International Immunology Advance Access published April 29, 2008

International Immunology 2008; 1 of 10
doi:10.1093/intimm/dxn037

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

Yuichi Kondo[1,2,*], Tomohiro Yoshimoto[1,3,*], Koubun Yasuda[1,3], Shizue Futatsugi-Yumikura[1,3], Mai Morimoto[1,4], Nobuki Hayashi[1,3], Tomoaki Hoshino[5], Jiro Fujimoto[2] and **Kenji Nakanishi[1,3]**

[1]Department of Immunology and Medical Zoology and [2]Department of Surgery, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan
[3]Collaborative Development of Innovation Seeds, Japan Science and Technology Corporation, Saitama 332-0012, Japan
[4]Department of Internal Medicine, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan
[5]Division of Respirology, Neurology and Rheumatology, Department of Medicine, Kurume University School of Medicine, Kurume, Fukuoka 830-0011, Japan

Keywords: basophils, IL-33, innate immunity, ST2, T_h2 cytokines

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-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 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-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

*These authors contributed equally to this study.

Correspondence to: K. Nakanishi; E-mail: nakaken@hyo-med.ac.jp

Transmitting editor: T. Hamaoka

Received 6 February 2008, *accepted* 26 March 2008

(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 $RAG\text{-}2^{-/-}$ 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 $IL\text{-}13^{-/-}$ mice were generated by backcrossing B6X129 $IL\text{-}13^{-/-}$ mice (23) with C57BL/6. BALB/c $RAG\text{-}2^{-/-}$ 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 $Fc\varepsilon R^+$/*c-Kit*$^-$ cells (basophils) and $Fc\varepsilon R^+$/*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-1α, IL-1β, 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-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 (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 (1×10^5 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 $OVA_{323-339}$ (1 μM) in the presence of irradiated T cell-depleted BALB/c splenocytes (1×10^6 ml^{-1}) in 24-well plate in a total 1-ml volume of medium for 7 days as described previously (25). Polarized T_h2 (1×10^5/0.2 ml per well) were re-cultured with IL-2 (100 pM) and $OVA_{323-339}$ (1 μM) and irradiated T cell-depleted BALB/c splenocytes (1×10^5/0.2 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 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/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 β-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 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 T_h2 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 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-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 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 FcεRI⁺/*c-Kit*⁻ cells and FcεRI⁺/*c-Kit*⁺ cells (1), and then highly purified both populations by FACS (Fig. 1A). Light and electron microscopical examination revealed that resultant FcεRI⁺/*c-Kit*⁻ cells and FcεRI⁺/*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

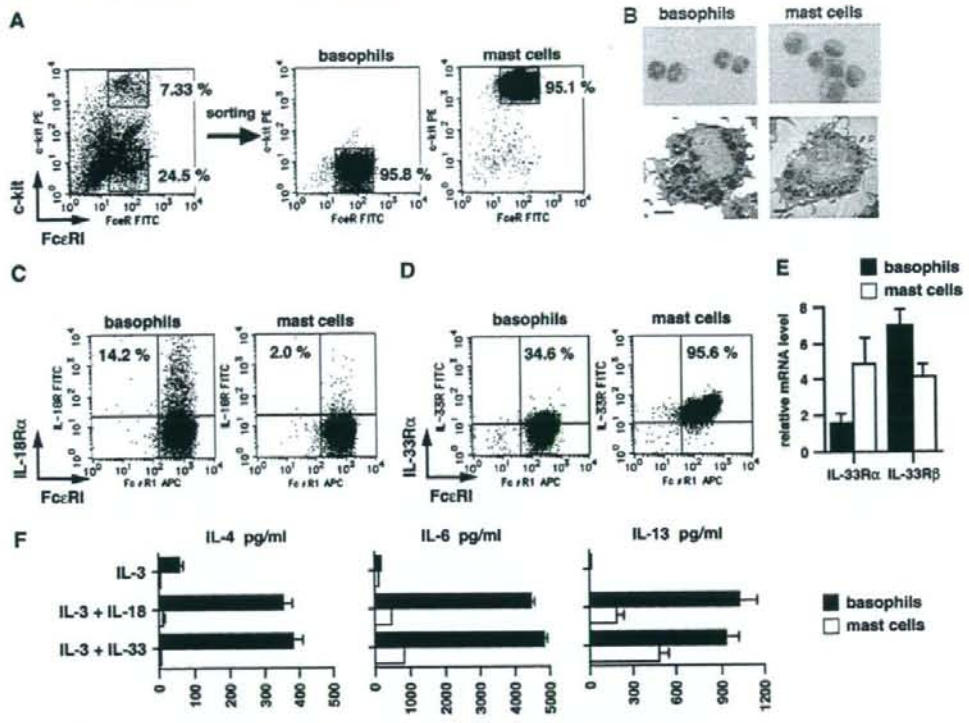

Fig. 1. IL-3 plus IL-33-induced Th2 cytokine production from bone marrow-derived basophils and mast cells. (A) Bone marrow cells from BALB/c mice cultured with IL-3 (10 U ml−1) for 10 days were analyzed for expression of FcεRI and *c-Kit* by flow cytometry and then sorted into FcεRI$^+$/*c-Kit*$^-$ (basophils) 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 (×100) (upper) and subjected to electron microscopic examination (lower). Scale bar, 1 μM. (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-33Rβ 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-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 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-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

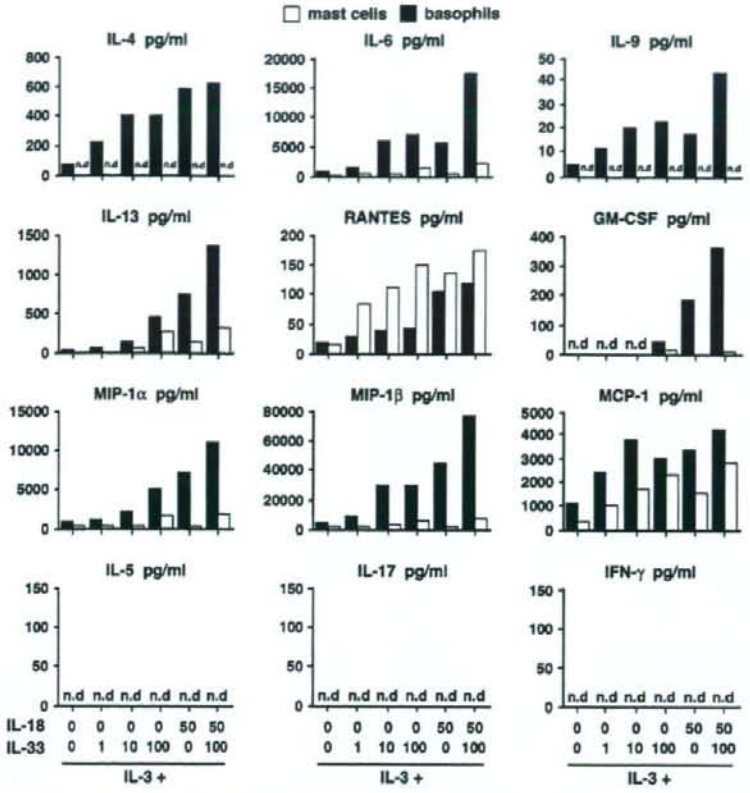

Fig. 2. IL-3 plus IL-33-induced cytokines and chemokines production from bone marrow-derived basophils and mast cells. The sorted basophils and mast cells (each 10^5/0.2 ml per well) as shown in Fig. 1 were re-stimulated with IL-18 (50 ng ml^{-1}), IL-33 (1–100 ng ml^{-1}) or IL-18 (50 ng ml^{-1}) plus 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-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 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.

