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 Th2-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-1α, MIP-1β 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-33Rβ-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 Th2-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 Th2 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 Th2 cytokines/IgE responses without help from antigen. It is well-known evidence that basophils and mast cells produce Th2 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-1β or IL-18, closely related IL-33 is also synthesized as a 31-kDa precursor form and becomes active after cleavage with caspase-1
IL-33 induces type 2 response without T cell help

IL-33 is an important cytokine that augments Th2 response. Enhancing their IL-4 production, suggesting that IL-33 is an important cytokine that augments Th2 response. Enhancing their IL-4 production, suggesting that IL-33 is an important cytokine that augments Th2 response. Enhancing their IL-4 production, suggesting that IL-33 is an important cytokine that augments Th2 response.

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 (D011.10) 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/Wv 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 FcRRI (MAR-1), streptavidin–APC and streptavidin–FITC were purchased from eBioscience (San Diego, CA, USA). Anti-mouse IL-1Rα 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 FcγRI 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 FcγRI/c-Kit+ cells (basophils) and FcγRI/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 (105/0.2 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-1α, IL-1β, IL-2, 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-1α, MIP-1β, 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 (100 ng ml⁻¹) and IL-33 (100 ng ml⁻¹) for 4 days. For generation of Tn2, sorted splenic CD4⁺CD62L⁺ T cells (1 x 10⁵ ml⁻¹) from DO11.10 mice were stimulated with IL-4 (1000 U ml⁻¹), anti-IL-12p40 (20 µg ml⁻¹), anti-IFN-γ (20 µg ml⁻¹), IL-2 (100 pM) and OVA₁₃₂-₁₃₉ (1 µM) in the presence of irradiated T cell-depleted BALB/c splenocytes (1 x 10⁶ ml⁻¹) in 24-well plate in a total 1-ml volume of medium for 7 days as described previously (25). Polarized Tn2 (1 x 10⁵/0.2 ml per well) were re-cultured with IL-2 (100 pM) and OVA₁₃₂-₁₃₉ (1 µM) and irradiated T cell-depleted BALB/c splenocytes (1 x 10⁵/0.2 ml per well) in the presence of IL-18 (50 ng ml⁻¹) or IL-33 (100 ng ml⁻¹) for 48 h. In the absence of culture, supernatants were harvested and tested for IL-4 and IL-13 contents by ELISA.

In vivo treatment of mice

Mice were daily injected intra-peritonally with PBS alone or with IL-33 (4 µg day⁻¹) for 4 days. In some experiments, mice were daily exposed intra-nasally to IL-33 (1 µg day⁻¹) in 50 µl 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/Wv 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) 3 days before IL-33 treatment) with mAb to CD4 (clone, RM4-5). The enriched CD4⁺ T cells from DO11.10 mice were purified by MicroBeads (anti-mouse CD4; clone RM4-5). The purity of sorted cells was >98.5% after re-analysis.

Measurement of AHR

We measured AHR to β-methacholine (Mch) inhalation in mice by using Pulmos-1 (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 β-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-µm 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-18Rα chain and ST2 (IL-33Rα chain), sorted basophils and mast cells were further incubated with rat anti-mouse IL-18Rα 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-33Rα chain on T cells, freshly isolated splenic CD4⁺ T cells and polarized Tn2 were first treated with 10 µg ml⁻¹ anti-FcγRII/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 µg ml⁻¹ anti-FcγRII/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 gene was quantified by real-time PCR with TaqMan H⁻ Reverse Transcriptase (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-33Rα chain (assay ID: Mm01233982_m1), IL-33Rβ 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 Tn2 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 FcRIIα/c-Kit+ cells and FcRIIα/c-Kit⁻ cells (1), and then highly purified both populations by FACs (Fig. 1A). Light and electron microscopical examination revealed that resultant FcRIIα/c-Kit+ cells and FcRIIα/c-Kit⁻ cells are basophils and mast cells, respectively (Fig. 1B). Next, we examined the proportions of cells positive for IL-18Rα chain or IL-33Rα chain in each population (Fig. 1C and D). Basophils were composed of IL-18Rα+ cells.
IL-33Rα+ 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-33Rβ-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-33Rβ 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-γ and GM-CSF) and chemokines (RANTES, MIP-1α, MIP-1β and MCP-1) in response to IL-18 and/or IL-33 (Fig. 2). Basophils dose dependently 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-γ. 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
chemokines (RANTES and MCP-1), although productions of other chemokines (MIP-1α and MIP-1β) 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 Th2 cytokines via MyD88**

We wished to determine the pathway involved in IL-18- or IL-33-induced production of Th2 cytokines by basophils or mast cells in vitro. Since basophils are good producers of Th2 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 to IL-18 and/or IL-33. 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 Th2 cytokines in response to IL-33 (Fig. 3), revealing that IL-33 stimulates basophils in an ST2/MyD88-dependent manner. 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 Th2 cytokines via respective receptor and that MyD88 is an essential adapter molecule required for IL-18R or IL-33Rα chain-mediated Th2 cytokine production from basophils.

**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 10^6/0.2 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.
IL-33 induces T\(_\text{h}2\) cytokine production from basophils and T\(_\text{h}2\) 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, and on in vitro Th2-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\(_\text{h}2\) cytokines. Therefore, non-polarized CD4\(^+\) T cells showed completely different responsiveness to IL-18 or IL-33. However, non-polarized CD4\(^+\) T cells became responsive to IL-33 after their development into T\(_\text{h}2\) (Fig. 4B), which express IL-33R\(\alpha\) (Fig. 4C). Indeed, IL-33 dose dependently increased IL-5 and IL-13 production from T\(_\text{h}2\) without affecting their IL-4 production (Fig. 4B). In contrast, as we reported previously (7), IL-18 showed modest enhancing effect on T\(_\text{h}2\). These results clearly indicated that the effects of IL-18 and IL-33 on non-polarized CD4\(^+\) T cells and T\(_\text{h}2\) are entirely different.

Thus, we assumed the possibility that non-polarized CD4\(^+\) T cells and T\(_\text{h}2\) are target cells of IL-18 and IL-33 in vivo and produced T\(_\text{h}2\) 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 Th2 cytokine response in vivo in an ST2/MyD88-dependent manner. Thus, we injected IL-33 (4 μg) 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 Th2 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 CD4+ T cells and mast cells. 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), the 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 FcγR1 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 administration of IL-33. As shown in Fig. 5(C), wild-type and TRIF−/− mice failed to do so indicating that 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 NK cells induces AHR. As shown in Fig. 6(A), wild-type and RAG-2−/− mice developed AHR following intra-nasal administration of IL-33.

**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/WY mice and WBB6F1-W/WY mice depleted of CD4+ T cells (C) were daily injected intra-peritoneally with IL-33 (4 μg day−1) for 5 days. (D) RAG−/− and RAG−/− depleted of NK cells mice were daily exposed intra-nasally to IL-33 (1 μg 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/WY 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 anti-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.
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 Th2, which express IL-33Rα 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 Th2 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 Th2-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 (34.6%) in mast cells than that (95.6%) 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-33Rβ-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-33-producing innate immune cells in IL-33-treated mice.

Since Th2 also produce IL-13 in response to IL-33 in vitro (Fig. 4B), we examined the capacity of CD4+ T cell-depleted WBB6F1-W/Wv mice, having basophils but lacking mast cells and Th2, 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 FceR1 (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 γc−/−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 γc−/−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 Th2 response. Very recent study further suggests their involvement in induction of Th2 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 Th2 cytokines and TSLP, resulting in induction of Th2, 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
IL-33 induces type 2 response without T cell help

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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