
9894 T/C[w]	Intron 3		0.455	rs1929992
11607 T/C	Intron 4		0.432	rs1113573
11877 C/T[w]	Exon 5	Tyr163Tyr	0.433	rs10975519
12016 G/C	Intron 5		0.422	rs10975520
12514 T/C	Intron 5		0.427	rs7044343
13206 A/G	Intron 6		0.487	rs7871381
13316 C/A	Intron 6		0.371	rs1412421
13625 G/A	Intron 6		0.422	rs7047921
14187 G/T	Intron 6		0.420	rs1332290
14598 G/A	Exon 7	3′-UTR	0.409	rs1048274
23562 G/C	3′-Flanking region		0.455	rs10815397

Numbering according to the genomic sequence of IL-33 (AL353741.16) and position 1 is the A of the initiation codon. Major allele/minor allele.

[w]SNPs were genotyped in this study.

[z]Minor allele frequencies

[y]NCBI, number from the dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/SNP/).

SNP, single nucleotide polymorphisms; MAF, minor allele frequency.

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test at each locus. We then compared differences in allele frequencies and genotype distribution of the polymorphism between case and control subjects by using a 2 2 contingency w^2 test with one degree of freedom, and calculated odds ratios (ORs) with 95% confidence intervals (CIs). Serum total IgE and IL-33 levels were analysed as quantitative levels, and we investigated associations between these levels and genetic variations. Log-transformed individual serum IgE levels were analysed by one-way ANOVA. When the data for IL-33 levels were not distributed normally after log-transformation, they were analysed using non-parametric equivalents and summarized using the median. Multiple comparisons were first analysed by the Kruskal–Wallis test and then by individual testing by the Mann–Whitney U-test if significant. Correlations were analysed by Spearman's test. A P value of less than 0.05 was considered statistically significant.

Results

Linkage disequilibrium of the IL-33 gene

A total of 22 polymorphisms with a frequency 40.10 in IL-33 were contained in the public databases available at the NCBI dbSNP website (http://www.ncbi.nlm.nih.gov/SNP/) (Table 2). Two variants including a synonymous substitution (Tyr163Tyr) were in the exons, and four variants were in the 5⁰-flanking region of the IL-33 gene. Pairwise LD among the 22 SNPs was measured by different parameters, r^2 using the Haploview 3.2 program (http://www.broad.mit.edu/mpg/haploview/) (Fig. 1), and all the 22 SNPs were in strong LD (r^2 40.75). We finally selected polymorphism rs1929992 and rs10975519 (Tyr163Tyr) for association studies using tagger in the Haploview 3.2 program, and these two SNPs captured 22 of 22 alleles with a mean r^2 of 0.95 (r^2 40.91).

Association between polymorphisms in the IL-33 gene and susceptibility of Japanese cedar pollinosis

The locus was in Hardy–Weinberg equilibrium in the entire group. To test the association between the SNP and JC pollinosis, we compared differences in the allele frequency and genotype distribution of each polymorphism between case and control subjects by using contingency chi-square tests with one degree of freedom. ORs with 95% CIs were also calculated. In the population genotyped in this study, the MAF of rs1929992 (C=0.49) was higher than those in the HapMap JPT data set (C=0.46). We found a significant association between rs1929992 (T4C) and JC pollinosis (TT1 TC vs. CC: OR, 1.82; 95% CI, 1.00–3.31; P=0.048) (Table 3). The serum total IgE level was analysed as a quantitative level, and we investigated the association between this level and genetic variation. However, we could not find any association between the SNP and serum IgE level in this study (P=0.46 by ANOVA).