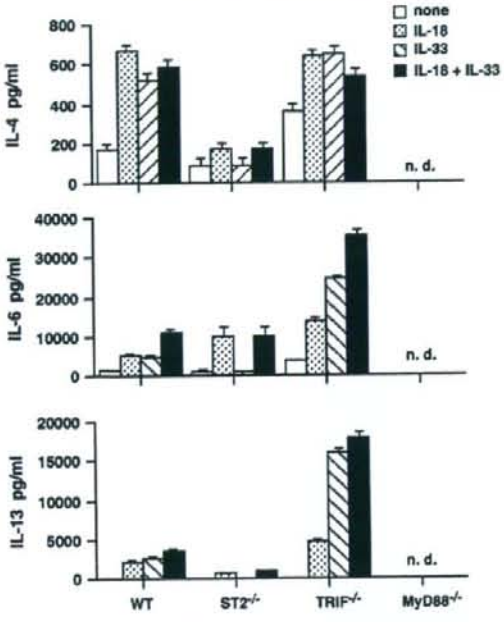

Fig. 3. ST2- and MyD88-dependent and TRIF-independent T_h2 cytokine production from IL-33 stimulated basophils. Bone marrow cells from C57BL/6 (WT), ST2$^{-/-}$, TRIF$^{-/-}$ and MyD88$^{-/-}$ mice cultured with IL-3 (10 U ml^{-1}) for 10 days were sorted into FcεRI$^+$/*c-Kit*$^-$ (basophils) cell populations 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.

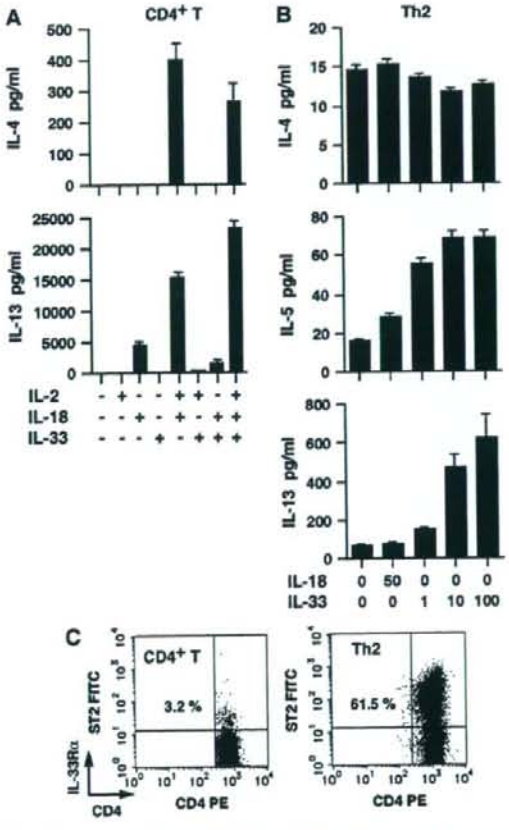

Fig. 4. T_h2 cytokine production from T cells cultured with IL-33. (A) Splenic CD4$^+$ T cells (10^5/0.2 ml per well) from BALB/c mice were cultured with medium alone or various combinations of IL-2 (200 pM), IL-18 (50 ng ml^{-1}) and IL-33 (100 ng ml^{-1}). After 4 days of culture, supernatants were 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 μM) and 1 × 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 μg ml^{-1}) and anti-IFN-γ (20 μg 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 μM) 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 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 $IL\text{-}13^{-/-}$ 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 $IL\text{-}13^{-/-}$ 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 $RAG\text{-}2^{-/-}$ 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 $WBB6F1\text{-}W/W^v$ 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 $RAG\text{-}2^{-/-}$ mice or NK cell-depleted $RAG\text{-}2^{-/-}$ 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 $IL\text{-}13^{-/-}$ 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 $RAG\text{-}2^{-/-}$ 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 $RAG\text{-}2^{-/-}$ 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 $RAG\text{-}2^{-/-}$ mice. As shown in Fig. 6(A), wild-type and $RAG\text{-}2^{-/-}$ mice developed AHR following intra-nasal

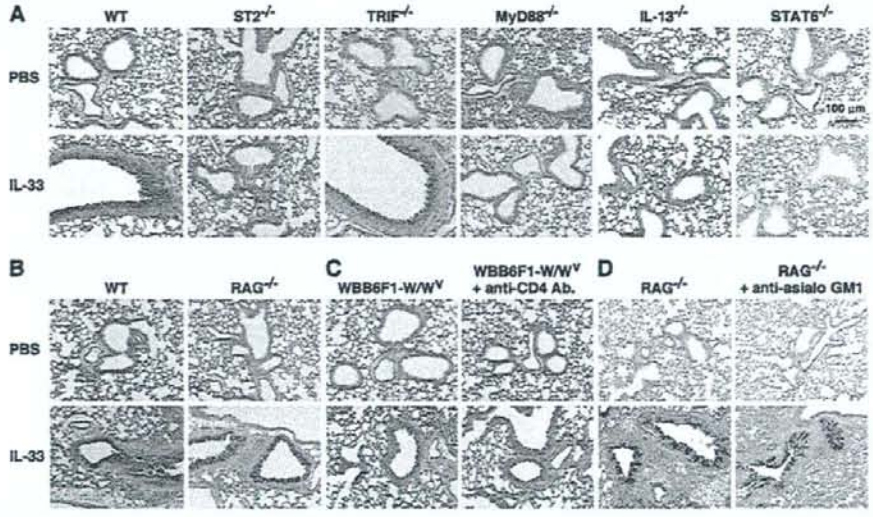

Fig. 5. IL-33-induced IL-13-dependent goblet cell hyperplasia. C57BL/6 (WT), $ST2^{-/-}$, $TRIF^{-/-}$, $MyD88^{-/-}$, $IL\text{-}13^{-/-}$ and $STAT6^{-/-}$ mice (A), BALB/c (WT) and $RAG\text{-}2^{-/-}$ ($RAG^{-/-}$) (B) and $WBB6F1\text{-}W/W^v$ mice and $WBB6F1\text{-}W/W^v$ mice depleted of $CD4^+$ 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, $WBB6F1\text{-}W/W^v$ 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.

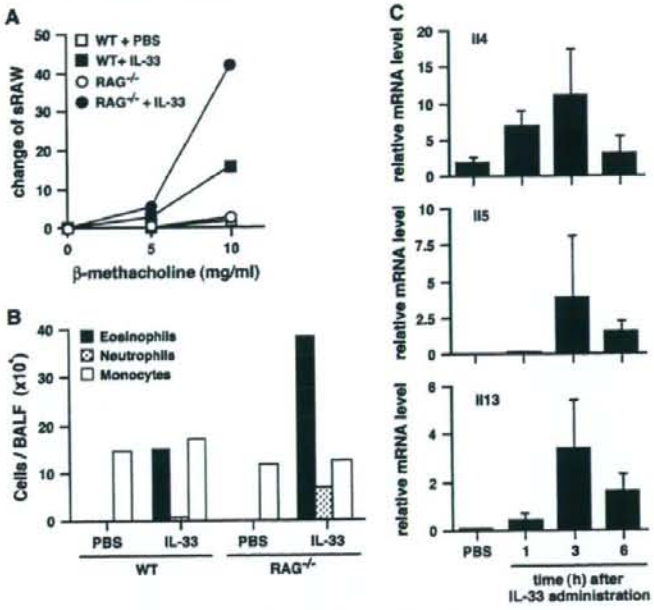

Fig. 6. IL-33-induced AHR and airway inflammation. (A and B) BALB/c (WT) and RAG$^{-/-}$ mice (four to six mice per group) were daily exposed intra-nasally to PBS alone or IL-33 (1 μg day^{-1}) in 50 μl of PBS for 4 days. (A) AHR in response to increased concentrations of inhaled β-Mch was measured in 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 μg day^{-1}) at once. IL-33 (1 μg) 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-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 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-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-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 $\gamma c^{-/-}$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 $\gamma c^{-/-}$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.

Funding

Grant-in-Aid for Scientific Research on Priority Areas (520); Hitech Research Center grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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

References

1 Yoshimoto, T., Tsutsui, H., Tominaga, K. *et al.* 1999. IL-18, although antiallergic when administered with IL-12, stimulates IL-4 and histamine release by basophils. *Proc. Natl Acad. Sci. USA* 96:13962.

2 Yoshimoto, T., Min, B., Sugimoto, T. *et al.* 2003. Nonredundant roles for CD1d-restricted natural killer T cells and conventional CD4+ T cells in the induction of immunoglobulin E antibodies in response to interleukin 18 treatment of mice. *J. Exp. Med.* 197:997.

3 Hoshino, T., Wiltrout, R. H. and Young, H. A. 1999. IL-18 is a potent coinducer of IL-13 in NK and T cells: a new potential role for IL-18 in modulating the immune response. *J. Immunol.* 162:5070.

4 Yoshimoto, T., Mizutani, H., Tsutsui, H. *et al.* 2000. IL-18 induction of IgE: dependence on CD4+ T cells, IL-4 and STAT6. *Nat. Immunol.* 1:132.

5 Kawakami, T. and Galli, S. J. 2002. Regulation of mast-cell and basophil function and survival by IgE. *Nat. Rev. Immunol.* 2:773.

6 Schmitz, J., Owyang, A., Oldham, E. *et al.* 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity.* 23:479.

7 Sugimoto, T., Ishikawa, Y., Yoshimoto, T., Hayashi, N., Fujimoto, J. and Nakanishi, K. 2004. Interleukin 18 acts on memory T helper cells type 1 to induce airway inflammation and hyperresponsiveness in a naive host mouse. *J. Exp. Med.* 199:535.

8 Yoshimoto, T., Takeda, K., Tanaka, T. *et al.* 1998. IL-12 upregulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-gamma production. *J. Immunol.* 161:3400.

9 Hoshino, K., Kashiwamura, S., Kuribayashi, K. *et al.* 1999. The absence of interleukin 1 receptor-related T1/ST2 does not affect T helper cell type 2 development and its effector function. *J. Exp. Med.* 190:1541.

10 Lohning, M., Stroehmann, A., Coyle, A. J. *et al.* 1998. T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc. Natl Acad. Sci. USA* 95:6930.

11 Moritz, D. R., Rodewald, H. R., Gheyselinck, J. and Klemenz, R. 1998. The IL-1 receptor-related T1 antigen is expressed on immature and mature mast cells and on fetal blood mast cell progenitors. *J. Immunol.* 161:4866.

12 Xu, D., Chan, W. L., Leung, B. P. *et al.* 1998. Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J. Exp. Med.* 187:787.

13 Nakanishi, K., Yoshimoto, T., Tsutsui, H. and Okamura, H. 2001. Interleukin-18 regulates both Th1 and Th2 responses. *Annu. Rev. Immunol.* 19:423.

14 Born, T. L., Thomassen, E., Bird, T. A. and Sims, J. E. 1998. Cloning of a novel receptor subunit, AcPL, required for interleukin-18 signaling. *J. Biol. Chem.* 273:29445.

15 Ali, S., Huber, M., Kollewe, C., Bischoff, S. C., Falk, W. and Martin, M. U. 2007. IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells. *Proc. Natl Acad. Sci. USA* 104:18660.

16 Chackerian, A. A., Oldham, E. R., Murphy, E. E., Schmitz, J., Pflanz, S. and Kastelein, R. A. 2007. IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *J. Immunol.* 179:2551.

17 Yoshimoto, T. and Nakanishi, K. 2006. Roles of IL-18 in basophils and mast cells. *Allergol. Int.* 55:105.

18 Kaisho, T. and Akira, S. 2003. Regulation of dendritic cell function through toll-like receptors. *Curr. Mol. Med.* 3:759.

19 Takeda, K., Kaisho, T. and Akira, S. 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21:335.

20 Sokol, C. L., Barton, G. M., Farr, A. G. and Medzhitov, R. 2008. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat. Immunol.* 9:310.

21 Adachi, O., Kawai, T., Takeda, K. *et al.* 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity.* 9:143.

22 Yamamoto, M., Sato, S., Hemmi, H. *et al.* 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 301:640.

23 McKenzie, G. J., Emson, C. L., Bell, S. E. *et al.* 1998. Impaired development of Th2 cells in IL-13-deficient mice. *Immunity* 9:423.

24 Kitamura, Y., Go, S. and Hatanaka, K. 1978. Decrease of mast cells in W/Wv mice and their increase by bone marrow transplantation. *Blood* 52:447.

25 Yoshimoto, T., Yoshimoto, T., Yasuda, K., Mizuguchi, J. and Nakanishi, K. 2007. IL-27 suppresses Th2 cell development and Th2 cytokines production from polarized Th2 cells: a novel therapeutic way for Th2-mediated allergic inflammation. *J. Immunol.* 179:4415.