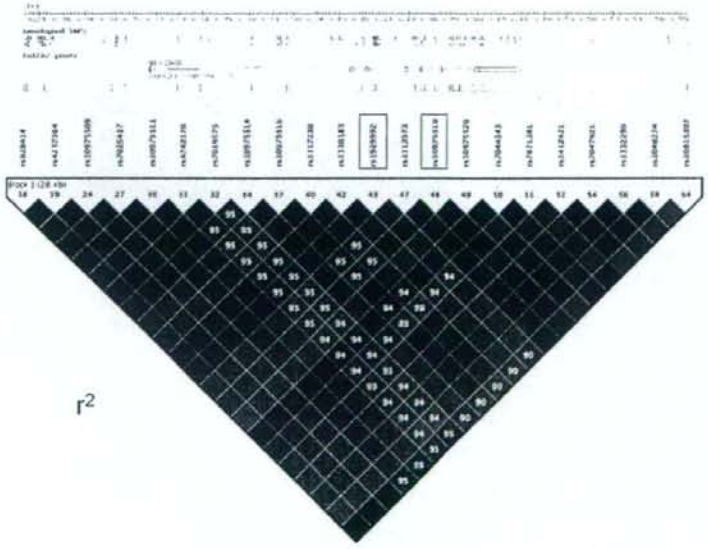

Fig. 1. Pairwise linkage disequilibrium between 22 SNPs as measured by r^2 estimated by the Haploview 3.2 program using the HapMap JPT data set. The boxed polymorphisms, rs1929992 and rs10975519, were genotyped in this study.

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Patients with Japanese cedar pollinosis display higher interleukin-33 serum levels than healthy control subjects

To evaluate whether dysregulation at the IL-33 protein level might be a characteristic feature of JC pollinosis, we conducted ELISA assays of sera of patients with JC pollinosis (n = 170) and healthy control subjects (n = 100). Patients with JC pollinosis exhibited significantly higher serum levels of the IL-33 protein (P = 0.0018) (Fig. 2). The median serum IL-33 concentration of JC pollinosis patients was 549 pg/mL, compared with 361.8 pg/mL for controls. In addition, we examined the serum IL-33 level in infectious rhinitis as non-allergic rhinitis. The median serum IL-33 concentration of subjects with infectious rhinitis was 241.3 pg/mL. There was no significant difference of the serum IL-33 level between healthy control subjects and those with infectious rhinitis. Although total serum IgE and IL-33 levels were analysed as quantitative phenotypes, there was no significant association between the total serum IgE level and serum IL-33 level (P = 0.095 by Spearman's test). We also examined whether the IL-33 genotype affected the serum level of IL-33, but we could not find any significant association between the genotype and serum IL-33 level (P = 0.58 by the Kruskal–Wallis test).

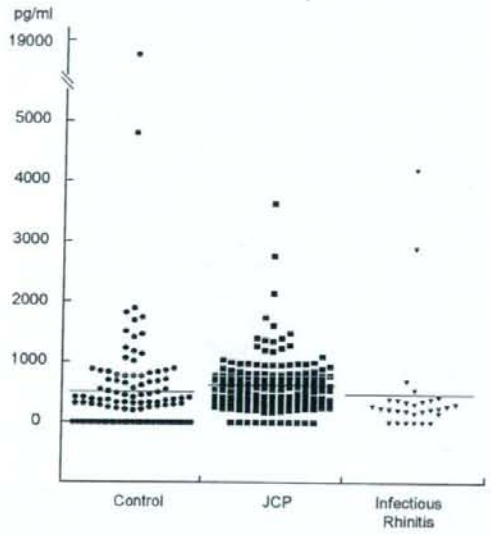

Fig. 2. Comparison of serum IL-33 levels among controls, patients with JC pollinosis and those with infectious rhinitis. Horizontal bars indicate the median value of each group. JCP, Japanese Cedar pollinosis.

Discussion

To determine the role of the IL-33 gene in the pathogenesis of JC pollinosis, we conducted an association study using the sequence variation of the IL-33 gene and compared serum IL-33 levels between subjects with JC pollinosis and controls. We found a significant association between JC pollinosis susceptibility and IL-33 polymorphism and higher serum IL-33 levels in subjects with JC pollinosis. Although IL-33 has been thought to play an important role in allergic diseases, this is the first study providing evidence for its involvement in such a disease. We consider the results to be hypothesis generating as the findings in this study need to be confirmed in another population with a larger size.

Recent studies have reported important roles of non-lymphoid cell-derived cytokines such as IL-33 and TSLP in the induction of Th2 differentiation [9, 19]. IL-33 is highly expressed in normal human bronchial epithelial cells and airway smooth muscle cells [9]. It induces Th2-type responses and Th2-associated cytokines IL-4, IL-5 and IL-13 by signalling through IL1RL1 [9, 20]. A recent study has shown that IL-33 induces IL-13 production by mast cells independently of IgE–FcεRI signals in mice. These findings suggest important roles for IL-33 in mast cell- and Th2 cytokine-associated immune disorders [21].