26 Sasaki, Y., Yoshimoto, T., Maruyama, H. *et al.* 2005. IL-18 with IL-2 protects against Strongyloides venezuelensis infection by activating mucosal mast cell-dependent type 2 innate immunity. *J. Exp. Med.* 202:607.

27 Yoshimoto, T., Okamura, H., Tagawa, Y. I., Iwakura, Y. and Nakanishi, K. 1997. Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-gamma production from activated B cells. *Proc. Natl Acad. Sci. USA* 94:3948.

28 Kawai, T., Adachi, O., Ogawa, T., Takeda, K. and Akira, S. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11:115.

29 Yoshimoto, T. and Paul, W. E. 1994. CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to *in vivo* challenge with anti-CD3. *J. Exp. Med.* 179:1285.

30 Ishikawa, Y., Yoshimoto, T. and Nakanishi, K. 2006. Contribution of IL-18-induced innate T cell activation to airway inflammation with mucus hypersecretion and airway hyperresponsiveness. *Int. Immunol.* 18:847.

31 Min, B., Prout, M., Hu-Li, J. *et al.* 2004. Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J. Exp. Med.* 200:507.

32 Herz, U., Lacy, P., Renz, H. and Erb, K. 2000. The influence of infections on the development and severity of allergic disorders. *Curr. Opin. Immunol.* 12:632.

33 Lemanske, R. F. 2004. Viral infections and asthma inception. *J. Allergy Clin. Immunol.* 114:1023.

34 Schwarze, J., Hamelmann, E., Bradley, K. L., Takeda, K. and Gelfand, E. W. 1997. Respiratory syncytial virus infection results in airway hyperresponsiveness and enhanced airway sensitization to allergen. *J. Clin. Invest.* 100:226.

doi: 10.1111/j.1365-2222.2008.03114.x

ORIGINAL PAPER Asthma and Rhinitis

Clinical and Experimental Allergy, 38, 1875–1881

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

M. Sakashita*†, T. Yoshimoto‡, T. Hirota*, M. Harada*, K. Okubo§, Y. Osawa†, S. Fujieda†, Y. Nakamura¶, K. Yasuda‡, K. Nakanishi‡ and M. Tamari*

*Laboratory for Genetics of Allergic Diseases, SNP Research Center, RIKEN, Kanagawa, Japan, †Department of Otorhinolaryngology, Fukui Medical University, Matsuoka, Fukui, Japan, ‡Department of Immunology and Medical Zoology, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan, §Department of Otolaryngology, Nippon Medical School, Tokyo, Japan and ¶Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Clinical and Experimental Allergy

Correspondence:
Mayumi Tamari, Laboratory for Genetics of Allergic Diseases, SNP Research Center, RIKEN, Kanagawa 230-0045, Japan.
E-mail: tamari@src.riken.jp
Cite this as: M. Sakashita, T. Yoshimoto, T. Hirota, M. Harada, K. Okubo, Y. Osawa, S. Fujieda, Y. Nakamura, K. Yasuda, K. Nakanishi and M. Tamari, *Clinical and Experimental Allergy*, 2008 (38) 1875–1881.

Summary

Background IL-33, an IL-1-like cytokine, is a ligand for IL1RL1, which is an important effector molecule of type 2 T helper responses. Although IL-33/IL1RL1 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
Submitted 28 August 2007; revised 3 February 2008, 31 July 2008; accepted 3 September 2008

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 > 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]. IL1RL1 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 IL1RL1, 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-κB and mitogen-activated protein kinases, and drives production of Th2-associated cytokines from *in vitro* polarized Th2 cells via IL1RL1 [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 IL1RL1 have shown its regulatory functions in the development and effector phases of Th2 responses [10]. The *IL1RL1* gene encodes a soluble-secreted protein, IL1RL1, and a transmembrane protein, ST2L [11]. In murine models of allergic airway inflammation, increases in endogenous IL1RL1 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 IL1RL1 acts as a negative regulator of Th2 cytokine production via IL-33 signalling in allergic airway inflammation. In asthmatic patients, serum levels of soluble IL1RL1 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 IL1RL1 distal promoter region are associated with atopic dermatitis. The genetic variants regulate IL1RL1 expression, and immunohistochemical staining of a skin biopsy specimen from an atopic dermatitis patient showed IL1RL1 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

were immunized with rhIL-33 (200 μg/body) with CFA, and boosted with rhIL-33 (200 μg/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 μg/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-1β, IL-2, IL-4, IL-12, IL-18, TNF-α, IFN-γ 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 χ^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 (%)‡	NCBI§
−5345 G/A	5′-Flanking region		0.477	rs928414
−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†	Intron 3		0.455	rs1929992
11607 T/C	Intron 4		0.432	rs1113573
11877 C/T†	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.
†SNPs were genotyped in this study.
‡Minor allele frequencies
§NCBI, number from the dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/SNP/).
SNP, single nucleotide polymorphisms; MAF, minor allele frequency.

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 χ^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 >0.10 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 5′-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 ($r^2 > 0.75$). 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 ($r^2 > 0.91$).

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 (T > C) and JC pollinosis (TT+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).

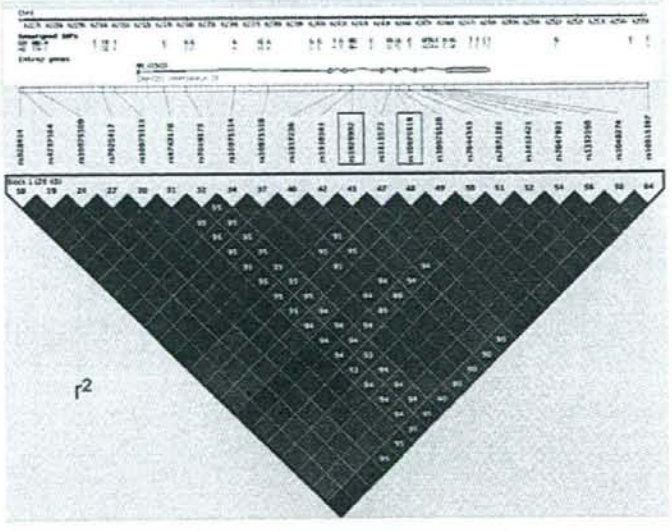

Fig. 1. Pairwise linkage disequilibrium between 22 SNPs as measured by r^2 estimated by the Haploview 3.2 program using the HapMap JPT data set. The boxed polymorphisms, rs1929992 and rs10975519, were genotyped in this study.

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).

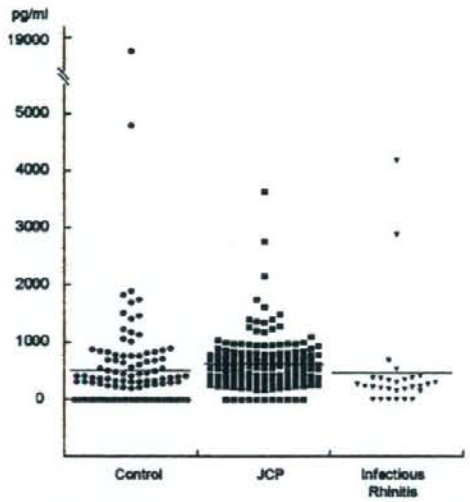

Fig. 2. Comparison of serum IL-33 levels among controls, patients with JC pollinosis and those with infectious rhinitis. Horizontal bars indicate the median value of each group. JCP, Japanese Cedar pollinosis.

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. CC+TC in rs1929992, CC vs. CT+TT in rs10975519).

†Recessive model (TT+TC vs. CC in rs1929992, CC+CT vs. TT in rs10975519).

‡Allelic model (T allele vs. C allele in rs1929992, C allele vs. T allele in rs10975519).

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-γ 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′-flanking region, which is often involved in transcriptional regulation of the gene. Several transcription factors are involved in asthmatic inflammation, including NF-κB, 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′ 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.

Acknowledgements

We thank all the participants in the study. We also thank Makiko Shimizu-Terada, Hiroshi Sekiguchi, Aya Jodo-Ito, Yuko Taki and Nami Kawaraichi. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare of Japan.

References

1 Okawa T, Konno A, Yamakoshi T, Numata T, Terada N, Shima M. Analysis of natural history of Japanese cedar pollinosis. *Int Arch Allergy Immunol* 2003; 131:39–45.
2 Romagnani S. The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both? *Immunology* 2004; 112:352–63.
3 Romagnani S. Lymphokine production by human T cells in disease states. *Annu Rev Immunol* 1994; 12:227–57.
4 Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature* 1996; 383:787–93.
5 Xu D, Chan WL, Leung BP *et al.* Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J Exp Med* 1998; 187:787–94.

6 Lohning M, Stroehmann A, Coyle AJ *et al.* T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc Natl Acad Sci U S A* 1998; 95:6930–5.

7 Coyle AJ, Lloyd C, Tian J *et al.* Crucial role of the interleukin 1 receptor family member T1/ST2 in T helper cell type 2-mediated lung mucosal immune responses. *J Exp Med* 1999; 190:895–902.

8 Townsend MJ, Fallon PG, Matthews DJ, Jolin HE, McKenzie AN. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J Exp Med* 2000; 191:1069–76.

9 Schmitz J, Owyang A, Oldham E *et al.* IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005; 23:479–90.

10 Trajkovic V, Sweet MJ, Xu D. T1/ST2 – an IL-1 receptor-like modulator of immune responses. *Cytokine Growth Factor Rev* 2004; 15:87–95.

11 Yanagisawa K, Takagi T, Tsukamoto T, Tetsuka T, Tominaga S. Presence of a novel primary response gene ST2L, encoding a product highly similar to the interleukin 1 receptor type 1. *FEBS Lett* 1993; 318:83–7.

12 Oshikawa K, Yanagisawa K, Tominaga S, Sugiyama Y. Expression and function of the *ST2* gene in a murine model of allergic airway inflammation. *Clin Exp Allergy* 2002; 32:1520–6.

13 Lambrecht BN, De Veerman M, Coyle AJ, Gutierrez-Ramos JC, Thielemans K, Pauwels RA. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J Clin Invest* 2000; 106:551–9.

14 Oshikawa K, Kuroiwa K, Tago K *et al.* Elevated soluble ST2 protein levels in sera of patients with asthma with an acute exacerbation. *Am J Respir Crit Care Med* 2001; 164:277–81.

15 Cookson WO, Moffatt MF. Genetics of asthma and allergic disease. *Hum Mol Genet* 2000; 9:2359–64.

16 Palmer LJ, Cookson WO. Using single nucleotide polymorphisms as a means to understanding the pathophysiology of asthma. *Respir Res* 2001; 2:102–12.

17 Ober C, Hoffjan S. Asthma genetics 2006: the long and winding road to gene discovery. *Genes Immun* 2006; 7:95–100.

18 Shimizu M, Matsuda A, Yanagisawa K *et al.* Functional SNPs in the distal promoter of the *ST2* gene are associated with atopic dermatitis. *Hum Mol Genet* 2005; 14:2919–27.

19 Ziegler SF, Liu YJ. Thymic stromal lymphopoietin in normal and pathogenic T cell development and function. *Nat Immunol* 2006; 7:709–14.

20 Dinarello CA. An IL-1 family member requires caspase-1 processing and signals through the ST2 receptor. *Immunity* 2005; 23:461–2.

21 Ho LH, Ohno T, Oboki K *et al.* IL-33 induces IL-13 production by mouse mast cells independently of IgE-FcεRI signals. *J Leukoc Biol* 2007; 82:1481–90.

22 Yoshimoto T, Takeda K, Tanaka T *et al.* IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-gamma production. *J Immunol* 1998; 161:3400–7.

23 Higa S, Hirano T, Mayumi M *et al.* Association between interleukin-18 gene polymorphism 105A/C and asthma. *Clin Exp Allergy* 2003; 33:1097–102.

24 Kruse S, Kuehr J, Moseler M *et al.* Polymorphisms in the *IL 18* gene are associated with specific sensitization to common allergens and allergic rhinitis. *J Allergy Clin Immunol* 2003; 111:117–22.

25 Novak N, Kruse S, Potreck J *et al.* Single nucleotide polymorphisms of the *IL18* gene are associated with atopic eczema. *J Allergy Clin Immunol* 2005; 115:828–33.

26 Shin HD, Kim LH, Park BL *et al.* Association of interleukin 18 (IL18) polymorphisms with specific IgE levels to mite allergens among asthmatic patients. *Allergy* 2005; 60:900–6.

27 Verhaeghe B, Gevaert P, Holtappels G *et al.* Up-regulation of IL-18 in allergic rhinitis. *Allergy* 2002; 57:825–30.

28 Gajewska BU, Swirski FK, Alvarez D *et al.* Temporal–spatial analysis of the immune response in a murine model of ovalbumin-induced airways inflammation. *Am J Respir Cell Mol Biol* 2001; 25:326–34.

29 Suzuki A, Yamada R, Chang X *et al.* Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat Genet* 2003; 34:395–402.

30 Hirota T, Suzuki Y, Hasegawa K *et al.* Functional haplotypes of IL-12B are associated with childhood atopic asthma. *J Allergy Clin Immunol* 2005; 116:789–95.

31 Barnes PJ, Adcock IM. Transcription factors and asthma. *Eur Respir J* 1998; 12:221–34.

32 Siroux V, Curt F, Oryszczyn MP, Maccario J, Kauffmann F. Role of gender and hormone-related events on IgE, atopy, and eosinophils in the epidemiological study on the genetics and environment of asthma, bronchial hyperresponsiveness and atopy. *J Allergy Clin Immunol* 2004; 114:491–8.