Table 3. Association between polymorphisms of IL-33 and Japanese cedar (JC) pollinosis

Genotype	Cases (n = 170)	Controls (n = 100)	Allele	Cases (n = 170)	Controls (n = 100)	Genotype P	Dominant P	Recessive* P	Allelic† P
rs1929992									
TT	44 (26.0)	32 (32.3)	T	162 (47.9)	112 (56.6)	0.13	0.27	0.048	0.053
TC	74 (43.8)	48 (48.4)	C	176 (52.1)	86 (43.4)				
CC	51 (30.2)	19 (19.2)							
rs10975519									
CC	52 (30.6)	36 (36.0)	C	177 (52.1)	119 (59.5)	0.20	0.36	0.074	0.093
CT	73 (42.9)	47 (47.0)	T	163 (47.9)	81 (40.5)				
TT	45 (26.5)	17 (17.0)							

Dominant model (TT vs. CC1 TC in rs1929992, CC vs. CT1 TT in rs10975519).
*Recessive model (TT1 TC vs. CC in rs1929992, CC1 CT vs. TT in rs10975519).
†Allelic model (T allele vs. C allele in rs1929992, C allele vs. T allele in rs10975519).

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Structurally, IL-33 is related to IL-18, and intensive studies of the relationship between allergic inflammation and IL-18 have been conducted. IL-18 was the first cytokine demonstrated to activate T cells to produce abundant IFN-g without T cell receptor (TCR) engagement [22]. Furthermore, genetic association studies of the IL-18 gene have provided evidence for an association with atopic diseases [23–26]. Verhaeghe et al. reported the up-regulation of IL-18 in nasal secretions in allergic rhinitis and the persistence of elevated IL-18 concentrations until after the season [27]. Increased IL-33 concentrations were observed in subjects with JC pollinosis in the present study; however, there was no significant difference in the serum IL-33 level between controls and subjects with infectious rhinitis. Up-regulation of the IL-33 level appears to be characteristic of JC pollinosis. Further analyses of the involvement and interactions of those structurally similar cytokines in allergic inflammation should also be conducted.

Recent reports have shown that IL1RL1 is a reliable marker of Th2 lymphocytes in allergic airway inflammation [7, 13, 28]. Elevated levels of the soluble form of IL1RL1 in the circulation of patients with asthma with acute exacerbation have been reported [14]. The study has also shown that a differential rise of serum IL1RL1 level that correlates well with the severity of asthma exacerbation [14]. In a murine model of allergic airway inflammation, serum murine (m) IL1RL1 protein levels increased after allergen exposure, and pre-treatment with soluble mIL1RL1 protein significantly inhibited the Th2 cytokine production [12]. Other studies have shown that administration of either a monoclonal antibody against IL1RL1 or a recombinant IL1RL1 fusion protein attenuates eosinophilic inflammation of the airways and suppresses IL-4 and IL-5 production in vivo following adoptive transfer of Th2 cells [6, 7]. These findings suggest that blocking IL1RL1 pathways would be therapeutically efficacious as a new treatment for allergic diseases, and expression of soluble IL1RL1 could serve as a physiological mechanism to down-regulate Th2-driven immunopathology [10]. In this study, we did not measure the serum soluble IL1RL1 levels, and further examination of the relationship between serum IL-33 and soluble IL1RL1 is needed to clarify their functions in Th2 inflammation. The genetic factors of the IL-33 gene or serum IL-33 level might provide valuable information for selecting appropriate therapeutic options.

We showed here a significant association between susceptibility to JC pollinosis and a polymorphism. In this study, we selected polymorphisms using HapMap information, and did not examine the functional effects of polymorphisms in strong LD with the related variant. Previous studies have shown that polymorphisms in exons often contribute to their transcript stability [29, 30]. Variants rs10975519 (Tyr163Tyr) and rs1048274 in the exon might affect the expression level or mRNA stability of the IL-33 gene. In addition, four genetic variations were in the 5^0-flanking region, which is often involved in transcriptional regulation of the gene. Several transcription factors are involved in asthmatic inflammation, including NF-kB, activator protein-1 (AP-1), nuclear factor of activated T cells (NF-AT), cyclic AMP response element-binding protein (CREB) and signal transduction-activated transcription factors (STAT) [31]. Using the TRASFAC system, we surveyed whether SNPs in the 5^0 region of the IL-33 gene create transcription factor binding sites. However, we could not find any SNP that changed the affinity of those transcription factors. The functions of these linked polymorphisms remain to be elucidated. Demonstrating the alteration of gene functions as the result of polymorphisms is necessary to further validate the involvement of the IL-33 gene in the pathogenesis of JC pollinosis. Furthermore, there were gender differences in the population in this study, and several studies have suggested that sex affects the asthma phenotype, possibly via hormone-related events [32, 33]. If there is a sex-related difference in the association of IL-33 with JC pollinosis, looking at females only might be informative.