33 Levy H, Raby BA, Lake S *et al.* Association of defensin beta-1 gene polymorphisms with asthma. *J Allergy Clin Immunol* 2005; 115:252–8.

Inhibition of collagen-induced platelet aggregation by anopheline antiplatelet protein, a saliva protein from a malaria vector mosquito

Shigeto Yoshida,[1] Toshiki Sudo,[2] Masashi Niimi,[3] Lian Tao,[3] Bing Sun,[3] Junichi Kambayashi,[3] Hiroyuki Watanabe,[1] Enjie Luo,[1] and Hiroyuki Matsuoka[1]

[1]Division of Medical Zoology, Department of Infection and Immunity, Jichi Medical University, Tochigi, Japan; [2]First Institute of New Drug Discovery, Otsuka Pharmaceutical, Tokushima, Japan; and [3]Otsuka Maryland Medicinal Laboratories, Rockville, MD

During blood feeding, mosquitoes inject saliva containing a mixture of molecules that inactivate or inhibit various components of the hemostatic response to the bite injury as well as the inflammatory reactions produced by the bite, to facilitate the ingestion of blood. However, the molecular functions of the individual saliva components remain largely unknown. Here, we describe anopheline antiplatelet protein (AAPP) isolated from the saliva of *Anopheles stephensi*, a human malaria vector mosquito. AAPP exhibited a strong and specific inhibitory activity toward collagen-induced platelet aggregation. The inhibitory mechanism involves direct binding of AAPP to collagen, which blocks platelet adhesion to collagen and inhibits the subsequent increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$). The binding of AAPP to collagen effectively blocked platelet adhesion via glycoprotein VI (GPVI) and integrin $\alpha_2\beta_1$. Cell adhesion assay showed that AAPP inhibited the binding of GPVI to collagen type I and III without direct effect on GPVI. Moreover, intravenously administered recombinant AAPP strongly inhibited collagen-induced platelet aggregation *ex vivo* in rats. In summary, AAPP is a malaria vector mosquito-derived specific antagonist of receptors that mediate the adhesion of platelets to collagen. Our study may provide important insights for elucidating the effects of mosquito blood feeding against host hemostasis. (Blood. 2008; 111:2007-2014)

Introduction

The saliva of blood-sucking arthropods contains a number of pharmacologically active molecules that counteract the host defenses triggered by blood feeding, such as inhibitors of the clotting cascade, platelet aggregation and vasodilators.[1] Mosquitoes search for blood by repeatedly thrusting their mouthparts into the host's deep network of skin vessels. This probing causes vessel laceration by the mouthparts. After laceration of blood vessels, platelets are generally exposed to subendothelial collagen in the damaged vessel walls, and subsequently adhere to the collagen, become activated, release their granular contents and form aggregates, with the result that the injuries are plugged by platelets.[2] Thus, platelet activation by collagen is an initial step in normal hemostasis. However, mosquitoes can successfully engorge on their hosts within a half minute because antihemostatic components of their saliva facilitate location of blood vessels.[3] Although mosquito saliva is known to show inhibitory activity toward platelet aggregation in response to adenosine diphosphate (ADP), collagen, and thrombin,[3] a limited number of saliva components involved in the inhibition of platelet aggregation have been characterized. Specifically, *Aedes aegypti* apyrase inhibits ADP-induced platelet aggregation by metabolizing ADP released from injured cells,[4] while *Anopheles albimanus* anopheline inhibits thrombin-mediated blood coagulation.[5] However, no inhibitors of collagen-induced platelet aggregation have been identified to date.

In anopheline mosquitoes, saliva and the salivary glands have been extensively studied due to their direct involvement in the transmission of malaria parasites to human hosts.[6,7] Recently, the salivary gland transcriptosome and proteosome (termed the sialome) of anopheline mosquitoes have been reported.[8-10] In a previous study that characterized a salivary gland-specific promoter by using a transgenic *Anopheles stephensi*,[11] we cloned a gene encoding a putative saliva protein that was specifically expressed in the female salivary glands. Sialome searches revealed that the gene product is a homolog of a 30-kDa *A aegypti* allergen of unknown function[12] and well conserved among anopheline mosquitoes as a member of the GE-rich protein family.[8-10,13] In the present paper, we demonstrate that this *A stephensi* homolog protein specifically inhibits collagen-induced platelet aggregation. Therefore, we have renamed it anopheline antiplatelet protein (AAPP). To the best of our knowledge, this is the first report of a mosquito saliva component that is capable of specifically inhibiting collagen-induced platelet aggregation in vitro and ex vivo.

Methods

Preparation of a salivary gland extract

A stephensi mosquitoes (SDA 500 strain) were maintained at Jichi Medical University as described previously.[14] The salivary glands were dissected from unfed female *A stephensi* mosquitoes, transferred to 1.5-mL conical polypropylene tubes containing cooled PBS and disrupted by sonification

Submitted June 25, 2007; accepted November 22, 2007. Prepublished online as *Blood* First Edition paper, December 4, 2007; DOI 10.1182/blood-2007-06-097824.

S.Y. and T.S. contributed equally to this study.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

on ice. After centrifugation at 15 000*g* for 5 minutes, the resulting supernatant was assayed for platelet aggregation.

Materials

Fibrillar type I collagen from equine tendon (Horm), for use in platelet aggregation, cell adhesion, and Ca^{2+} influx assays, was from Nycomed Pharma (Munich, Germany); nonfibrillar type I collagen from rat tail collagen, for use under static conditions, was from BD Biosciences (San Jose, CA); collagen type III, collagen type IV, ADP, bovine serum albumin (BSA), normal goat serum, platelet-activating factor (PAF) and A23187 were from Sigma-Aldrich (St Louis, MO); epinephrine was from Daiichi Pharmaceuticals (Tokyo, Japan); U-46 619 was from Cayman Chemicals (Ann Arbor, MI); convulxin was from Alexis Biochemicals (San Diego, CA). Thrombin receptor-activating peptide (TRAP) was synthesized by Sawadey Technology (Tokyo, Japan). Collagen-related peptide (CRP) was synthesized by Peptide Institute (Osaka, Japan). Antihuman GPVI monoclonal antibody (mAb) OM-2 has previously been described.[15] Anti-(human integrin α_2-subunit) mAb 6F1[16] was a generous gift from Dr B. J. Coller (Mount Sinai Hospital, New York, NY).

Recombinant proteins

A cDNA encoding AAPP was cloned from an *A stephensi* salivary gland cDNA library by immunoscreening with a rabbit anti-*A stephensi* salivary gland immune serum as described previously.[11] The cDNA fragment encoding amino acids 22 to 269 of AAPP, which lacked the signal peptide, was amplified from the aapp cDNA by PCR using the primers pAnSG-F7 (5′-CCATGGCGTCCGACGAGACTACGGATCAAGAA-3′) and pAnSG-R1 (5′-GCGGCCGCCTCTGAATCACGCTTTTCGACGATGC-3′). The PCR product was inserted into the *NcoI/NotI* sites of pET32-b (+) (Invitrogen, Carlsbad, CA) and expressed as a thioredoxin fusion protein. As a control, recombinant thioredoxin (rTrx) was produced by using pET32-b (+) without any insert. rAAPP and rTrx were produced and solubilized under denaturing conditions using 6 M guanidine-HCl, and then purified by affinity chromatography on a Ni-NTA column (Qiagen, Valencia, CA) followed by dialysis against PBS as described previously.[17] The purity of the recombinant proteins was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the protein yield was estimated by comparison with BSA standards.

Indirect immunofluorescence

The salivary glands were dissected in PBS, fixed in 4% paraformaldehyde for 20 minutes at 4°C and dried on poly-L-lysine–treated slides (MAS Coated Slides; Matsunami, Tokyo, Japan). The glands were then incubated with 10% normal goat serum in PBS for 1 hour at room temperature, followed by incubation with a mouse anti-rAAPP immune serum. After extensive washing, the salivary glands were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Biosource, Camarillo, CA). The salivary glands were mounted onto glass slides, covered with a drop VECTASHIELD (Vector, Laboratories, Burlingame, CA), and examined by fluorescence microscopy.

SDS-PAGE, silver staining, and immunoblotting

Salivary glands were solubilized in Laemmli buffer[18] containing 2% 2-mercaptoethanol and boiled for 5 minutes. The proteins were separated by SDS-PAGE using a 10% gel, and then stained with a Silver Stain Kit (GE Healthcare UK, Chalfont St Giles, United Kingdom) or electrophoretically transferred to an Immobilon Transfer Membrane (Millipore, Bedford, MA). For immunoblotting, the membrane was treated with a mouse anti-rAAPP immune serum. A Polypeptide band recognized by the serum was detected with biotinylated antimouse IgG (H + L; Vector Laboratories), followed by color development with 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt/nitroblue tetrazolium chloride substrate (Life Technologies, Rockville, MD) as described previously.[19]

RT-qPCR

Total RNA was isolated from mosquito salivary glands and carcasses using the RNeasy Mini columns (Qiagen). The *aapp* and *ubiquitin* mRNAs were quantified by reverse-transcription quantitative polymerase chain reaction (RT-qPCR), with ABI PRISM 7700 Sequence Detection System and the SYBR Green Master Mix Kit (Applied Biosystems, Foster City, CA). The primers used for detection of the *aapp* and *ubiquitin* genes were as follows: pAAPP-RT-F1: 5′-CGACCTGGTCGGCAGTATCT-3′, and pAAPP-RT-R1, 5′-CGAGTCGAAGCATTCCTTAATCTT-3′; pAnsUbi-RT-F1, 5′-CGCAAGTGCTACGCTCGTC-3′, and pAnsUbi-RT-R1, 5′-TGGTGT-GACCGCACTTCTTC-3′. These primers were designed using Primer Express Software v. 1.5 (Applied Biosystems). The fold induction of the mRNA was determined from the threshold cycle values normalized for the ubiquitin gene expression and then normalized to the value derived from the aapp gene in the salivary glands of male mosquitoes.

Platelet aggregation assay

Platelet aggregation with human platelet-rich plasma (PRP), obtained by centrifuging citrated blood, was investigated as described previously.[20] Platelet number was determined by an automatic counter, and PRP were prepared to 3×10^8 cells/mL with platelet-poor plasma (PPP). To measure changes in the light transmission rate, PRP samples (200 μL) were incubated with stirring for 2 minutes at 37°C in the presence or absence of rAAPP, then with 22.2 μL of platelet agonists for 5 minutes at 37°C. The intensity of light transmission over 5 minutes was then measured using an aggregometer (MCM Hematracer 313M, Model:PAM-12C, SSR Engineering, Tokyo, Japan). The baseline was set with PRP and the maximum possible increase in light transmission (platelet aggregation rate: 100%) was set with PPP. The percentage inhibition was calculated based on the maximum aggregation rate of the test samples relative to the appropriate buffer control.

Preparation of human washed platelets

Blood was isolated from an arm vein of healthy human volunteers. The blood was drawn with a 21-G needle into a plastic syringe containing 0.1 vol 3.8% trisodium citrate solution, and centrifuged at 180*g* for 10 minutes. PRP was removed and mixed with one-fourth volume of ACD buffer (44.8 mM sodium citrate, 20.9 mM citric acid, 74.1 mM glucose, pH 5.0). After the centrifugation, the platelet pellet was resuspended in washing buffer (113 mM NaCl, 4 mM KCl, 24 mM NaH_2PO_4, 4 mM Na_2HPO_4, 0.2 mM EGTA, 0.1% glucose pH 6.0), and recentrifuged at 500*g* for 20 minutes to create a platelet pellet. The final pellet was resuspended in incubation buffer (134 mM NaCl, 12 mM $NaHCO_3$, 0.34 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5 mM Hepes, 5 mM glucose, pH 7.4).

AAPP binding assay

Binding of rAAPP to collagen was measured in 96-well collagen-coated microtiter plates (Nunc, Rochester, NY). After blocking with blocking buffer (PBS containing 5% BSA) for 1 hour at room temperature, various concentrations of rAAPP was added and incubated for 1 hour at room temperature. Binding of rAAPP to collagen was detected using the ExpressDetector Nickel-HRP (KPL, Gaithersburg, MD), which can bind to the His-tag at the C-terminal of rAAPP, according to the manufacturer's protocol. As a control, rTrx was used.

Platelet adhesion assay

Ninety-six–well microtiter plates were coated with 40 μg/mL of collagen for 1 hour at room temperature, followed by blocking with the blocking buffer for 1 hour. Control wells were coated with the blocking buffer only. The wells were rinsed 3 times with PBS. rAAPP was serially diluted, and 50 μL of each dilution was added to the plates and incubated for 30 minutes at room temperature. Next, 50 μL of human washed platelet suspension (6×10^8 cells/mL) was added to each well and incubated for 45 minutes at room temperature. Nonadherent platelets were removed, and the wells were washed 3 times with PBS. The number of adherent platelets was determined using the Dc protein assay kit (Nippon Bio-Rad, Tokyo, Japan).

GPVI-expressing Jurkat cells

The human GPVI cDNA was cloned from a cDNA library of the human placenta (Clontech, Palo Alto, CA) and inserted into a lenti virus vector,[21] kindly provided by Dr X. Wu (University of Alabama, Birmingham, AL), and transfected with Jurkat cells. A stable GPVI-expressing Jurkat cell line was established by a method of Kappes et al[21] and maintained in RPMI1640 10% FCS medium supplemented with 0.5 μg/mL Puromycin (Sigma-Aldrich). Surface expression of GPVI was confirmed by flow cytometry using antihuman GPVI mAb OM-2. Nontransfected Jurkat cells do not express endogenous GPVI.