Our data strongly support the important role of IL-33 in JC pollinosis. Further investigation of the connections between genotypes and the functional role of IL-33 during allergic events may provide additional targets for therapeutic interventions and would be helpful to clarify the aetiology of allergic diseases.

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Change of subunit composition of mitochondrial complex II (succinate–ubiquinone reductase/quinol–fumarate reductase) in *Ascaris suum* during the migration in the experimental host

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Abstract

The mitochondrial metabolic pathway of the parasitic nematode *Ascaris suum* changes dramatically during its life cycle, to adapt to changes in the environmental oxygen concentration. We previously showed that *A. suum* mitochondria express stage-specific isoforms of complex II (succinate–ubiquinone reductase: SQR/quinol–fumarate reductase: QFR). The flavoprotein (Fp) and small subunit of cytochrome *b* (CybS) in adult complex II differ from those of infective third stage larval (L3) complex II. However, there is no difference in the iron–sulfur cluster (Ip) or the large subunit of cytochrome *b* (CybL) between adult and L3 isoforms of complex II. In the present study, to clarify the changes that occur in the respiratory chain of *A. suum* larvae during their migration in the host, we examined enzymatic activity, quinone content and complex II subunit composition in mitochondria of lung stage L3 (LL3) *A. suum* larvae. LL3 mitochondria showed higher QFR activity (~160 nmol/min/mg) than mitochondria of *A. suum* at other stages (L3: ~80 nmol/min/mg; adult: ~70 nmol/min/mg). Ubiquinone content in LL3 mitochondria was more abundant than rhodoquinone (~1.8 nmol/mg versus ~0.9 nmol/mg). Interestingly, the results of two-dimensional blue-native/sodium dodecyl sulfate polyacrylamide gel electrophoresis analyses showed that LL3 mitochondria contained larval Fp (Fp^L) and adult Fp (Fp^A) at a ratio of 1:0.56, and that most LL3 CybS subunits were of the adult form ($CybS^A$). This clearly indicates that the rearrangement of complex II begins with a change in the isoform of the anchor CybS subunit, followed by a similar change in the Fp subunit.
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Keywords: *Ascaris suum* lung-stage L3 (LL3); Complex II; Quinone; NADH–fumarate reductase; Quinol–fumarate reductase (QFR); Oxidative stress

1. Introduction

During the life cycle of the parasitic nematode *Ascaris suum*, it transitions from aerobic to anaerobic metabolism in parallel with changes in the environmental oxygen concentration (Fig. 1) [1–6]. In aerobic metabolism, which is used by *A. suum* larvae during their development from fertilized egg to third stage larvae (L3), phosphoenolpyruvate (PEP) is converted to pyruvate by pyruvate kinase, and pyruvate is converted to CO_2 and H_2O via the tricarboxylic acid (TCA) cycle, generating a large amount of ATP by aerobic oxidative phosphorylation [7]. Adult *A. suum* worms, which live in a low-oxygen environment, use the anaerobic phosphoenolpyruvate carboxykinase (PEPCK)-

Abbreviations: Fp, flavoprotein subunit; Ip, iron–sulfur cluster subunit; CybL, large subunit of cytochrome *b*; CybS, small subunit of cytochrome *b*; Fp^L, larval Fp; Fp^A, adult Fp; $CybS^L$, larval CybS; $CybS^A$, adult CybS; L3, third stage larva; LL3, lung stage L3; SDH, succinate dehydrogenase; SQR, succinate–ubiquinone reductase; QFR, quinol–fumarate reductase; UQ, ubiquinone; dUQ, decyl UQ; RQ, rhodoquinone; dRQ, decyl RQ; HPLC, high performance liquid chromatography; BN-PAGE, blue-native polyacrylamide gel electrophoresis; SDS-PAGE, sodium dodecyl sulfate-PAGE; CBB, Coomassie brilliant blue.

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