Cell adhesion assay

Ninety-six–well microtiter plates were coated with various concentrations of collagen or rAAPP. The wells were blocked with the blocking buffer at room temperature for 1 hour, then rinsed once in PBS. GPVI-expressing Jurkat cells were washed with PBS twice and resuspended in the blocking buffer at 1×10^6 cells/mL. One hundred microliters of cell suspension was added to each well and incubated at room temperature for 1 hour. The wells were washed with PBS 3 times to remove nonadherent cells. Adherent cells were quantified by the BCA protein assay kit (Pierce, Rockford, IL).

GPVI- and $\alpha_2\beta_1$-mediated platelet adhesion assay

Ninety-six–well microtiter plates were coated with insoluble equine tendon fibrillar type I collagen or soluble rat tail type I (nonfibrillar) collagen maintained in acetate buffer, pH 4.5. Various concentrations of rAAPP were added to the 96-well plates immobilized with the fibrillar type I collagen with Tyroad's-Hepes buffer (136.7 mM NaCl, 13.8 mM, $NaHCO_3$, 0.36 mM $NaH_2PO_4{\cdot}H_2O$, 2.6 mM KCl, 1.0 mM $MgCl_2{\cdot}6H_2O$, 5.5 mM glucose, 0.25% BSA, pH 7.4) containing 50 μM EDTA. Similarly, various concentrations of rAAPP were added to the 96-well plates immobilized with the nonfibrillar type I collagen with Tyroad-Hepes buffer and incubated for 1 hour. After washing with PBS 3 times, washed platelet suspension (10^5 cells/μL) were allowed to adhere under static conditions to each well for 1 hour. As positive controls, washed platelet suspension were pre-incubated with antihuman GPVI mAb OM-2 (1 μg/mL) or anti-(human integrin α_2-subunit) mAb 6F1 (5 μg/mL). Adherent platelets were quantified fluorimetrically as described by Nakamura et al.[22] Results are representative of 3 independent experiments and expressed as the mean of triplicate reading plus or minus SEM for the indicated concentrations.

Measurement of intracellular Ca^{2+} concentration

Ca^{2+} concentration ($[Ca^{2+}]i$) transients were monitored by fura-2 fluorescence. Human PRP was incubated with 5 μM fura-2 AM (Dojindo, Tokyo, Japan) for 60 minutes at 37°C. After 2 washes with the wash buffer, the platelets were resuspended to 3×10^8 cells/mL with the incubation buffer. After a 1-minute incubation with various concentrations of rAAPP, the platelets were stimulated with collagen (0.5 μg/mL) and the fura-2 fluorescence was measured at an excitation wavelength of 340/380 nm and emission wavelength of 500 nm using a Hitachi F-2000 fluorescence spectrophotometer.

Ex vivo platelet aggregation of rats

Platelet aggregation of rats was investigated as described previously.[23] Crl:SD rats (Charles River Japan, Tokyo, Japan) were used in this study. An rAAPP solution (0.1, 0.3, or 1.0 mg/kg) or rTrx control solution was administered intravenously into the tail vein. 10 minutes after the administration, 6 mL of blood was sampled from the inferior vena cava of each rat under ether anesthesia using a 21-G needle and a plastic syringe containing 0.1 vol. of 3.18% trisodium citrate solution. Platelets were prepared at 10^9 cells/mL with autologous PPP. Platelet aggregation was measured 60 minutes after blood collection. All care and handling of the animals was in accordance with the Guidelines for Animal Care and Use prepared by Otsuka Pharmaceutical.

Statistical analyses

Results are presented as the means plus or minus standard error of the mean (SEM). The protein concentrations and inhibitory ratios of platelet aggregation were transformed into logarithms and logits, respectively. IC_{50} values and 95% confidence limits were calculated by log-logit regression analysis. The SAS system (Release 8.1; SAS Institute Japan, Tokyo, Japan) was employed for these statistical analyses.

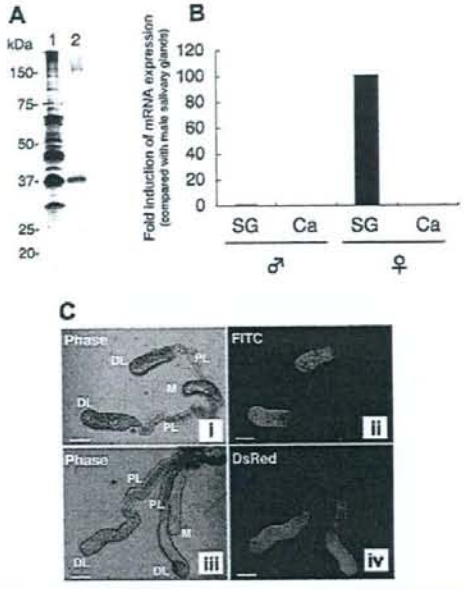

Figure 1. Female salivary gland–specific expression of AAPP. (A) Western blotting for AAPP in the salivary glands of female mosquitoes. A homogenate of 2 pairs of female salivary glands was subjected to 10% SDS-PAGE. Lane 1, silver staining; lane 2, Western blotting using a mouse anti-rAAPP immune serum. (B) RT-qPCR of *aapp* mRNA. Total RNA was isolated from salivary glands (SG) and carcasses (Ca) of male and female mosquitoes. The expression of *aapp* mRNA was examined for its tissue- and sex-specificity using RT-qPCR. The fold induction was determined from threshold cycle values normalized for *ubiquitin* mRNA expression and then normalized to the values of each gene from the male salivary gland mRNA. (C) Immunostaining of salivary glands with the anti-rAAPP immune serum. The paired salivary glands of mosquitoes are present in the thorax flanking the esophagus. Each gland consists of 3 lobes: a distal lateral lobe (DL), proximal lateral lobe (PL), and medial lobe (M). (i) phase-contrast image of the salivary glands of a wild-type mosquito; (ii) immunostaining of the salivary glands of a wild-type mosquito with the anti-rAAPP immune serum; (iii) phase-contrast image of the salivary glands of a DsRed transgenic mosquito; and (iv) DsRed expression in the salivary glands of a DsRed transgenic mosquito. Scale bars: 100 μm.

Results

AAPP is a predominant protein in the female salivary glands

The complete AAPP cDNA has an ORF encoding 269 amino acid residues. The gene product has characteristic features of acidic secreted proteins (pI = 3.8) containing 10 unique repeats of a 6-amino acid unit (GEEGGA) or related sequences (Figure S1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). We named this protein anopheline antiplatelet protein (AAPP), according to its biologic function identified in the present study.

rAAPP was produced as a thioredoxin fusion protein by using an *E coli* expression system. Anti-rAAPP immune serum specifically reacted with a salivary gland protein with an apparent molecular mass of around 37 kDa (Figure 1A lane 2), which corresponds to one of the most abundant proteins found in the